ABSTRACT

LASHNITS, ERIN WRIGHT. Epidemiology and Modeling *Bartonella* in a One Health Context. (Under the direction of Dr. Edward Breitschwerdt)

As emerging zoonotic vector borne pathogens, members of the genus *Bartonella* are increasingly recognized as causes of disease in human and veterinary medicine. The domestic cat is the reservoir host for multiple species of zoonotic *Bartonella*, the most well-studied of which is *Bartonella henselae*, the etiologic agent of a syndrome historically termed "cat scratch disease" (CSD). Often in human infection with zoonotic *Bartonella* spp. the source of infection remains unknown or speculative, and some reports indicate potential for other epidemiologically important hosts and vectors that are not cats and fleas (particularly ticks). Further understanding of transmission and pathogenesis of *Bartonella* spp. is most effectively gained with a One Health approach.

With this background, the research presented here describes the epidemiologic landscape of *Bartonella* spp. exposure in dogs, characterizing potential risk factors for exposure, and describing possible transmission scenarios. Additionally, the clinical research studies included here evaluated one potential disease manifestation in dogs, and another in people, among the many currently proposed for investigation.

Epidemiology and Modeling *Bartonella* in a One Health Context

by
Erin Lashnits

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APPROVED BY:

Edward Breitschwerdt Committee Chair	Cristina Lanzas
Ricardo Maggi	Andrew Stringer

DEDICATION

To Mrs. Fisher, my fourth grade teacher, who taught me the importance of a well-researched report; and to Mr. Corsilia, my ninth grade English teacher, who taught me how to write an essay.

BIOGRAPHY

Erin Lashnits is a member of the Intracellular Pathogens Research Laboratory at NCSU CVM, which studies flea and tick-transmitted pathogens. The lab's current major focus is on *Bartonella* species, emerging zoonotic pathogens of One Health importance. Dr. Lashnits's current research involves epidemiology of *Bartonella* infection in a One Health context, specifically modeling *Bartonella* transmission as a zoonotic vector-borne disease at the interface of humans, companion animals, and the environment.

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CHAPTER 1

Introduction

As emerging zoonotic vector borne pathogens, members of the genus *Bartonella* are increasingly recognized as causes of disease in human and veterinary medicine. While human *Bartonella* species – *B. bacilliformis* in South America and *B. quintana* worldwide – have been recognized as the cause of disease for generations, the potential for zoonotic *Bartonella* species to cause disease in humans has only been realized over the past few decades, with the first report of *B. henselae* as a cause of cat scratch disease in 1990.[1] It has taken even longer for *Bartonella* spp. to be recognized as etiologic agents of infectious disease in veterinary medicine, with the first case of bartonellosis in a dog reported in 1995.[2]

Given this short history, transmission has been comparatively well-studied for the human-adapted *Bartonella* spp. (*B. quintana* and *B. bacilliformis*), but relatively little is still known about transmission of zoonotic *Bartonella* spp.[3] The domestic cat is the reservoir host for multiple species of zoonotic *Bartonella*, the most well-studied of which is *Bartonella henselae*, the etiologic agent of a syndrome historically termed "cat scratch disease" (CSD).[4,5] Bartonellosis has historically been defined by CSD, which includes a triad of a cat scratch or bite, fever, and regional lymphadenopathy. However, with the advent of increasingly sensitive molecular and microbiological tools with which to diagnose *Bartonella* spp. infection, there are increasing reports of atypical manifestations of bartonellosis (historically termed "atypical CSD"), including severe and persistent neurological and neuropsychological manifestations, endocarditis, and other non-specific signs of chronic illness.[6–8] The cat flea is the major vector for transmission of *B. henselae* among cats, but the exact role of the cat flea in the transmission of *B. henselae* to humans – and whether fleas act as mechanical or biological vectors – has not

been determined. Often in human infection with zoonotic *Bartonella* spp. the source of infection remains unknown or speculative, and some reports indicate potential for other epidemiologically important hosts and vectors that are not cats and fleas (particularly ticks). [8,9]

Further understanding of transmission and pathogenesis of *Bartonella* spp. is most effectively gained with a One Health approach. While investigating *Bartonella* infection in cats helps to elucidate transmission risk in ecological and epidemiological studies, as well as for individual human patients, accurately diagnosing dogs may help by providing a natural model system in which to study zoonotic *Bartonella* species. As has been seen with Lyme Disease and in the realm of toxicology, dogs may have similar exposures to their human companions, thereby potentially serving as a sentinel for emerging infectious diseases affecting both species.[10–12] In addition, as an incidental hosts for *B. henselae* and other cat- or wildlife-adapted *Bartonella* spp., dogs and humans appear to exhibit similar disease manifestations and immunological responses, enhancing the dog as a naturally occurring model for human bartonellosis.[13,14] Similar zoonotic *Bartonella* spp. to those that infect humans also infect pet dogs, and it has been suggested that dogs may serve not only as a sentinel of human exposure risk, but also as a source of infection for human cases of bartonellosis.[15,16] Investigating *Bartonella* spp. infection in both cats and dogs is, therefore, of paramount importance to informing our understanding of human *Bartonella* spp. transmission and infection and ultimately protecting human health.

With this background, the research presented here aimed to describe the epidemiologic landscape of *Bartonella* spp. exposure in dogs, characterize potential risk factors for exposure, and describe possible transmission scenarios. Additionally, the clinical research studies included here evaluated one potential disease manifestation in dogs, and another in people, among the many currently proposed for investigation.

CHAPTER 2

Descriptive epidemiology of *Bartonella* exposure in dogs

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Bartonella Seroepidemiology in Dogs from North America, 2008–2014

E. Lashnits, M. Correa, B.C. Hegarty, A. Birkenheuer, and E.B. Breitschwerdt

Background: Improved understanding of *Bartonella* species seroepidemiology in dogs may aid clinical decision making and enhance current understanding of naturally occurring arthropod vector transmission of this pathogen.

Objectives: To identify demographic groups in which *Bartonella* exposure may be more likely, describe spatiotemporal variations in *Bartonella* seroreactivity, and examine co-exposures to other canine vector-borne diseases (CVBD).

Animals: A total of 15,451 serology specimens from dogs in North America were submitted to the North Carolina State University, College of Veterinary Medicine Vector Borne Disease Diagnostic Laboratory between January 1, 2008, and December 31, 2014.

Methods: *Bartonella henselae*, *Bartonella koehlerae*, and *Bartonella vinsonii* subspecies *berkhoffii* indirect fluorescent antibody (IFA) serology results, as well as results from a commercial assay kit screening for *Dirofilaria immitis* antigen and *Ehrlichia* species, *Anaplasma phagocytophilum*, and *Borrelia burgdorferi* antibodies, and *Ehrlichia canis*, *Babesia canis*, *Babesia gibsoni*, and *Rickettsia* species IFA results were reviewed retrospectively.

Results: Overall, 3.26% of dogs were *Bartonella* spp. seroreactive; *B. henselae* (2.13%) and *B. koehlerae* (2.39%) were detected more frequently than *B. vinsonii* subsp. *berkhoffii* (1.42%, $P < 0.0001$). Intact males had higher seroreactivity (5.04%) than neutered males (2.87%, $P < 0.0001$) or intact or spayed females (3.22%, $P = 0.0003$). Mixed breed dogs had higher seroreactivity (4.45%) than purebred dogs (3.02%, $P = 0.0002$). There was no trend in seasonal seroreactivity; geographic patterns supported broad distribution of exposure, and co-exposure with other CVBD was common.

Conclusions and Clinical Importance: *Bartonella* spp. exposure was documented throughout North America and at any time of year. Male intact dogs, mixed breed dogs, and dogs exposed to other CVBD have higher seroreactivity to multiple *Bartonella* species.

Key words: Canine; Seroreactivity; Zoonoses.

Members of the genus *Bartonella*, fastidious gram-negative rod-shaped hemotropic and endotheliotropic bacteria, are important emerging pathogens in dogs and humans worldwide.[1–3] For the past 2 decades, an increasingly diverse number of *Bartonella* species have been isolated or detected using PCR in a wide range of animals including cats, dogs, and humans, as well as many wildlife reservoir and arthropod vector species.[4] *Bartonella* persists in erythrocytes and vascular endothelial cells, causing chronic relapsing bacteremia.[2,5–8]

Abbreviations:

CI	confidence interval
CVBD	canine vector-borne diseases
IFA	immunofluorescent antibody
OR	odds ratio
VBDDL	Vector Borne Disease Diagnostic Laboratory

Worldwide, domestic dogs can be infected with at least 10 *Bartonella* species.[3,9] *Bartonella vinsonii* subsp. *berkhoffii*, *B. henselae*, and *B. koehlerae* represent the most frequent species found infecting dogs in North America.[10] All 3 of these species have been implicated as pathogenic in cases of endocarditis in dogs[7,11–13] and have been associated with other clinical abnormalities in dogs including vasoproliferative diseases, vasculitis, myocarditis, polyarthritis, granulomatous disease (lymphadenitis, rhinitis, hepatitis), epistaxis, and neurologic diseases.[14–23] However, because they are emerging pathogens in dogs, the spectrum of diseases associated with *Bartonella* infection has not been fully elucidated.

Bartonella species are primarily arthropod vector transmitted.[4,24,25] A wide variety of *Bartonella* species have coevolved with their specific vertebrate reservoirs hosts, among which transmission occurs via the arthropod vectors that typically infest these reservoirs (eg, cats are the primary reservoir host for *B. henselae* and *B. henselae* is transmitted between cats by the cat flea *Ctenocephalides felis*).[2,4,24] To date, no definitive vector has been identified for natural transmission of *Bartonella* to dogs. However, on the basis of case reports,[4,19,26–28] serosurveys,[29–36] surveys of arthropod vectors,[37–41] and experimental data (Lappin and Breitschwerdt, unpublished data),[42–44] ticks (including *Ixodes*

From the Department of Clinical Sciences and the Intracellular Pathogens Research Laboratory, Institute for Comparative Medicine, College of Veterinary Medicine, North Carolina State University, Raleigh, NC (Lashnits, Correa, Hegarty, Birkenheuer, Breitschwerdt).

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Corresponding author: E. Breitschwerdt, College of Veterinary Medicine, North Carolina State University, 1060 William Moore Dr., Raleigh, NC 27607; e-mail: ed_breitschwerdt@ncsu.edu.

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spp. and *Rhipicephalus sanguineus*), and fleas (*C. felis* and *Ctenocephalides canis*) have been proposed as potential vectors for *Bartonella* spp. transmission in dogs.

To date, a limited number of *Bartonella* seroepidemiologic studies have been performed involving large numbers of dogs from different regions of North America. *Bartonella* seroepidemiologic studies can provide important information about temporospatial distribution, disease prevalence, and potentially may help elucidate modes of transmission. Regional and seasonal differences in *Bartonella* spp. seroreactivity, as well as associations with other vector-borne pathogens across dog populations, can indirectly implicate potential arthropod vectors. In addition, seroreactivity data can guide clinical decision making. For example, coinfection with multiple vector-borne pathogens can cause more severe manifestations of disease, and determining exposure to *Bartonella* in dogs suspected of other CVBD is warranted.[45,46]

To better understand the epidemiology and distribution of *Bartonella* infection in dogs in North America, we analyzed a large diagnostic laboratory database. The purpose of our study was to identify *Bartonella* seroreactivity differences among demographic groups, describe variations in temporal and geographic patterns of *Bartonella* seroreactivity, and examine co-exposure between *Bartonella* and other vector-borne pathogens. Improved understanding of seroepidemiologic patterns may aid clinical decision making, as well as increase our understanding of transmission by arthropod vectors in naturally infected dogs.

Materials and Methods

Canine serum samples submitted to the North Carolina State University, College of Veterinary Medicine, Vector Borne Disease Diagnostic Laboratory (VBDDL), over a 7-year period between January 1, 2008, and December 31, 2014, were selected for study. Samples originated from veterinary hospitals in North America for diagnostic immunofluorescent antibody (IFA) testing for *Bartonella* and other vector-borne diseases. Available patient information included date of sample collection, date of sample receipt, signalment, and veterinary practice location. Test results were retrospectively reviewed, and the extracted data were analyzed. This a convenience sample given that the NCSU VBDDL is 1 of several laboratories where canine *Bartonella* serology samples can be submitted in North America. Samples were excluded if a sample from the same dog was submitted within the prior 5 weeks, to exclude convalescent samples.

Serum samples included in the study were submitted by the attending clinician to the VBDDL for individual serologic tests for ≥ 1 *Bartonella* spp., or for a comprehensive vector-borne pathogen serology panel. The VBDDL is not informed as to the motivation for testing, and thus, this information was not available in the data. Between January 2008 and July 2011, only *B. henselae* and *B. vinsonii* subsp. *berkhoffii* were used as antigens for IFA testing. After July 2011, the serology panel was amended to include *B. koehlerae*. Before July 2012, comprehensive panels included a SNAP 4Dx; starting in July 2012, this was changed to a SNAP 4Dx PLUS[a] test. Other antigens included in comprehensive serology panels for dogs included *Ehrlichia canis*, *Babesia canis*, *Babesia gibsoni*, and *Rickettsia* species. A subset of samples also was submitted for *Bartonella* alpha proteobacteria growth medium (BAPGM) culture enrichment and polymerase chain reaction, performed as previously described.[47]

All IFA antigens were grown in vitro at the VBDDL. *Bartonella* strains were isolated from naturally infected cats or dogs with species characterizations made using PCR amplification and DNA sequence analysis techniques. A canine isolate of *B. vinsonii* subsp. *berkhoffii* genotype I (NCSU 93CO-01, ATCC type strain #51672) and feline isolates of *B. henselae* H-1 strain (NCSU 93FO-23) and *B. koehlerae* (NCSU 09FO-01) were passed from agar plate grown cultures into a *Bartonella*-permissive cell line, DH82 cells (a canine monocytoid cell line) to obtain antigens for IFA testing; the same isolates were used across all years of this study (2008–2014). For each antigen, heavily infected cell cultures were spotted onto 30-well teflon-coated slides, air-dried, acetone fixed, and stored frozen. Serum samples diluted in phosphate-buffered saline solution containing normal goat serum, Tween-20, and powdered nonfat dry milk to block nonspecific antigen binding sites were screened at dilutions of 1:16 to 1:64. All sera that were reactive at a titer of 1:64 were further tested with 2-fold dilutions out to 1:8,192. Fluorescein-conjugated goat anti-dog IgG was used to visualize bacteria within cells using a fluorescent microscope. To avoid confusion with possible nonspecific binding found at low dilutions, a cutoff of 1:64 was used to define a seroreactive titer.

Regions were based on address provided with sample submission and defined by US census region as follows: Pacific—WA, OR, CA; Mountain—ID, NV, MT, WY, UT, CO, AZ, NM; West North Central—ND, SD, NE, KS, MN, IA, MO; West South Central—OK, AR, TX, LA; East North Central—WI, IL, IN, OH, MI; East South Central—KY, TN, MS, AL; South Atlantic—MD, DE, WV, VA, NC, SC, GA, FL; Middle Atlantic—NY, PA, NJ; New England—ME, NH, VT, MA, CT. Dogs from AK and HI (n = 8) were not included in these regions. Canada was considered as 1 region. Breed groups were defined using AKC breeds; breeds that are not considered by the AKC were grouped with mixed breed dogs. Seasonality was based on month: autumn: September, October, and November; winter: December, January, and February; spring: March, April, and May and; summer: June, July, and August.

Descriptive statistics were obtained, and seroreactivity to each *Bartonella* species was compared for different demographic, regional, and chronologic variables using the chi-square test. Logistic regression was used to identify univariate associations between *Bartonella* seroreactivity and selected comparison groups. Possible effects on the odds ratios (ORs) of the low event per variable were checked using the Firth adjustment, also known as the penalization approach.[48] ORs and 95% confidence intervals (CIs) for the ORs were estimated. Maps were created using ArcGIS.[b] Boundaries were created from publicly available data from the US Census Bureau[49] and Statistics Canada,[50] using the North American Datum (NAD) 1983 geographic coordinate system with Geodetic Reference System (GRS) 1980 spheroid. For each *Bartonella* spp., the minimum number of samples needed to detect a single positive sample was calculated based on the overall seroreactivity for that species in North America. States were excluded from seroreactivity maps if the number of samples submitted from a state was lower than the minimum number calculated above. Data analysis was performed using SAS/STAT software[c] and OpenEpi.[d] Statistical significance was considered at a P value of ≤0.05.

Results

Over 7 years, from 2008 through 2014, 15,451 individual canine serum samples from 15,295 dogs were submitted to the VBDDL for *Bartonella* IFA serology as previously described. Of these, 14,935 dogs (96.7%) were tested for both *B. henselae* and *B. vinsonii* subsp.

berkhoffii antibodies; 4,517 dogs (29.2%) were tested for *B. henselae*, *B. vinsonii* subsp. *berkhoffii*, and *B. koehlerae* antibodies. The highest number of samples originated from the South Atlantic region (6,548, 42.4%); the fewest samples came from the New England region (367, 2.4%). The region was not reported for 13 samples (0.08%). The largest number of samples was submitted in 2009 (2,581, 16.7%) and the smallest number in 2012 (1,780, 11.5%). The breeds most frequently represented in the study population included mixed breed dogs (2,608, 16.9%), Labrador Retrievers (1,603, 10.4%), and Golden Retrievers (858, 5.5%), with dogs from each remaining breed (188 breeds) making up <5% of the study population. Breed was not reported for 1 sample. Ages ranged from 4 weeks to 20 years, with a median of 6.0 years; the age was not reported for 642 dogs. There were 7,482 males (5,855 neutered, 78%) and 7,691 females (6,752 spayed, 88%). Sex was not reported for 278 samples (1.8%). Breed, sex, region, date of submissions, and seroreactivity are summarized in Table 1.

On the basis of IFA seroreactivity, 504 (3.26%) dogs were seroreactive to ≥1 *Bartonella* spp. Seroreactivity to *B. henselae* (2.13%) and *B. koehlerae* (2.39%) antigens was detected more frequently than seroreactivity to *B. vinsonii* subsp. *berkhoffii* (1.42%, $P < 0.0001$) antigen (Fig 1).

The youngest seroreactive dog was 4 weeks of age, and the oldest was 20 years of age, with a median age of 6 years. The median age for both seropositive and seronegative dogs was 6.0 years.

There was no statistically significant difference in overall seroreactivity based upon sex (248 seroreactive females and 250 seroreactive males). However, intact male dogs were more likely to be seroreactive (5.04%) than neutered males (2.87%; OR, 1.80; 95% CI, 1.37–2.35) or intact or spayed females (3.22%; OR, 1.59; 95% CI, 1.23–2.05; also see Table 2). When the

Table 1. Summary of samples submitted for *Bartonella* serology and number seroreactive to each antigen.

	Bh			*Bvb*			*Bk*			Any spp.	% Of
	Tested	*Bh*+	% *Bh*+	Tested	*Bvb*+	% *Bvb*+	Tested	*Bk* +	% *Bk* +	Tested	Total
All	15,017	320	2.1	15,365	218	1.4	4,521	108	2.4	15,451	—
Sex											
F	893	19	2.1	931	12	1.3	257	7	2.7	939	6.1
FS	6,597	144	2.2	6,715	90	1.3	1,999	49	2.5	6,752	43.7
M	1,556	47	3.0	1,612	45	2.8	398	9	2.3	1,627	10.5
MC	5,702	106	1.9	5,829	68	1.2	1,746	40	2.3	5,855	37.9
Breed											
Herding	1,747	39	2.2	1,784	38	2.1	518	15	2.9	1,797	11.6
Hound	1,663	41	2.5	1,697	25	1.5	483	8	1.7	1,714	11.1
Mixed	2,533	80	3.2	2,593	52	2.0	784	27	3.4	2,608	16.9
Nonsporting	1,035	20	1.9	1,060	13	1.2	305	2	0.7	1,067	6.9
Sporting	3,568	61	1.7	3,649	33	0.9	1,003	23	2.3	3,660	23.7
Terrier	1,245	19	1.5	1,270	15	1.2	420	14	3.3	1,276	8.3
Toy	1,541	24	1.6	1,581	9	0.6	494	6	1.2	1,591	10.3
Working	1,684	36	2.1	1,730	33	1.9	514	13	2.5	1,737	11.2
Region											
Canada	465	11	2.4	469	3	0.6	64	1	1.6	472	3.1
E. N. Central	2,051	42	2.0	2,059	19	0.9	489	8	1.6	2,063	13.4
E. S. Central	532	10	1.9	541	6	1.1	212	5	2.4	544	3.5
Mid-Atlantic	1,067	20	1.9	1,155	19	1.6	398	6	1.5	1,164	7.5
Mountain	627	10	1.6	657	12	1.8	129	5	3.9	661	4.3
New England	356	14	3.9	363	6	1.7	152	5	3.3	367	2.4
Pacific	521	15	2.9	612	11	1.8	210	5	2.4	619	4.0
S. Atlantic	6,421	116	1.8	6,508	77	1.2	1,827	37	2.0	6,548	42.4
W. N. Central	477	11	2.3	487	5	1.0	189	7	3.7	494	3.2
W. S. Central	2,487	71	2.9	2,501	60	2.4	844	29	3.4	2,506	16.2
Year											
2008	2,456	138	5.6	2,506	53	2.1	—	—	—	2,516	16.3
2009	2,460	50	2.0	2,556	52	2.0	—	—	—	2,581	16.7
2010	2,029	33	1.6	2,111	35	1.7	—	—	—	2,140	13.9
2011	1,987	13	0.7	2,064	18	0.9	51	1	2.0	2,076	13.4
2012	1,729	14	0.8	1,771	14	0.8	117	14	12.0	1,780	11.5
2013	1,920	29	1.5	1,921	20	1.0	1,917	60	3.1	1,921	12.4
2014	2,436	43	1.8	2,436	26	1.1	2,436	33	1.4	2,437	15.8
Month											
December–February	3,454	76	2.2	3,533	65	1.8	982	34	3.5	3,554	23.0
March–May	3,829	79	2.1	3,921	47	1.2	1,082	25	2.3	3,940	25.5
June–August	3,994	83	2.1	4,072	59	1.4	1,221	24	2.0	4,096	26.5
September–November	3,740	82	2.2	3,839	47	1.2	1,236	25	2.0	3,861	25.0

Bh, *B. henselae*; *Bvb*, *B. vinsonii* subsp. *berkhoffii*; *Bk*, *B. koehlerae*; Any, seroreactive to any 1 or more *Bartonella* spp.

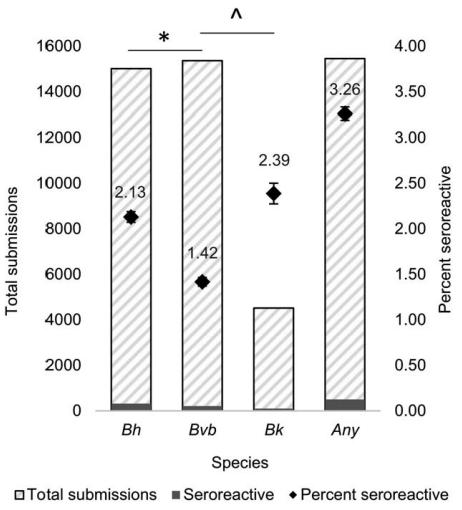

Fig. 1. *Bartonella* spp. seroreactive dogs, 2008–2014. *Bh*, *B. henselae*; *Bvb*, *B. vinsonii* subsp. *berkhoffii*; *Bk*, *B. koehlerae*; Any, positive to any one or more species. Numbers represent the percent of dogs seroreactive to each *Bartonella* species (right side scale). Error bars represent standard error for the percent of dogs seroreactive to each *Bartonella* species. Statistically significant differences ($P \leq 0.05$) between percent of dogs seroreactive to each species represented by * and ^.

proportion of dogs seroreactive to each species of *Bartonella* was determined using 2 × 2 tables, male intact dogs had higher seroreactivity than male neutered dogs or female intact or spayed dogs for both *B. henselae* and *B. vinsonii* subsp. *berkhoffii*, but not for *B. koehlerae*. There was no difference in seroreactivity between female intact and female spayed dogs, either in overall seroreactivity or when analyzed for each individual *Bartonella* species.

Mixed or non-AKC breed dogs were more likely to be seroreactive to any *Bartonella* spp. (4.45%) than were purebred dogs (3.02%; OR, 1.49; 95% CI, 1.21–1.85). When compared to mixed breed dogs, multiple categories of pure breed dogs were less likely to be *Bartonella* spp. seroreactive (Table 2). The actual ORs are presented in Table 2, given the negligible differences using the maximum likelihood estimates with logistic regression and logistic regression with the Firth bias reduction for the possible effect of low event per variable.

Overall proportions of seroreactive dogs by region are shown in Figure 2. For any *Bartonella* species, the highest proportions of seroreactive dogs in the study population were found in the New England, Pacific, and West South Central regions (5.18, 4.52, and 4.39%, respectively), whereas the lowest seroreactivity was found in the South Atlantic and East South Central regions (2.75 and 2.76%). *Bartonella henselae* had the highest proportion of seroreactive dogs in the New England region (3.93%), and lowest in the Mountain region

Table 2. Odds ratios for main effects based on logistic regression for seroreactivity to any of the 3 *Bartonella* spp. tested.

	OR	95% CI	*P* Value
Sex			
Versus MI			
F	0.64	0.42–0.98	0.0409*
FS	0.62	0.47–0.81	0.0004*
MC	0.55	0.42–0.73	<0.0001*
Breed			
Versus mixed			
Herding	0.78	0.5–1.06	0.1101
Hound	0.77	0.56–1.06	0.1082
Nonsporting	0.56	0.37–0.85	0.0065*
Sporting	0.53	0.40–0.70	<0.0001*
Terrier	0.64	0.44–0.93	0.0197*
Toy	0.45	0.30–0.66	<0.0001*
Working	0.74	0.54–1.02	0.0653
Region			
Versus S. Atlantic			
Canada	1.19	0.69–2.03	0.7554
E. N. Central	1.05	0.78–1.41	0.1350
E. S. Central	0.95	0.55–1.66	0.2416
Mid-Atlantic	1.11	0.77–1.60	0.3798
Mountain	1.27	0.81–2.00	0.9722
New England	2.03	1.24–3.30	0.0381*
Pacific	1.66	1.10–2.49	0.1662
W. N. Central	1.31	0.79–2.18	0.9145
W. S. Central	1.62	1.26–2.06	0.0338*

Season of submission did not contribute significantly to the model.

P values were obtained using analysis of maximum likelihood estimates and Wald chi-square test. Statistical significance indicated by * at $P \leq 0.05$.

(1.59%). *Bartonella vinsonii* subsp. *berkhoffii* had the highest proportion of seroreactive dogs in the West South Central region (2.4%) and lowest in Canada and East North Central regions (0.64 and 0.92%). *Bartonella koehlerae* had the highest proportion of seroreactive dogs in the Mountain and West North Central regions (3.88 and 3.7%) and lowest in the Middle Atlantic, Canada, and East North Central regions (1.51, 1.56, and 1.64%, respectively). Based on logistic regression, region was a significant factor for seroreactivity against any *Bartonella* spp. ($P = 0.0036$). With this model, dogs from the New England, Pacific, and West South Central regions were more likely than dogs from the South Atlantic region to be seroreactive against any of the 3 *Bartonella* spp. antigens (Table 2).

Seroreactivity varied by state and species (Fig 3). When states with low numbers of submissions were removed, state-by-state percentage seroreactive for *B. henselae* ranged from 0% (NM, 0/71) to 6.67% (Washington, 4/60), for *B. vinsonii* subsp. *berkhoffii* ranged from 0% (NM, 0/71 and IN, 0/300) to 3.8% (OK, 3/79), and for *B. koehlerae* ranged from 0% (VA, 0/171) to 6.59% (MO, 6/91).

Overall seroreactivity varied by year (Fig 4), with the highest overall seroreactivity in 2008 (6.92%), and lowest in 2011 (1.2%; OR, 6.095; 95% CI, 3.991–9.308). Seroreactivity was particularly high for *B. henselae* in 2008

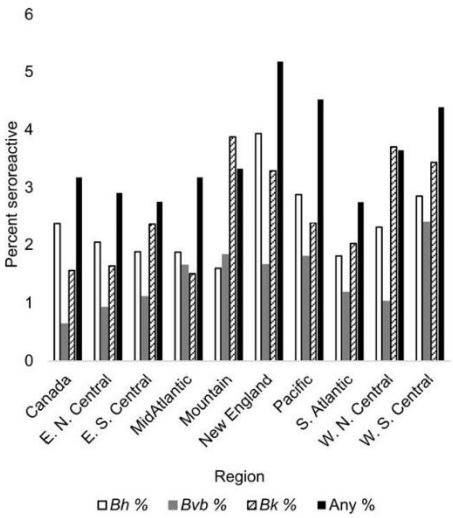

Fig. 2. *Bartonella* spp. seroreactivity by region. *Bh*, *B. henselae*; *Bvb*, *B. vinsonii* subsp. *berkhoffii*; *Bk*, *B. koehlerae*; Any, positive to any one or more species.

(5.62%) compared to 2011 and 2012 (0.65 and 0.81%), and only increased slightly again in 2013 and 2014 (1.51 and 1.77%). Similarly, *B. vinsonii* subsp. *berkhoffii* seroreactivity was highest in 2008 (2.11%), decreased to its lowest in 2011 and 2012 (0.87 and 0.79%), and increased slightly again in 2013 and 2014 (1.04 and 1.07%). *Bartonella koehlerae* serology was not offered before July 2011, but the highest annual seroreactive rate for *B. koehlerae* was in 2012 (11.97%), before it too decreased in 2013 and 2014 (3.13 and 1.35%). There was no significant trend in seroreactivity by month and no seasonal trend either for overall seroreactivity or seroreactivity to each of the *Bartonella* spp. (Fig 4). The highest overall seroreactivity was in June (4.85%) and the lowest in July (1.82%). Season did not contribute significantly to the logistic regression model.

Of dogs tested for *Bartonella*, 13,803 also had concomitant SNAP 4Dx or SNAP 4Dx PLUS testing performed, indicating 2.12% positive for *Anaplasma platys/phagocytophilum*, 4.59% positive for *B. burgdorferi*, and 5.36% positive for *E. canis/ewingii*. Odds ratios for coinfection between *Bartonella* and other vector-borne pathogens are presented in Table 3. Dogs that were *B. henselae* seroreactive had increased risk of being *E. canis* (by IFA), *E. canis/E. ewingii* (by SNAP test), *B. burgdorferi*, *A. platys*, *A. phagocytophilum*, *B. canis*, and *Rickettsia* spp. seroreactive. Dogs that were

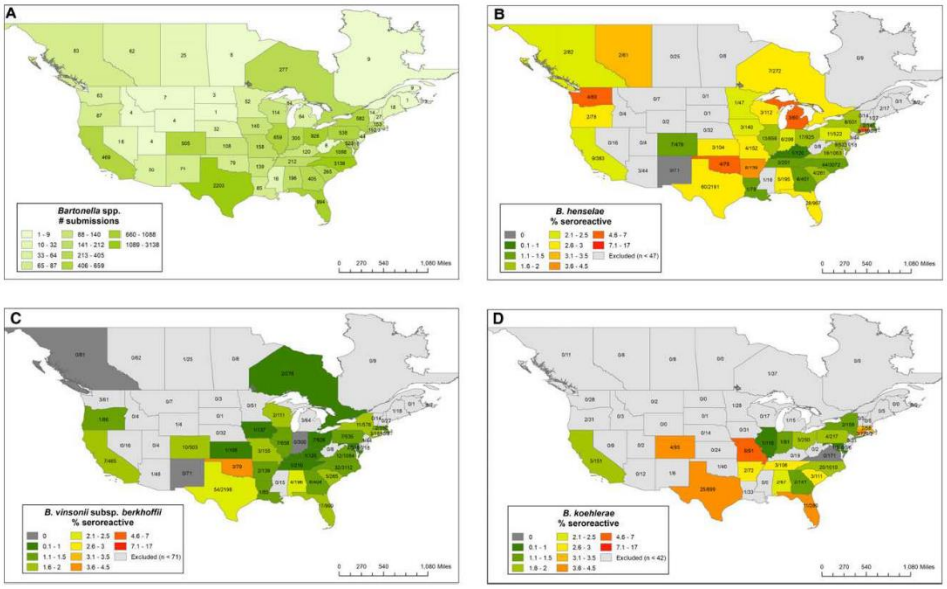

Fig. 3. (**A**) Map showing the total number of samples per state/province submitted for *Bartonella* spp. serology during the study period (2008–2014). (**B–D**) Maps of *Bartonella* spp. seroreactivity in North America. Colors depict the percent of dogs seroreactive for each species, ratios shown within each state or province show number of positive samples in the numerator and total number of samples in the denominator; states with low sample sizes are excluded (shown in gray). Alaska, Hawaii, and Canadian provinces for which no samples were submitted are not shown. (**B**) *B. henselae* seroreactivity. (**C**) *B. vinsonii* subsp. *berkhoffii* seroreactivity. (**D**) *B. koehlerae* seroreactivity.

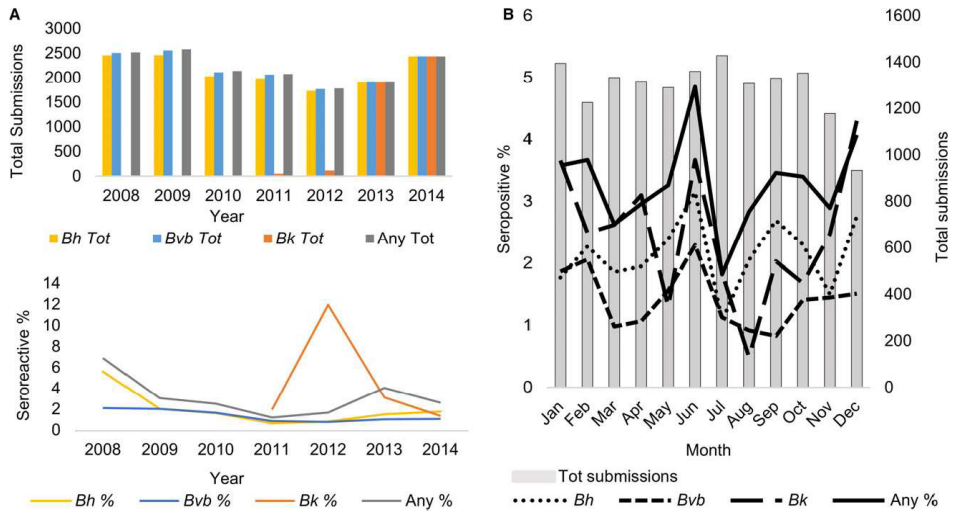

Fig. 4. Annual and monthly trends in *Bartonella* spp. seroreactivity. *Bh*, *B. henselae*; *Bvb*, *B. vinsonii* subsp. *berkhoffii*; *Bk*, *B. koehlerae*; Any, positive to any one or more species. (**A**) Trends by year. Top panel shows total submissions by year; bottom panel shows percent seroreactive by year. *B. koehlerae* was added to the comprehensive serology panel in July 2011. (**B**) Trends by month.

B. vinsonii berkhoffii seroreactive had increased risk of being *E. canis* (by IFA), *E. canis*/*E. ewingii* (by SNAP test), *B. burgdorferi*, *Dirofilaria immitis*, *B. canis*, and *Rickettsia* spp. seroreactive. Dogs that were *B. koehlerae* seroreactive had increased risk of being *E. canis* (by IFA), *E. canis*/*E. ewingii* (by SNAP test), *D. immitis*, and *Rickettsia* spp. seroreactive. All 34 *B. gibsoni* seroreactive dogs were *Bartonella* spp. seronegative.

Coinfections with different *Bartonella* spp. were common (Fig 5). Of 4,517 dogs tested for all 3 *Bartonella* spp., 159 (3.52%) were seroreactive to ≥1 species. The majority of these seroreactive dogs was seroreactive to *B. koehlerae* alone (67/159, 42%) or *B. henselae* alone (33/159, 21%), but 23 (14%) were seroreactive to all 3 *Bartonella* spp. antigens. Very few dogs were seroreactive to *B. vinsonii* subsp. *berkhoffii* alone (7/159, 4%). Dogs that were *B. vinsonii* subsp. *berkhoffii* or *B. koehlerae* seroreactive had an increased likelihood of being *Bartonella* PCR or *Bartonella* alpha proteobacteria growth medium (BAPGM) culture positive compared to dogs seronegative for those *Bartonella* spp. (OR, 5.72; 95% CI, 1.67–19.60; $P = 0.0017$ and OR, 18.69; 95% CI, 5.65–61.86; $P < 0.0001$, respectively). However, *B. henselae* seroreactive dogs were no more likely than *B. henselae* seronegative dogs to be *Bartonella* PCR or BAPGM culture positive (OR, 2.44; 95% CI, 0.57–10.45; $P = 0.2139$).

Discussion

Overall, 3.26% of dogs in our study were *Bartonella* spp. seroreactive, a percentage that is comparable to seroreactivity patterns for other CVBDs among US canine population-wide serosurveys.[5,34,51,52] For comparison, based on the Companion Animal Parasite Council publicly available data for 2014 (the final year of our study), the seroprevalence for the contiguous United States, of *B. burgdorferi*, ehrlichiosis, and anaplasmosis was 6.35, 3.01, and 2.97%, respectively (https://www.capcvet.org/parasite-prevalence-maps). Seroreactivity to *B. henselae* (2.13%) or *B. koehlerae* (2.39%) antigen was detected significantly more frequently than seroreactivity to *B. vinsonii* subsp. *berkhoffii* (1.42%) antigen. Although it was previously thought that *B. vinsonii* subsp. *berkhoffii* was the most common *Bartonella* to infect dogs, recent evidence from 2 studies,[9,30] as well as the results presented here, refutes that assumption.

In our study, male intact dogs had significantly higher seroreactivity (5.04%) than either female dogs (3.22%) or male neutered dogs (2.87%). Male intact status previously has been reported as a high risk category for heartworm disease in dogs.[5,53,54] Mechanistically, lifestyle or socioeconomic factors, rather than a biologic phenomenon, is considered the most likely reason for male intact status as a marker of heartworm disease risk. Additionally, mixed or non-AKC registered breed dogs were more likely to be *Bartonella* spp. seroreactive (4.45%) than purebred dogs (3.02%). It is unknown what underlies either of these risk factors for *Bartonella* infection, and further studies are warranted to investigate confounding factors.

Geographic patterns of seroreactivity did not correspond with other regional CVBD patterns (https://www.capcvet.org/parasite-prevalence-maps). In contrast to previous studies,[30,32] no *Bartonella* species was found

Table 3. Co-exposure between *Bartonella* spp. and other CVBD pathogens.

	OR	95% CI	*P* Value
B. henselae			
Lyme SNAP	2.44	1.59–3.76	<0.0001*
Anaplasma SNAP	2.58	1.42–4.66	0.0012*
Ehrlichia SNAP	1.68	1.05–2.67	0.0277*
E. canis IFA	3.34	2.31–4.85	<0.0001*
Babesia canis IFA	3.93	1.70–9.06	0.0005*
Rickettsia IFA	4.38	3.23–5.93	<0.0001*
HW SNAP	1.36	0.33–5.55	0.6694
B. vinsonii subsp. *Berkhoffii*			
Lyme SNAP	2.42	1.36–4.33	0.002*
Anaplasma SNAP	1.52	0.56–4.15	0.4067
Ehrlichia SNAP	2.79	1.67–4.68	<0.0001*
E. canis IFA	6.00	3.96–9.10	<0.0001*
Babesia canis IFA	5.94	2.37–14.86	<0.0001*
Rickettsia IFA	5.78	3.95–8.47	<0.0001*
HW SNAP	3.90	1.22–12.50	0.0135*
B. koehlerae			
Lyme SNAP	1.95	0.83–4.55	0.1171
Anaplasma SNAP	2.44	0.87–6.84	0.0787
Ehrlichia SNAP	2.33	1.27–4.27	0.0052*
E. canis IFA	3.45	1.84–6.50	<0.0001*
Babesia canis IFA	2.39	0.57–10.05	0.2196
Rickettsia IFA	2.72	1.57–4.71	0.0002*
HW SNAP	7.62	1.70–34.12	0.0018*
Any *Bartonella* spp.			
Lyme SNAP	2.42	1.69–3.46	<0.0001*
Anaplasma SNAP	2.00	1.16–3.46	0.0115*
Ehrlichia SNAP	1.97	1.37–2.83	0.0002*
E. canis IFA	3.31	2.43–4.51	<0.0001*
Babesia canis IFA	3.50	1.68–7.26	0.0003*
Rickettsia IFA	4.34	3.37–5.59	<0.0001*
HW SNAP	3.41	1.56–7.44	0.001*

OR, odds ratio.

ORs represent odds of seroreactivity to each CVBD for sample seroreactive to each *Bartonella* species antigen (or any *Bartonella* spp.), compared to samples not seroreactive to each *Bartonella* antigen (or any *Bartonella* spp.). ORs obtained using Cochran-Mantel-Haenszel test for categorical data.

Statistical significance indicated by * at $P \leq 0.05$.

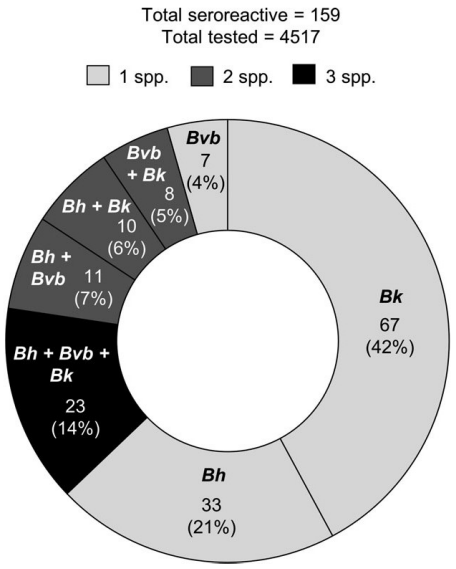

Fig. 5. Coinfection between *Bartonella* spp. *Bh, B. henselae*; *Bvb, B. vinsonii* subsp. *berkhoffii*; *Bk, B. koehlerae*. Numbers within each section show the number of dogs seroreactive to the particular combination of *Bartonella* species represented; percentages in parentheses show the proportion of dogs tested for all 3 *Bartonella* species that were seroreactive to that particular combination of species. Shades show the number of different species to which a dog was seroreactive.

to be most common in dogs from the Southeast or in warmer climates. Rather, seroreactivity was distributed broadly across the North American regions from which samples originated. The largest number of samples originated from the South Atlantic region (42% of samples), which was expected because of the location of the VBDDL in North Carolina. Extrapolations to underrepresented regions (Canada, Mountain and Pacific regions, New England, and areas of the Midwest) should be done with caution given the lower sample numbers from these regions (300–700 samples per region; see Table 1). However, even when excluding states with low sample numbers, there were states with apparently higher exposure that were different for each *Bartonella* species, including *B. henselae* in WA (4/56 seroreactive) and CT (9/141 seroreactive), and *B. koehlerae* in MO (6/91 seroreactive). Because of this finding, it appears important to evaluate each *Bartonella* spp. separately based on their disparate geographic patterns. Future studies using multivariate analysis or statistical modeling could integrate climate and land-use data to identify possible locations with higher *Bartonella* exposure. Clinicians should be aware that *Bartonella* infections in dogs can be seen throughout North America.

No seasonal trend in seroreactivity was found, with seroreactivity varying with no discernable pattern throughout the year. The lack of seasonality may reflect transmission by different vectors at various time points throughout the year, variability among individual dogs in the time required to seroconvert, the duration of infection at the time of testing, or other factors that were not examined in our study. However, if there is no seasonal trend for dogs acquiring *Bartonella* infection, exposure to ≥1 vector is equally likely to occur year-round. Clinicians should be aware that it is possible to detect *Bartonella* seroreactive dogs in North America during any season of the year.

The high risk for co-exposure with *Bartonella* and other vector-borne pathogens has been reported previously[14,26,27,31,33–36] and is consistent with the results of our study. In conjunction with male intact status, sequential or concurrent infection with another vector-borne pathogen may be a marker for lifestyle behaviors that influence a dog's risk of *Bartonella* exposure, including failure to effectively administer flea and

tick prevention products, outdoor exposure, ability to roam, and increased contact with reservoir hosts (eg, feral cats, wild canids such as coyotes, or their ectoparasites).[5,31,55,56] Co-exposure or coinfection with known tick-borne pathogens continues to support ticks as possible vectors for *Bartonella* transmission. As significant rates of coinfection were found for all *Bartonella* spp., and particularly for *B. vinsonii* subsp. *berkhoffii* and *B. henselae*, our data do not specifically implicate any single vector, but provide supportive evidence for many previously proposed vectors including *Rhipicephalus sanguineous*,[26,27,31,35,39,44] *Ixodes* spp,[28,33,36–38,40–43] *Dermacentor variabilis*/*andersonii*,[14,30,31,34,38] or *Amblyomma americanum*.[31,35] However, given the likelihood of CVBD co-exposures and coinfections,[57] screening for *Bartonella* infection should be considered in dogs infected with, or exposed to, other CVBD pathogens. This is particularly important in sick dogs, because treatment with doxycycline, which is indicated for several other vector-borne diseases, does not appear to be effective in eliminating *Bartonella* infection.[58] Thus, doxycycline treatment failure could lead to chronic illness or incomplete resolution of clinical signs or clinicopathologic abnormalities.[23,27,59]

Interestingly, *B. koehlerae* seroreactivity, unlike seroreactivity to *B. henselae* or *B. vinsonii* subsp. *berkhoffii*, was not significantly associated with either *Anaplasma* spp. or *B. burgdorferi* seroreactivity, 2 agents known to be transmitted by *Ixodes* ticks. Based on state-by-state seroreactivity rates, *B. koehlerae* exposure in dogs also appears to be more common in areas of the Rocky Mountains and Midwest where *Ixodes* ticks are uncommon, and less common in the Northeast and Middle Atlantic where *B. burgdorferi* transmission by *Ixodes scapularis* ticks is widespread. Based on this finding, studies focusing on vectors other than *Ixodes* spp. ticks should be considered for *B. koehlerae*. *Bartonella koehlerae* previously has been detected in cat fleas (*C. felis*),[6,60,61] and flea transmission should be considered for *B. koehlerae* in dogs as well.

Several limitations are inherent to retrospective seroprevalence studies. Although the motivation for submission of samples to the VBDDL is not specified on submission forms, typically most testing is performed diagnostically for sick dogs or when screening blood donors; therefore, our study sample does not represent a random sample from the general dog population in North America. The decision to submit a sample for testing may be biased by both owners and veterinarians, based on previous experience with or knowledge of *Bartonella*, as well as perception of vector-borne disease risk in certain locations or seasons. Whether testing was done to confirm a suspected clinical diagnosis, to rule out a possible underlying etiology for a clinical syndrome typically associated with *Bartonella* or another vector-borne disease, or to screen a healthy dog (eg, blood donors, military or other working dogs), is unknown. These samples, however, do not include experimental animals from research institutions, but rather diagnostic submissions only. Limited knowledge of, and access to, *Bartonella* serology testing by both dog owners and veterinarians may lead to dogs not being tested by serology for this emerging infectious disease. The population examined in our study may overestimate or underestimate the true prevalence of exposure in healthy or sick populations of dogs. Additionally, several laboratories across the country perform *Bartonella* serology testing, but we have no reason to believe that samples would be preferentially submitted to any particular laboratory for reasons related to likelihood of positive test, so this possibility likely contributes little bias to our sample.

In addition to sample submission bias, there are limitations inherent in using serology as a diagnostic test. Serology is the current gold standard for determination of exposure to *Bartonella* for both diagnostic and serosurvey purposes, but this modality has limitations.[6] Dogs experimentally infected with single species of *Bartonella* did not develop cross-reactive antibodies against other species,[62] but the extent to which serologic cross-reactivity versus co-exposure to multiple *Bartonella* species occurs in naturally infected dogs is unknown. Previous studies have shown poor associations between seroreactivity and bacteremia,[63,64] with antibody reactivity to *Bartonella* species antigens detected in ≤50% of dogs and humans in which active infection with *B. vinsonii* subsp. *berkhoffii* and *B. henselae* can be documented.[9,10] Therefore, IFA antibody testing lacks sensitivity, and, if detected, the presence of antibodies can only be used to infer prior exposure.[9,65] The seroreactivity data described our study could underestimate the true infection rate in a given population, and do not provide information on active or subclinical infection.

In summary, we report the largest North American retrospective seroepidemiologic study targeting 3 *Bartonella* species in dogs by IFA testing. The overall *B. henselae* and *B. koehlerae* seroreactivity for the dogs tested in our study was similar to that reported for other CVBD in population-wide serosurveys, whereas lower overall *B. vinsonii* subsp. *berkhoffii* seroreactivity was found. Dogs appear to be exposed to *Bartonella* spp. throughout most of North America, and seroreactivity can be detected at any time of year. Dogs exposed to other CVBD, male intact dogs, and mixed breed dogs are at higher risk for *Bartonella* exposure. Fleas and several tick species are proposed vectors for bartonellosis in dogs; our seroepidemiologic analyses suggest there may be multiple vectors or nonvectorial transmission for *Bartonella* infection in dogs, or that the primary vector may depend on local geographic, environmental, or reservoir host factors.

Footnotes

[a] Canine SNAP 4Dx or SNAP 4Dx PLUS, IDEXX Laboratories, Westbrook, ME

[b] ArcMap ArcMap v. 10.4.1, Environmental Systems Research Institute, Redlands, CA

[c] SAS v. 9.4, SAS Institute 2002–2012, SAS, Cary, NC
[d] Version 3.01, www.OpenEpi.com

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Conflict of Interest Declaration: Authors declare no conflict of interest.

Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

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CHAPTER 3

Modeling potential drivers of *Bartonella* exposure in dogs

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Ecological and Socioeconomic Factors Associated with *Bartonella henselae* Exposure in Dogs Tested for Vector-Borne Diseases in North Carolina

Erin W. Lashnits,[1] Daniel E. Dawson,[2] Edward Breitschwerdt,[3] and Cristina Lanzas[2]

Abstract

Bartonella henselae is a zoonotic vector-borne pathogen affecting both humans and dogs. Little is known about the epidemiology of *B. henselae* in dogs, including risk factors associated with exposure. The objectives of this study were to map the current distribution of *B. henselae* in dogs in North Carolina (NC) and to identify ecological and socioeconomic factors influencing *B. henselae* seroreactivity.

Results from 4446 *B. henselae* serology samples from dogs in NC submitted by veterinarians for clinical diagnostic testing to the North Carolina State University College of Veterinary Medicine Vector Borne Disease Diagnostic Laboratory between January 1, 2004 and December 31, 2015 were retrospectively reviewed. These results were used to generate a map of *B. henselae* seroreactivity. To account for sparsely sampled areas, statistical smoothing using head banging and areal interpolation kriging was performed. Using previously described risk factors for exposure to canine tick-borne diseases, eight multivariable logistic regression models based on biologically plausible hypotheses were tested, and a final model was selected using an Akaike's Information Criterion weighted-average approach.

Seroreactivity among dogs tested for vector-borne disease was variable across the state: higher along the southern/eastern coastal plains and eastern Piedmont, and lower in the western mountains. Of 25 explanatory factors considered, the model combining demographic, socioeconomic, climatic, and land use variables fits best. Based on this model, female intact sex and increasing percentage of the county with low-intensity development and evergreen forest were associated with higher seroreactivity. Conversely, moderate development, increasing median household income, and higher temperature range and relative humidity were associated with lower seroreactivity. This model could be improved, however, by including local and host-scale factors that may play a significant role in dogs' exposure.

Keywords: canine, seroreactivity, tick, flea, zoonoses, vector-borne

Background

Members of the bacterial genus *Bartonella* are important emerging pathogens in dogs and humans worldwide (Harms and Dehio 2012, Breitschwerdt et al. 2017). There are >30 named species, more than half of which have been associated with human and animal diseases (Breitschwerdt et al. 2014, 2017). One of the most common zoonotic species of *Bartonella* in humans and dogs is *Bartonella henselae*, the causative agent of human cat scratch disease (CSD) (Breitschwerdt et al. 2014, Regier et al. 2016, Lashnits et al. 2018). In humans, *B. henselae* is transmitted by inoculation of infected flea feces through the patient's skin through cat scratch (Zangwill 2013, Regier et al. 2016). Although the cat flea, *Ctenocephalides felis*, serves as the primary arthropod vector for transmission of *B. henselae* among the domestic cat reservoir, the primary vector for transmission to dogs is unknown (Billeter et al. 2008, Angelakis et al. 2010, Mosbacher et al. 2011). Ticks (primarily *Ixodes* spp., but also *Dermacentor* spp., *Amblyomma americanum*, and *Rhipicephalus sanguineus*) and fleas (*C. felis* and *Pulex* spp.) have been proposed as vectors for *B. henselae* in dogs based on case reports, serosurveys, surveys of arthropod vectors, and laboratory transmission studies investigating *Bartonella* spp.

[1]Comparative Biomedical Sciences Graduate Program, [2]Population Health and Pathobiology, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina.
[3]Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina.

transmission (Pappalardo et al. 1997, 2000, Breitschwerdt et al. 1998, Kordick et al. 1999, Chang et al. 2001, Honadel et al. 2001, Chang et al. 2002, Adelson et al. 2004, MacDonald et al. 2004, Morozova et al. 2004, Solano-Gallego et al. 2004, Henn et al. 2005, Holden et al. 2006, Foley et al. 2007, Wikswo et al. 2007, Billeter et al. 2008, 2012, Cotté et al. 2008, Reis et al. 2011, Yancey et al. 2014, Lashnits et al. 2018, Regier et al. 2017, Duplan et al. 2018). There is also evidence suggesting that occasionally human *B. henselae* infection may be due to tick transmission, including with reports of CSD, in patients with no reported cat contact, or with reported tick exposure or Lyme disease (Lucey et al. 1992, Zangwill et al. 1993, Arnez et al. 2003, Podsiadły et al. 2003, Breitschwerdt et al. 2007, Billeter et al. 2008, Angelakis et al. 2010, Rigaud et al. 2016, Donà et al. 2018).

Climatic conditions, geographical factors, and socioeconomic factors have been associated with the prevalence of tick-borne diseases in dogs, including *Anaplasma* spp. (McMahan et al. 2016), *Borrelia burgdorferi* (Watson et al. 2017), and *Ehrlichia* spp. (Liu et al. 2017). Although *B. burgdorferi* and *Ehrlichia* spp. have different tick vectors, modeling studies suggest higher exposure to either of these diseases in locations with lower population density and more forest (away from urban centers) (Liu et al. 2017, Watson et al. 2017). However, no such analysis for *Bartonella* exposure in dogs has been published.

The availability of a large amount of *Bartonella* serology data from a national diagnostic laboratory (North Carolina State University College of Veterinary Medicine Vector Borne Disease Diagnostic Laboratory [NCSU-VBDDL], North Carolina State University, NC) has previously allowed us to investigate trends across dog populations and over many years, to identify demographic and geographical risk factors associated with *Bartonella* spp. exposure in dogs (Solano-Gallego et al. 2004, Yancey et al. 2014, Lashnits et al. 2018). However, ecological and socioeconomic factors associated with *B. henselae* exposure in dogs have not previously been studied.

The goal of this study was, therefore, to provide further insight into ecological and socioeconomic factors associated with *B. henselae* exposure in dogs, using NCSU-VBDDL clinical diagnostic serology data from dogs residing in North Carolina (NC) and suspected of having one or more canine vector-borne diseases (CVBDs). NC is a logical choice to identify large-scale patterns of association between *Bartonella* exposure and ecological and socioeconomic factors, because it is a large and diverse state, with wide variation in these factors (see Supplementary Fig. S1), as well as having the largest number of sample submissions for *Bartonella* diagnostic testing each year.

The specific aims of this study were to map the current spatial distribution of *B. henselae* in dogs in NC and to characterize ecological and socioeconomic factors associated with *B. henselae* exposure based on NCSU-VBDDL serology data. We hypothesized that risk factors previously associated with vector-borne diseases of dogs, including climatic conditions, geographical factors, and societal factors, are associated with *B. henselae* exposure in dogs.

Materials and Methods

Study design, setting, and participants

We performed a retrospective cross-sectional observational analysis of dog blood samples submitted to the NCSU-VBDDL for *B. henselae* serology. The NCSU-VBDDL routinely tests sera for antibodies against *B. henselae* as an individual serological test or as a part of comprehensive panel that includes multiple *Bartonella* spp. as well as other CVBDs. Samples originate from veterinary hospitals and practices throughout North America.

In this study, we included samples from dogs submitted from either from the North Carolina State University Veterinary Hospital or other veterinary clinics located throughout NC between January 1, 2004, and December 31, 2015. If dogs had multiple tests submitted, only one test per year was included. If multiple samples were submitted within 1 year, samples were excluded after the first positive result. If no samples were positive, the chronologically first sample was chosen and the others excluded. Dogs enrolled with the NCSU Veterinary Hospital Blood Bank were identified by manual review of medical records, and excluded.

Data source for outcome variable

Samples were tested for *B. henselae* H-1 strain through immunofluorescent antibody (IFA) as previously described, using a cutoff of 1:64 to define a seroreactive titer (Hegarty et al. 2014).

Map creation

To characterize the spatial distribution of *B. henselae* in dogs tested for vector-borne disease in NC during the study period, a map of the average percentage of samples seroreactive over all sample years, for each county, was created using ArcGIS (ArcMap v. 10.4.1; Environmental Systems Research Institute [ESRI], Redlands, CA). Boundaries were created from publicly available data from the U.S. Census Bureau (United States Census Bureau 2017) and ESRI using the North American Datum 1983 geographic coordinate system with Geodetic Reference System 1980 spheroid.

Two smoothing techniques were applied to the empiric map. First, a weighted head-banging algorithm (NCI 2016) was used to reduce the influence of sparsely sampled counties (Wang et al. 2014, McMahan et al. 2016). Missing values were replaced with the average proportion of *B. henselae* seropositive dogs for adjacent counties with sampling. Parameters used were 6 nearest neighbors, 4 triples, 10 iterations, and 135 degrees angle. Second, to aid visualization, we smoothed the map into a continuously variable surface using areal interpolation kriging with baseline parameters in the geostatistical analyst extension of ArcGIS (Wang et al. 2014, McMahan et al. 2016). Maps for explanatory factors, averaged over all study years, were also created (Supplementary Fig. S1).

Data sources for explanatory variables

Patient information available from the NCSU-VBDDL included date of sample collection, signalment (age, breed, gender), and veterinary practice location. County of sample origin was assigned based on owner's zip code if available, or veterinary clinic location if not.

Previous studies have examined risk factors for exposure to CVBDs (Stich et al. 2014, Wang et al. 2014, McMahan et al. 2016). These factors were initially investigated for analysis, and included climate factors (annual temperature,

precipitation, and humidity); socioeconomic factors (median household income, population density, and estimate of number of dogs per county); and geographic factors (elevation and land cover). In addition, the presence or absence of *Ixodes* spp. ticks on a county-wide scale across the United States. was recently reported (Eisen et al. 2016a), and this presence/absence data were used as an additional factor. Year of sample submission was initially explored, but ultimately not included as an explanatory factor since it does not provide any mechanistic information about the underlying drivers of exposure. A list of considered factors and the publicly available data sources are provided in Table 1, and the range of values for these variables within NC is given in Table 2 and Supplementary Figure S1.

Detailed data collection and management information are available in Supplementary Data. All data management and analyses were performed in R 3.3.1 (R Core Team 2016).

Descriptive statistics

To better characterize differences between seroreactive and nonseroreactive dogs, descriptive statistics were obtained for gender, breed, and county-level tick reporting. Differences between seroreactive and nonseroreactive dogs were calculated using chi-squared tests.

Model development

To evaluate ecological and socioeconomic factors associated with *B. henselae* exposure, we followed a model selection approach in which we combined explanatory variables representing biologically plausible hypotheses (Johnson and Omland 2004). There were 25 explanatory variables initially considered, and a subset of these variables was included in each hypothesis-based model (Fig. 1). We first evaluated all pairwise correlations among the explanatory variables, using Pearson's correlation coefficients, to minimize statistical issues associated with collinearity. Combinations of excessively correlated explanatory variables were avoided in the hypothesis-based models.

The combinations of explanatory variables for each hypothesis-driven model are shown in Fig. 1. Dog gender was included in all hypothesis-based models, based on previous studies showing significant differences in *Bartonella* spp. exposure in different genders (Henn et al. 2005, Lashnits et al. 2018). The basis for the specific combinations of variables in each hypothesized model is as follows:

1. In model 1, we hypothesize that host factors, primarily dog demographics, are most important in explaining the variation in *B. henselae* exposure in dogs. Dog demographics include gender and breed, but not age, based on previous studies investigating demographics (Pappalardo et al. 1997, Honadel et al. 2001, Henn et al. 2005, Foley et al. 2007, Lashnits et al. 2018). Based on a recent and large-scale seroepidemiologic study of *Bartonella* spp. exposure in dogs (Lashnits et al. 2018), we hypothesize that the odds of exposure is highest in male intact mixed breed dogs.
2. In model 2, we explore the hypothesis that climatic factors alone are most important in explaining the variation in *B. henselae* exposure in dogs. The hypothesis for this model is that exposure is highest in areas with high relative humidity and in less extreme climates (lower temperature range).
3. In model 3, we consider the hypothesis that socioeconomic and development factors are most important in explaining the variation in *B. henselae* exposure in dogs. The hypothesis is that the highest seroreactivity is found where there is high median household income and high levels of development.
4. In model 4, we assume access to active farmland is most important in explaining the variation in *B. henselae* exposure in dogs. This model is based on a case–control study performed in the southeast United States in the 1990s, indicating that *Bartonella vinsonii* subsp. *berkhoffii* exposure was higher in dogs in rural environments, particularly on farms (Pappalardo et al. 1997). For this model, land covers including crops and pasture are tested, with the hypothesis that exposure is highest in counties with a high percentage of farmland.
5. In model 5, we assume that different types of forest cover are most important in explaining the variation in *B. henselae* exposure in dogs. In NC, the forest type follows an elevation gradient from the coastal eastern counties, which are predominantly evergreen forest, to the mountainous western counties, which are predominantly deciduous forest. A previous study of landscape risk factors for tick borne diseases of dogs in northern California found the highest seroprevalence for *Bartonella vinsonii* subsp. *berkhoffii* in evergreen forests (Foley et al. 2007). Based on this, the hypothesis is that exposure is highest in counties with large proportions of mixed or evergreen forest.
6. In model 6, we take into account multiple different land uses to explain the variation in *B. henselae* exposure in dogs. Including all land use categories produces excessive collinearity, particularly with all levels of development and all forest types. Therefore, high-intensity development and mixed forest were not included. The hypothesis is that the highest seroreactivity will be seen in areas with large percentage of forest, grass/shrub, and development.
7. In model 7, we include multiple categories of factors to explain the variation in *B. henselae* exposure in dogs. Particular types of land cover (forest and grass/shrub) as well as climate variables (temperature range and relative humidity) were included based on previous studies of factors important in predicting other CVBDs (Springer et al. 2015, Hahn et al. 2016, Alkishe et al. 2017, Eisen et al. 2018, Soucy et al. 2018). Whether *Ixodes* spp. ticks had been previously reported in each county was also included (Eisen et al. 2016a). The hypothesis is that exposure is highest in counties with established *Ixodes* spp. ticks, high percentage of land dedicated to forest or grass/shrub, and low temperature ranges with high relative humidity.
8. In model 8, we also include multiple categories of factors that may be associated with positive *B. henselae* serology, but we leave out the direct assessment of reported presence of *Ixodes* spp. ticks and instead include a measure of development. The hypothesis is that exposure is highest in counties with high percentage of land dedicated to forest or grass/shrub, low temperature ranges with high relative humidity, and high levels of development and income.

Table 1. Candidate Explanatory Variables. Candidate Factors with Abbreviations for Explanatory Variables Included in Models, Units, Spatial Resolution, and Data Sources

Category	*Factor*	*Abbreviations*	*Scale*	*Years*	*Source*
Demographic	Sex	SEX	Individual	—	NCSU-VBDDL
	Breed	BRD			
Climate	Mean, minimum, and maximum temperatures, temperature range (°F)	TR	County	Annual	PRISM Climate Group http://prism.oregonstate.edu
	Mean precipitation (inches)		County	Annual	PRISM Climate Group
	Mean dew point temperature (°F)		County	Annual	PRISM Climate Group
	Relative humidity	RH	County	Annual	Calculated from dew point and mean temperature
Geographic	Elevation (ft. above sea level)	ELEV	County	1986	North Carolina Geodetic Survey www.ncgs.state.nc.us
	Land cover (12 classes)	GS	30 meters	2006, 2011	National Land Cover Database www.mrlc.gov
	Grass and shrub	FOR			
	Forest	DEV			
	Development	WET			
	Wetland and water	CR			
	Crops	PST			
	Pasture				
Socioeconomic	Population density (persons/sq. mi)	PD	County	2009, 2010, 2015	U.S. Census Bureau, American Community Survey and 2010 Census www.socialexplorer.com
	Median household income ($)	INC	County	2009, 2010, 2015	U.S. Census Bureau, American Community Survey and 2010 Census
	Number of dogs		County	2009, 2010, 2015	U.S. Census Bureau, American Community Survey and 2010 Census U.S. Pet Ownership and Demographics Sourcebook (AVMA, 2012)
Tick vector presence	*Ixodes* spp. tick presence reported	TICK	County	1998, 2015	Eisen et al. 2016a

NCSU-VBDDL, North Carolina State University College of Veterinary Medicine Vector Borne Disease Diagnostic Laboratory.

TABLE 2. MEDIAN AND RANGE FOR COUNTY-LEVEL EXPLANATORY VARIABLES

Variable	*Abbreviation*	*Median*	*Range*
Climate			
Maximum annual temperature (°F)	MaxTemp	71.05	56.6–76.1
Mean annual temperature (°F)	MeanTemp	60.1	47.8–65
Minimum annual temperature (°F)	MinTemp	49	38–55.6
Mean annual dew point (°F)	DP	47	38.9–55.4
Annual precipitation (inches)	Precip	48.4	27.08–97.51
Temperature range (°F)	TempRange	22.2	15.1–28.6
Relative humidity (%)	MeanRH	63.40	51.40–75.71
Socioeconomic			
Population density (100 persons/sq. mi.)	PD	1.094	0.0858–18.904
Number of dogs (per county)	DogEst	13,751.6	986.7–258,254.5
Median household income ($1000/year)	Inc	39.642	27.487–67.309
Geographic			
Elevation (100 ft. above sea level)	Elev	4.35	0.01–35.82
Land use (% of county)			
Developed–high	DevHi	0.10	0–4.34
Developed–medium	DevMed	0.40	0.01–8.94
Developed–open+low	DevOpLow	7.53	1.27–49.34
Evergreen forest	EvFor	9.63	0.50–34.57
Deciduous forest	DecFor	28.95	0–84.01
Mixed forest	MixFor	2.00	0.02–7.44
Grass+shrub	GS	8.00	0.44–21.07
Pasture	Pst	7.09	0–38.04
Crops	Crop	1.30	0–46.27
Wetland+open water	Wet	4.62	0.12–92.86

Median and range over all 100 North Carolina counties for all study years (2004–2015). Land use type represented by the percentage of each county with specified land use type.

We used logistic regression to quantify the log odds of *B. henselae* exposure. The dependent variable was positive (vs. negative) *B. henselae* IFA sample.

Model selection and assessment

For each of the eight models, model p value was calculated based on ANOVA test compared with a null model, and goodness of fit (GOF) was assessed using the Hosmer–Lemeshow GOF test with the "Resource Selection" package (Subhash et al. 2017) and McFadden's Pseudo-R2 using the "pscl" package (Jackman 2017). Akaike's Information Criterion (AIC) was calculated for each model, and the relative importance of each model was assessed by assigning AIC weights (Anderson 2008) using the "MuMIn" package (Barton 2018). A ninth model, the final weighted model, was selected based on averaging the models within ΔAIC of 9 (Anderson 2008), allowing for evaluation of the relative support in the data for each model, and therefore quantitatively measure support for each model (Johnson and Omland 2004). Odds ratios (ORs) and 95% confidence intervals for the ORs were estimated for the final weighted-average model. Unless otherwise stated, $p \leq 0.05$ was considered statistically significant.

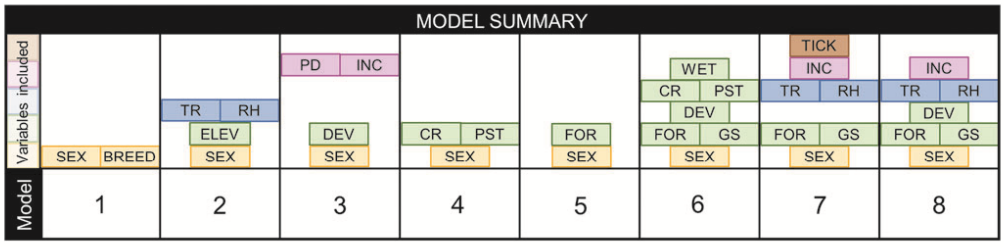

FIG. 1. Hypothesis model structures. Model summary, showing variables included in each hypothesis model. *Colored boxes* show individual explanatory variables included in each hypothesis-based model, with color based on the variable category. *Yellow*, demographic variable; *blue*, climate variables; *green*, geographic variables; *pink*, socioeconomic variables; *brown*, tick vector presence. BRD, breed group; CR, crops; DEV, developed land (open/low or moderate); ELEV, elevation; FOR, forest (evergreen, deciduous, or mixed); GS, grass and shrub; INC, median household income; PD, population density; PST, pasture; RH, relative humidity; TR, temperature range; TICK, *Ixodes* spp. ticks established, reported, or not reported; WET, wetland and open water.

Results

During the 12-year study period, there were 4446 blood samples tested for *B. henselae*, comprising 4343 unique dogs (demographic characteristics available in Supplementary Table S1). There were samples submitted from 88 counties (out of 100 counties in NC). Within a given year, the number of sampled counties ranged from 35 counties in 2007 to 59 counties in 2004. The counties from which the highest number of samples was submitted included Wake, Durham, and Mecklenburg; samples from these three counties made up 55% of the sample size. The largest number of samples (583, 13.1%) was submitted in 2015, the smallest number (137, 3.1%) in 2007 (Fig. 2).

There were 136 dogs (3.1%) that had serological evidence of *B. henselae* exposure. Test results by county of origin are shown in Fig. 3 (top panel). The smoothed map showing estimated percentages of dogs with seroreactivity to *B. henselae* across NC over the entire study period, based on headbanging and areal interpolation kriging, is shown in Fig. 3 (bottom panel). There are areas of higher seroreactivity on the coast as well as through the middle of the state, with areas of lower seroreactivity in the western part of the state and through the middle of the coastal plains.

Female intact dogs had higher seroreactivity (5.5%) compared with the other genders (male castrated 1.9%, $p=0.0002$; male intact 2.0%, $p=0.148$; female spayed 2.3%, $p=0.0324$). There were no statistically significant differences in seroreactivity when compared between American Kennel Club (AKC) breed groups, or when comparing specific breeds that made up >5% of the samples. There was higher seroreactivity in counties with *Ixodes* spp. ticks reported (4.35%) than in counties with *Ixodes* spp. ticks established or not reported (2.69% and 2.53%, respectively; $p=0.0225$). Seroreactivity was highest in 2004 (12.5%), and otherwise ranged from 0% in 2012 to 4.9% in 2005; overall annual seroreactivity is shown in Fig. 2.

The most informative model was model 8, which included as explanatory factors dog gender, median household income, relative humidity, temperature range, and percentage of land with evergreen forest, grass/shrub, open or low-intensity development, and moderate intensity development. This model had the lowest AIC (1152.7) and an AIC weight of 0.9 (Table 3), with a McFadden pseudo-R2 of 0.072 (where a value of ≥ 0.2 indicates an excellent fit) (McFadden 1979). The Hosmer–Lemeshow GOF test had a nonsignificant p value ($p=0.2998$), indicating that this model appropriately fit the data. The next best model was model 7, with an AIC weight of 0.08. Models 1 and 3–6 had ΔAIC >9, and,

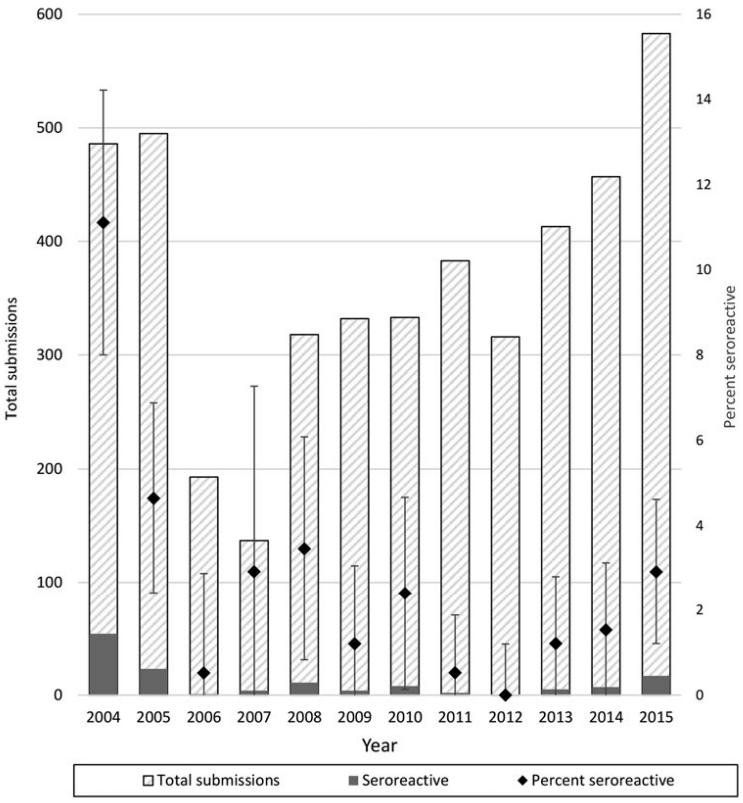

FIG. 2. *Bartonella henselae* seroreactivity by year during the study period. Total IFA submissions on left axis; positive samples in *solid gray*, negative samples in *striped gray*. Percentage of samples seroreactive per year on right axis; error bars represent 95% confidence intervals for the estimated proportions, and are cut off at 0%. IFA, immunofluorescent antibody.

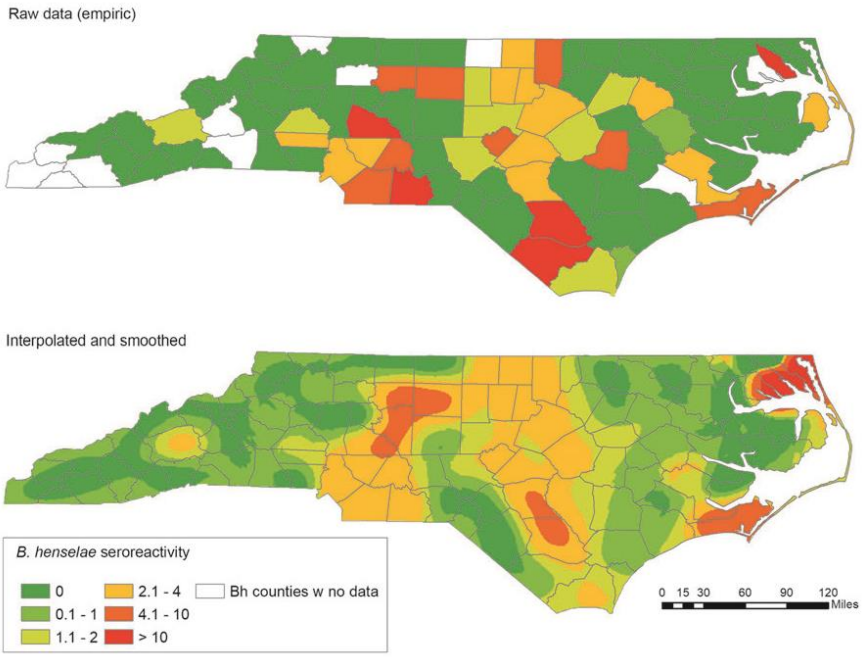

FIG. 3. Map of *B. henselae* seroreactivity in dogs. *Top panel* shows the empiric map (raw data), with the percentage of seroreactive dogs in each county. *Bottom panel* shows the smoothed and interpolated map, with estimated percentage of seroreactive dogs.

therefore, did not contribute to the AIC weighted average. Results of the initial eight hypothesis-based models are shown in Table 4. The weighted-average model (Table 5) showed that female intact or unknown gender status and increasing percentage land cover with open or low-intensity development or evergreen forest were independently associated with increased log odds of *B. henselae* exposure. Conversely, increasing percentage of moderate intensity developed land, increasing median household income, increasing temperature range, and increasing relative humidity were independently associated with decreased log odds of *B. henselae* exposure.

Discussion

This study provides a statistical modeling approach to understanding *B. henselae* exposure in dogs suspected of vector-borne disease across NC. There was variable seroreactivity across the state, with areas of apparent higher exposure along the coastal counties in the east, in the southern coastal plains counties, and in the eastern Piedmont counties. There was lower seroreactivity in the western mountain counties. Of the initial hypotheses for associations between explanatory variables and seroreactivity, the data provided the most support for a combination of patient demographic

TABLE 3. AKAIKE'S INFORMATION CRITERION WEIGHTED MODEL AVERAGE

Model	*Rank*	*DF*	*logLik*	*AICc*	*ΔAIC*	*Weight*	*Pseudo R2*
8	1	12	−564.29	1152.66	0	0.9	0.072
7	2	11	−567.69	1157.43	4.78	0.08	0.067
2	3	9	−571.5	1161.05	8.39	0.01	0.060
3	4	8	−580.76	1177.54	24.89	0.00	0.045
6	5	13	−576.67	1179.42	26.76	0.00	0.052
4	6	7	−583.03	1180.08	27.42	0.00	0.041
5	7	8	−582.92	1181.87	29.21	0.00	0.041
1	8	14	−578.65	1185.4	32.74	0.00	0.048

Models listed in descending order of AIC.
AIC, Akaike's Information Criterion.

Table 4. Hypothesis-Based Model Results

	Estimate	*Standard error*	p	*OR*	*95% CI*
Model 8					
(Intercept)	16.3458	4.5227	0.0003*		
Gender					
MI	0.1690	0.3800	0.6565	1.18	0.54–2.42
FS	0.2524	0.2577	0.3273	1.29	0.78–2.16
FI	1.2709	0.3292	0.0001*	3.56	1.84–6.75
Unk	1.0531	0.2742	0.0001*	2.87	1.68–4.96
DevOpLow	0.0832	0.0318	0.0089*	1.09	1.02–1.16
DevMed	–0.3939	0.1616	0.0148*	0.67	0.49–0.92
Inc	–0.0391	0.0128	0.0022*	0.96	0.94–0.99
EvFor	0.0969	0.0320	0.0025*	1.10	1.03–1.17
GS	–0.0751	0.0459	0.1018	0.93	0.85–1.01
MeanRH	–0.1328	0.0439	0.0025*	0.88	0.8–0.95
TempRange	–0.5219	0.1019	<0.0001*	0.59	0.49–0.72
Model 7					
(Intercept)	18.5113	4.4983	<0.0001*		
Gender					
MI	0.1226	0.3794	0.7466	1.15	0.52–2.34
FS	0.2371	0.2569	0.3561	1.27	0.77–2.12
FI	1.1914	0.3278	0.0003*	3.33	1.72–6.29
Unk	1.2795	0.2608	<0.0001*	3.46	2.07–5.89
EvFor	0.0942	0.0318	0.0030*	1.11	1.04–1.19
GS	–0.0923	0.0464	0.0465*	0.91	0.82–0.99
MeanRH	–0.1667	0.0422	0.0001*	0.85	0.78–0.93
TempRange	–0.5047	0.1021	<0.0001*	0.61	0.50–0.74
Inc	–0.0362	0.0117	0.0019*	0.97	0.94–0.99
Tick status					
Not reported	–0.0409	0.3844	0.9153	0.96	0.43–1.97
Reported	0.1961	0.26344	0.4566	1.22	0.72–2.03
Model 2					
(Intercept)	15.6572	4.1363	0.0002*		
Gender					
MI	0.1416	0.3795	0.7092	1.15	0.52–2.35
FS	0.2450	0.2568	0.3401	1.28	0.78–2.14
FI	1.2598	0.3250	0.0001*	3.52	1.83–6.62
Unk	1.3705	0.2591	<0.0001*	3.94	2.39–6.62
TempRange	–0.4099	0.0899	<0.0001*	0.66	0.56–0.79
MeanRH	–0.1535	0.0403	0.0001*	0.86	0.79–0.93
Inc	–0.0229	0.0102	0.0246*	0.98	0.96–1.00
Elev	–0.0320	0.0261	0.2197	0.97	0.91–1.01
Model 3					
(Intercept)	–2.7989	0.7075	0.0001*		
Gender					
MI	0.0554	0.3767	0.8832	1.06	0.01–0.24
FS	0.2298	0.2560	0.3693	1.26	0.48–2.15
FI	1.1275	0.3198	0.0004*	3.09	0.77–2.10
Unk	1.3848	0.2653	<0.0001*	3.99	1.62–5.73
Inc	–0.0269	0.0153	0.0789	0.97	2.39–6.79
DevMed	–0.5035	0.2472	0.0417*	0.60	0.94–1.00
PopDens	0.2689	0.1304	0.0392*	1.31	0.37–0.96

(*continued*)

TABLE 4. (CONTINUED)

	Estimate	*Standard error*	p	*OR*	*95% CI*
Model 6					
(Intercept)	–4.8029	7.0339	0.4947		
Gender					
MI	0.1279	0.3775	0.7348	1.14	0.52–2.31
FS	0.2485	0.2562	0.3321	1.28	0.78–2.14
FI	1.2050	0.3235	0.0002*	3.34	1.74–6.24
Unk	1.1748	0.2790	<0.0001*	3.24	1.89–5.65
DevMed	–0.5380	0.2148	0.0123*	0.58	0.38–0.89
DevOpLow	0.1081	0.0772	0.1614	1.11	0.96–1.29
DecFor	–0.0128	0.0736	0.8622	0.99	0.85–1.14
EvFor	0.0501	0.0912	0.5832	1.05	0.88–1.25
Crop	0.0070	0.0738	0.9241	1.01	0.87–1.16
Pst	0.0250	0.0731	0.7327	1.03	0.89–1.18
GS	–0.0661	0.0785	0.3999	0.94	0.80–1.09
Wet	0.0111	0.0702	0.8741	1.01	0.88–1.16
Model 4					
(Intercept)	–4.0361	0.2500	<0.0001*		
Gender					
MI	0.0691	0.3759	0.8542	1.07	0.49–2.17
FS	0.2355	0.2558	0.3573	1.27	0.77–2.11
FI	1.1232	0.3190	0.0004*	3.07	1.62–5.70
Unk	1.5154	0.2552	<0.0001*	4.55	2.78–7.60
Crop	0.0006	0.0107	0.9526	1.00	0.98–1.02
Pst	0.0065	0.0123	0.5983	1.01	0.98–1.03
Model 5					
(Intercept)	–4.0828	0.3569	<0.0001*		
Gender					
MI	0.0625	0.3760	0.8680	1.06	0.49–2.16
FS	0.2364	0.2558	0.3555	1.27	0.77–2.11
FI	1.1139	0.3198	0.0005*	3.05	1.60–5.66
Unk	1.5359	0.2588	<0.0001*	4.65	2.82–7.80
EvFor	0.0145	0.0215	0.5001	1.01	0.97–1.06
DecFor	0.0010	0.0080	0.9005	1.00	0.98–1.02
MixFor	–0.0277	0.0591	0.6391	0.97	0.87–1.09
Model 1					
(Intercept)	–3.7057	0.2846	<0.0001*		
Gender					
MI	0.0465	0.3769	0.9018	1.05	0.48–2.13
FS	0.2466	0.2563	0.3361	1.28	0.78–2.14
FI	1.1385	0.3197	0.0004*	3.12	1.64–5.80
Unk	1.4410	0.2583	<0.0001*	4.22	2.56–7.09
Breed group					
Herding	–0.3751	0.3351	0.2629	0.69	0.35–1.32
Hound	–0.1409	0.3414	0.6799	0.87	0.44–1.68
Non AKC	0.1477	0.7598	0.8459	1.16	0.18–4.16
Nonsporting	–0.6257	0.4397	0.1547	0.53	0.21–1.21
Sporting	–0.0570	0.2686	0.8319	0.94	0.56–1.62
Terrier	–0.9167	0.5021	0.0679	0.40	0.13–0.99
Toy	–0.6664	0.4205	0.1130	0.51	0.21–1.13
Working	–0.2937	0.3412	0.3895	0.75	0.37–1.44
Unk	0.2217	0.6507	0.7333	1.25	0.28–3.93

Logistic regression models based on biologically plausible hypotheses for factors driving differences in *Bartonella henselae* exposure in dogs, with *B. henselae* seroreactivity as dependent variable. Models are listed in ranked order based on AIC. Baseline sex male castrated; baseline breed mixed; baseline tick status established. Models listed in descending order of AIC. Statistical significance considered at $p < 0.05$ for individual factors (indicated by *).

AKC, American Kennel Club; CI, confidence interval; OR, odds ratio; Unk, gender/breed not recorded.

TABLE 5. AKAIKE'S INFORMATION CRITERION WEIGHTED-AVERAGE MODEL RESULTS

Variable	*Estimate*	*Standard error*	p	*OR*	*95% CI*
Gender					
MI	0.1673	0.3801	0.6600	1.18	0.56–2.49
FS	0.2517	0.2576	0.3287	1.29	0.78–2.13
FI	1.2679	0.3294	0.0001*	3.55	1.86–6.78
Unk	1.0654	0.2786	0.0001*	2.9	1.68–5.01
EvFor	0.0959	0.0340	0.0048*	1.1	1.03–1.17
DevOpLow	0.0785	0.0363	0.0307*	1.09	1.02–1.16
DevMed	−0.3719	0.1813	0.0402*	0.67	0.49–0.93
Inc	−0.0386	0.0129	0.0028*	0.96	0.94–0.99
MeanRH	−0.1342	0.0442	0.0024*	0.87	0.8–0.95
TempRange	−0.5194	0.1027	0.0000*	0.59	0.49–0.73
Elev	−0.0005	0.0049	0.9260	0.97	0.92–1.02
GS	−0.0751	0.0468	0.1087	0.93	0.85–1.01
Tick status					
Not reported	−0.0017	0.0789	0.9828	0.96	0.45–2.04
Reported	0.0082	0.0666	0.9022	1.22	0.73–2.04

Estimates and standard error of slope, p value for each explanatory variable, and ORs with 95% CIs for explanatory variables included in AIC weighted-average logistic regression model.

*Statistical significance considered at $p < 0.05$ for individual factors.

Sex baseline MC, tick status baseline established; Unk, gender not recorded; EvFor, percentage of county with evergreen forest classification; DevOpLow, percentage of county with open or low-intensity development classification; DevMed, percentage of county with medium development classification; Inc, median household income/1000; MeanRH, mean relative humidity; TempRange, difference between annual average highest temperature and annual average lowest temperature (°F); Elev, county average elevation/100; GS, percentage of county with grass or shrub classification.

factors, owner socioeconomic factors, and climate and land use factors. This model could be improved, however, by including local and host-scale factors that may play a significant role in dogs' exposure. Unmeasured factors that may influence exposure include, among others, local effects of a dog's particular living environment; host factors including acaricide usage, immunocompromise or other comorbidities, or genetic susceptibility; and direct evidence for proposed arthropod vector abundance and activity including possible seasonal trends.

The likelihood of a positive IFA test is dependent on three basic categories of factors: vector presence, vector contact, and detection of exposure. As direct evidence for any of these three variables is lacking, indirect associations with socioeconomic and ecological variables were assessed in this study. Because of this, any interpretation of these findings with regard to their implication for vector transmission must be done with caution.

That said, these findings suggest that the variation in seroreactivity may reflect variation in exposure not only to fleas, the widely accepted vector for *B. henselae* transmission in cats and humans, but also potentially to ticks. Since climate and habitat are well known to play a key role in the prevalence and activity of many species of ticks, the model provides support for transmission through ticks on a population scale but does not specify a particular species of tick vector (Springer et al. 2015, Eisen et al. 2016b, Ogden and Lindsay 2016, Minigan et al. 2017).

Flea abundance depends on temperature and humidity, but the suitable climatic range is wide [temperatures between 37°F and 95°F, with relative humidity >33% (Traversa 2013)] and climate extremes sufficient to limit flea development are rarely found in NC based on our data. In addition, exposure to fleas, particularly *C. felis*, may be independent of climatic and habitat factors due to their ability to complete their entire lifecycle indoors (Gracia et al. 2008, Rust 2017). However, in some cases, flea infestation may have a seasonal component, and *C. felis* thrive in warm humid environments (Cruz-Vazquez et al. 2001, Gracia et al. 2008, Traversa 2013). This model does not, therefore, preclude the involvement of fleas (or other arthropod vectors) in transmission, but rather suggests that there is an additional more climate- and habitat-dependent route of transmission than fleas alone.

It is possible that the variation in seroreactivity reflects variation in exposure to both fleas and ticks, or even a nonvector-borne pathway of transmission. Future epidemiologic studies surveying the ectoparasites present on dogs and cats and investigating risk factors for vector exposure would help define the role of these potential vectors, and address the variables of both vector presence and vector contact.

In addition to highlighting the role of climate and habitat in *B. henselae* exposure, this model showed that some of the variability in exposure was due to patient gender. We hypothesized that male intact dogs would have highest *B. henselae* seroreactivity, but in this sample in fact female intact dogs had highest *B. henselae* seroreactivity. The explanation for gender differences in *Bartonella* spp. exposure remains controversial. Whether there is a biological component to being a female or intact dog that increases exposure, such as the possibility of sexual transmission of *B. henselae* or immunological differences in intact dogs, or whether being an intact female is a marker of another confounding lifestyle factor that increases exposure, such as living outdoors or lack of acaricide use, is unknown.

In a report of patients presented to a Pennsylvania teaching hospital, patient age, owner household income, and being neutered were associated with an increased likelihood of heartworm preventative compliance, but it is difficult to generalize these localized small-scale survey-based findings to wider scale or to use of flea and tick preventatives (Gates and Nolan 2010). However, gender differences in prevalence

of vector-borne disease have been previously found in the case of heartworm, with intact dogs more likely to have heartworm disease (Selby et al. 1980, Levy et al. 2007), so this result is in keeping with patterns of exposure for other CVBDs and infectious disease generally (Hoffman et al. 2013).

Finally, in this model as median household income increased, exposure to *B. henselae* decreased (in contrast to our hypothesis of a positive association between median household income and *B. henselae* seroreactivity). Thus, assuming that the knowledge of—and financial ability to test for—*Bartonella* spp. as pathogens in dogs is not associated with climatic or land-use variables, then the differences in seroreactivity across counties did not appear to be based solely on increased detection. This may be indicative of lifestyle factors in dogs residing in counties with lower average median household income, such as higher risk of contacting flea or tick vectors due to lower use of acaricides, or reduced access to veterinary care (Brown et al. 2012, LaVallee et al. 2017).

Counties with larger percentages of moderate development had lower *B. henselae* seroreactivity, and counties with larger percentages of low-level development or open developed space, or evergreen forest, had higher *B. henselae* seroreactivity. As defined by the NLCD, areas of moderate development mainly include buildings and impervious surfaces such as roads and sidewalks, in contrast to low-level development or open space, which most commonly includes large-lot single-family housing and vegetation such as parks or lawns (Homer et al. 2015).

Along with the possibility that lower income levels are associated with decreased detection, this pattern suggests a rural–urban gradient of exposure. For example, counties with the largest cities did not have the highest seroreactivity: Mecklenburg, containing the city of Charlotte, had an average seroreactivity of 3.2%, compared with the adjacent suburban to rural counties to the east, Union (4.2%) and Stanly (8.3%). However, this model also provided little support for an association between farms and *B. henselae* exposure in dogs on a statewide level, in contrast to a previous study showing increased exposure to *B. vinsonii* subsp. *berkhoffii* in dogs in rural environments or with access to farms (Pappalardo et al. 1997). In fact, no single variable explained the distribution of *B. henselae* exposure well, further highlighting the complexity of *B. henselae* disease ecology in dogs.

Limitations of this study include the limitations inherent in a retrospective serology study using a convenience sample. Although the motivation for submission of samples to the VBDDL is not specified on submission forms, typically most testing is performed diagnostically for sick dogs; therefore, our study sample does not represent a random sample from the general dog population in NC. The decision to submit a sample for testing may be biased by both owners and veterinarians, based on previous experience with or knowledge of *Bartonella*, as well as perception of vector-borne disease risk in certain locations or seasons. Whether testing was done to confirm a suspected clinical diagnosis, to rule out a possible underlying etiology for a clinical syndrome typically associated with *Bartonella* or another vector-borne disease, or to screen a healthy dog (*e.g.*, military or other working dogs), is unknown. These samples, however, do not include experimental animals from research institutions or blood donor dogs screened at NCSU, but rather diagnostic submissions only.

Limited knowledge of, and access to, *Bartonella* serology testing by both dog owners and veterinarians may lead to dogs not being tested by serology for this emerging infectious disease. The population examined in our study may overestimate or underestimate the true prevalence of exposure in healthy or sick populations of dogs. Because sampling was not uniformly distributed throughout the state, and there was scant data from rural counties and counties in the far western part of the state, extrapolations to these under-represented regions should be done with caution. However, even when excluding counties with low number of samples, there were areas with apparently higher exposure, including Granville (3/39 seroreactive), Wayne (3/45 seroreactive), and Mecklenburg (30/557 seroreactive), compared with areas with low exposure (Wilson county, 0/44 and Hanover, 2/187); these findings were confirmed with the smoothed map.

Travel histories for the dogs were not available, and it is possible that dogs in this sample were exposed to *B. henselae* in other locations besides their home county. Despite these limitations, the NCSU-VBDDL database provides one of the best sources for existing *Bartonella* spp. serology data in dogs to date. This study included data from >80% of the counties in the state and >4300 dogs, which is a fairly large and comprehensive sample for a retrospective study of this nature.

Although serology is the current gold standard for determination of exposure to *B. henselae* for both diagnostic and serosurvey purposes, this modality does have limitations (Perez et al. 2011, Brenner et al. 2012, Hegarty et al. 2014, Maggi et al. 2014). Previous studies have shown poor associations between seroreactivity and bacteremia (Brenner et al. 2012), with antibody reactivity to *Bartonella* species antigens detected in ≤ 50% of dogs in which active infection can be documented (Perez et al. 2011). Therefore, IFA antibody testing lacks sensitivity, and may underestimate the true prevalence of *B. henselae* exposure in dogs.

Finally, limitations are inherent in the statistical model itself. This model does not account for factors that are not routinely measured with publicly available data. Importantly, this model analyzed factors at the county level on an annual timescale, and there may be important drivers of exposure at smaller scales or seasonally that we were not able to assess (Robertson and Feick 2018). Household-level effects may drastically change exposure risk for dogs within similar environments, particularly when considering variation in acaricide use. Because of this, care must be taken in interpreting the results from this model, particularly at smaller spatial scales. Further studies should focus on methods to assess previously undefined factors, such as household-level risks for vector exposure, and different spatial scales.

Conclusions

In this study, we report a statistical model for *B. henselae* seroreactivity in dogs in NC, providing a better understanding of its endemic range and highlighting the importance of considering ecological factors when evaluating *B. henselae* exposure. The model with the best fit included demographic, socioeconomic, climatic, and landscape factors. The maps created herein may help inform public health and veterinary professionals in NC about *B. henselae* in their areas, and may suggest areas where humans are at increased risk for *B. henselae* exposure. Humans and dogs share environments

both indoors and outdoors, and are thus often exposed to similar vectors and vector-borne diseases. Indeed, if *B. henselae* in dogs shares similar ecology with that in people, it could be expected that seroreactivity in dogs may be correlated with exposure risk in humans. In the future, this model may be expanded to investigate transmission risk and explore alternative vectors for *B. henselae* in humans, used to evaluate possible consequences of ecological and socioeconomic changes to the range and prevalence of *B. henselae* in dogs, or expanded to wider geographic areas as serology data become available.

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Author Disclosure Statement

No competing financial interests exist.

Supplementary Material

Supplementary Data
Supplementary Figure S1
Supplementary Table S1

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Address correspondence to:
Erin W. Lashnits
Comparative Biomedical Sciences Graduate Program
College of Veterinary Medicine
North Carolina State University
1060 William Moore Drive
Raleigh, NC 27607

E-mail: ewlashni@ncsu.edu

CHAPTER 4

Bartonella outbreak investigation and transmission

Lashnits E, Neupane P, Maggi RG, Linder KE, Bradley JM, Balakrishnan N, Southern BL, McKeon GP, Chandrashekar R, Breitschwerdt EB. Detection of *Bartonella* spp. in dogs after infection with *Rickettsia rickettsii.* J Vet Intern Med. 2020 Jan;34(1):145-159. PMID: 31891215.

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STANDARD ARTICLE

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Detection of *Bartonella* spp. in dogs after infection with *Rickettsia rickettsii*

Erin Lashnits[1] | Pradeep Neupane[2] | Ricardo G. Maggi[2,3] | Keith E. Linder[4] | Julie M. Bradley[2] | Nandhakumar Balakrishnan[3] | Brittany L. Southern[5] | Gabriel P. McKeon[2,5] | Ramaswamy Chandrashekar[6] | Edward B. Breitschwerdt[2,3]

[1]Comparative Biomedical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina

[2]Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina

[3]Intracellular Pathogens Research Laboratory, Comparative Medicine Institute, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina

[4]Department of Population Health and Pathobiology, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina

[5]Laboratory Animal Resources, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina

[6]IDEXX Laboratories, Inc, Westbrook, Maine

Correspondence
Edward B. Breitschwerdt, College of Veterinary Medicine, North Carolina State University, 1060 William Moore Drive, Raleigh, NC 27607.
Email: ebbreits@ncsu.edu

Present address

Nandhakumar Balakrishnan, Department of Health and Human Services, Bureau of Laboratories, Division of Infectious Diseases, 3350 N Martin Luther King Jr Blvd, Lansing, MI 48906.

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Abstract

Background: Dynamics of infection by *Bartonella* and *Rickettsia* species, which are epidemiologically associated in dogs, have not been explored in a controlled setting.

Objectives: Describe an outbreak investigation of occult *Bartonella* spp. infection among a group of dogs, discovered after experimentally induced *Rickettsia rickettsii* (*Rr*) infection.

Animals: Six apparently healthy purpose-bred Beagles obtained from a commercial vendor.

Methods: Retrospective and prospective study. Dogs were serially tested for *Bartonella* spp. and *Rr* using serology, culture, and PCR, over 3 study phases: 3 months before inoculation with *Rr* (retrospective), 6 weeks after inoculation with *Rr* (retrospective), and 8 months of follow-up (prospective).

Results: Before *Rr* infection, 1 dog was *Bartonella henselae* (*Bh*) immunofluorescent antibody assay (IFA) seroreactive and 1 was *Rickettsia* spp. IFA seroreactive. After inoculation with *Rr*, all dogs developed mild Rocky Mountain spotted fever compatible with low-dose *Rr* infection, seroconverted to *Rickettsia* spp. within 4-11 days, and recovered within 1 week. When 1 dog developed ear tip vasculitis with intra-lesional *Bh*, an investigation of *Bartonella* spp. infection was undertaken. All dogs had seroconverted to 1-3 *Bartonella* spp. between 7 and 18 days after *Rr* inoculation.

Abbreviations: BAPGM ePCR, Bartonella alpha-proteobacteria growth medium enrichment PCR; *Bh* SA2, *Bartonella henselae* San Antonio 2 strain; *Bh*, *Bartonella henselae*; *Bk*, *Bartonella koehlerae*; *Bvb I*, *Bartonella vinsonii* subspecies *berkhoffii* genotype I; *Bvb*, *Bartonella vinsonii* subspecies *berkhoffii*; CVBD, canine vector-borne diseases; IFA, immunofluorescent antibody assay; ITS, internal transcribed spacer; LAR, Laboratory Animal Resources; NCSU-VBDDL, North Carolina State University College of Veterinary Medicine Vector Borne Diseases Diagnostic Laboratory; RMSF, Rocky Mountain spotted fever; *Rr*, *Rickettsia rickettsii*; SFGR, spotted fever group *Rickettsia* spp.

Between 4 and 8 months after *Rr* inoculation, *Bh* DNA was amplified from multiple tissues from 2 dogs, and *Bartonella vinsonii* subsp. *berkhoffii* (*Bvb*) DNA was amplified from 4 of 5 dogs' oral swabs.

Conclusions and Clinical Importance: Vector-borne disease exposure was demonstrated in research dogs from a commercial vendor. Despite limitations, our results support the possibilities of recrudescence of chronic subclinical *Bartonella* spp. infection after *Rr* infection and horizontal direct-contact transmission between dogs.

KEYWORDS

PCR, recrudescence, serology, transmission

1 | INTRODUCTION

The genus *Bartonella* consists of over 40 globally distributed species of alpha-proteobacteria, infecting a wide range of mammalian hosts including dogs.[1,2] Studies on *Bartonella* exposure in dogs have described an epidemiologic association between spotted fever group *Rickettsia* spp. (SFGR) and *Bartonella* species.[3-7] Based on infection of both fleas and ticks with *Bartonella* spp. and SFGR, it is assumed that the serologic association between these 2 pathogens represents exposure from coinfected vectors or sequential exposure to multiple infected vectors. As the dynamics of *Bartonella* spp. and SFGR seroreactivity in coexposed dogs have not previously been explored in a controlled setting, it is also possible that infection with *Rickettsia rickettsii* (*Rr*) could result in recrudescence of chronic subclinical *Bartonella* infection.

Vector transmission of different *Bartonella* species by sand flies, fleas, lice, ticks, and flies is reasonably well documented by laboratory and field studies[8-10]—and transmission by a variety of other vectors has been suspected—but defining a single natural vector for *Bartonella* transmission among dogs has proved difficult.[1,9-11] Nonvectorial routes of transmission of *Bartonella* spp. are also proposed. Being scratched by an infected, flea-infested cat—allowing inoculation of flea feces under the skin—is a well-known route of transmission for *Bartonella henselae* (*Bh*) to humans. Transmission of *Bartonella* spp. by needle stick and blood transfusion has been reported, demonstrating direct transmission via infected cells, blood, or interstitial fluid in the absence of passage through an arthropod vector.[12-16] There are also reports implicating transmission by bites or suggesting the possibility of viable *Bartonella* spp. bacteria in the mouth or saliva.[17-19] In Korea, *Bh* DNA was PCR-amplified from over 15% of pet canine saliva samples and almost 30% of toenail samples,[20] and in the United States 5 of 44 Golden Retrievers sampled had *Bartonella* spp. DNA on oral swabs.[21] *Bartonella henselae* DNA was found in the saliva of a man with angioedema of the tongue and in his healthy dog,[22] and in eastern China *Bartonella* exposure was associated with dog bites.[23] However, the extent to which saliva might be infectious has not been established and direct transmission among dogs has not been reported.

Despite the evidence of nonvectorial routes of transmission, in the absence of concurrent flea infestation, the risk of *Bartonella* transmission is currently considered minimal.[24,25] However, if transmission can occur directly between dogs—or from dogs to humans in the absence of vectors—this could be of substantial importance. Establishment of an experimental model of *Bartonella* spp. infection in non-reservoir hosts has thus far remained elusive,[26] so investigation of the potential for direct transmission of *Bartonella* spp. has been confined to epidemiologic associations and case reports.

The original study objective was to evaluate sequentially timed serological response to low-dose experimental *Rr* infection in laboratory-raised dogs. However, after completion of the *Rr* study, *Bh* DNA was detected in ear-tip vasculitis lesions in 1 dog. Subsequently, *Bartonella* spp. antibodies were documented in all dogs, either before or after the experimental *Rr* infection in a vector-free biocontainment facility. This unexpected circumstance provided an opportunity to investigate both the serologic response to coinfection with these 2 previously associated pathogens, as well as to investigate the potential for reactivation and non-vectorial transmission of *Bartonella* species. Therefore, the objective of this study was to describe an outbreak investigation of occult *Bartonella* spp. infection among a group of laboratory-reared dogs subsequent to experimentally induced *Rr* infection.

2 | METHODS

2.1 | Animals

The animals included in this study were 6 healthy purpose-bred laboratory-reared female Beagles age 6-12 months (to protect their identities, referred to here as *Shok*, *Kat*, *Tan*, *Cher*, *Pam*, and *Sax*). The dogs had received routine preventative care and vaccinations before arrival at the NCSU Laboratory Animal Resources (LAR) facility, including treatment with sulfamethoxazole (30 mg/kg PO daily) and fenbendazole (25 mg/kg PO daily) for 1 week for coccidiosis prior to transport. The dogs were reported by the vendor to be otherwise free from intestinal parasites; *Dirofilaria immitis* and *Brucella canis* testing were negative. The vendor's canine housing facility consists of indoor/outdoor concrete-floor runs. The vendor practices routine pest control for the facility environment, but study dogs were not treated with flea/tick preventatives while housed at the vendor. The study

was approved by the NCSU Institutional Animal Care and Use Committee (Protocol #16-206).

2.2 | Study timeline

The dogs were acquired from a commercial vendor and arrived at the NCSU LAR facility on December 19, 2016. The study timeline is divided into 3 phases: pre-inoculation (*PI phase*, December 19, 2016-March 19, 2017), before experimental intervention; *Rr* monitoring (*RM phase*, March 20, 2017-April 28, 2017), the approximately 6-week period after experimental inoculation with *Rr*, and extended follow up (*EF phase*, April 29, 2017-December 12, 2017) when the remaining dogs were housed in the LAR facility and evaluated as needed and based on test results. All dogs were routinely vaccinated with DAPP and Rabies vaccines on 6 July, 2017. One dog (*Tan*) was adopted on August 24, 2017. Due to concerns for zoonotic disease transmission, 4 dogs were transferred to other investigators for studies that required euthanasia (October 24, 2017-11 December, 2017). A gross necropsy was permitted for 1 of these 4 dogs (*Shok*, 11 December, 2017) but histopathology was not performed. One dog (*Kat*) remains a resident at NCSU LAR at the time of writing (approximately 2.5 years after arrival).

2.3 | Study setting

From the date of arrival on December 19, 2017 through April 28, 2017 (the PI and RM phases), all dogs were housed in individual runs with access restricted to LAR personnel and study investigators. Figure 1 shows a housing schematic. The run enclosures had solid concrete 4-ft walls. Metal chain-link fence extended from the wall tops to the ceiling. The front and back of each run was enclosed with chain-link fence, with front doors opening onto a common corridor run. Dogs were isolated from one another in these separated run enclosures, except during twice daily 5- to 10-minute periods when runs were being cleaned. While runs were cleaned, each dog had access to the common corridor; the fencing between the corridor and each dog's individual run allowed for nose-to-nose contact between a dog in the corridor and any other dog.

During the EF phase, dogs were moved to various locations within LAR (Figure 1B). Four dogs were housed as pairs (*Tan* and *Pam* together, and *Cher* and *Sax* together), and 2 dogs (*Shok* and *Kat*) were housed singly in the same room. During the EF phase only, all dogs were allowed short periods of outdoor access (5-20 minutes) as part of their daily enrichment activities in compliance with the IACUC protocol and welfare standards. During this period, dogs were allowed to interact directly during twice daily exercise periods for approximately 5-20 minutes, both in indoor common areas and an outdoor play area;

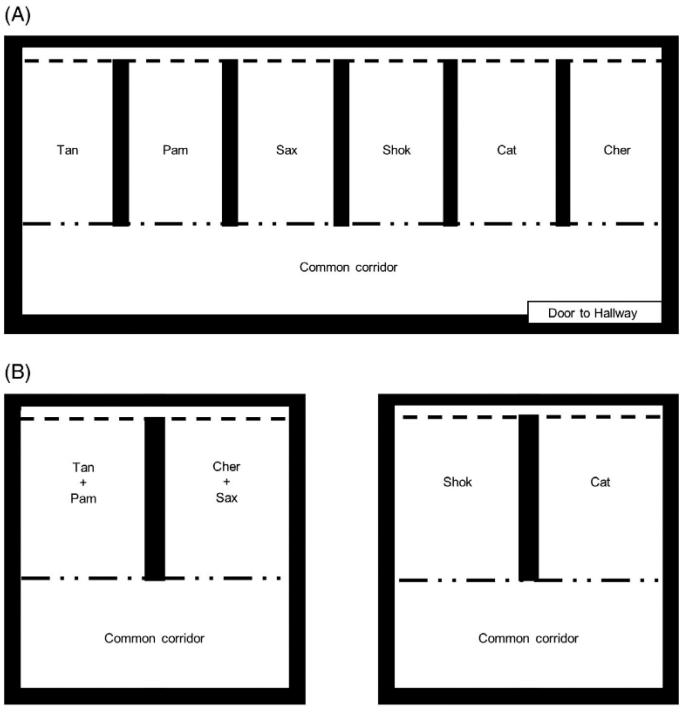

FIGURE 1 Schematic of dog housing in Laboratory Animal Resources. A, Housing during PI and RM phases. All dogs were housed in individual runs, with chain-link fence doors (broken dotted line) opening onto a common corridor run in the front, and chain-link fence (dashed line) in the rear. B, Housing during EF phase. Four dogs were housed as pairs, and 2 dogs were housed singly in the same room. Dashed line indicates chain-link fence, broken dotted line indicates chain-link door, thick black line indicates concrete walls and room borders. EF, extended follow up; PI, pre-inoculation; RM, *Rr* monitoring

detailed records of dog-dog interactions were not available for review from the EF phase, but it is possible that any dog could come into contact with any other dog during this time. During all study phases, dogs were observed at least twice daily by LAR staff. At no time was any arthropod or insect vector found on dogs or within the LAR biocontainment facility. During the RM phase, all personnel wore personal protective equipment including a laboratory coat or disposable coveralls, shoe covers/booties, and gloves.

2.4 | Rickettsia rickettsii experimental infection

According to the original objective of the study—to evaluate sequentially timed serological response to low-dose experimental *Rr* infection in dogs—each dog was inoculated with 3×10^5 $TCID_{50}$ (Median Tissue Culture Infectious Dose) of *Rr* via intradermal injection on March 20, 2017. Inoculum was prepared from frozen stocks of a canine *Rr* isolate derived from a clinical case of Rocky Mountain spotted fever (RMSF; NCSU-2008-CO4, "Murphy" strain).[27] The inoculum was prepared in the NCSU College of Veterinary Medicine Biosafety Level III Laboratory and the dose determined from previous experiments.[28,29]

To determine if the *Rr* cell culture inoculum used during the *Rr* experimental infection was contaminated with 1 or more *Bartonella* spp., DNA was extracted for PCR testing from the stored DH-82 *Rr* cell culture inoculum (see details in "Diagnostic methods" section). The inoculum was not cultured in BAPGM due to biosafety concerns involved with handling of this BSL-3 organism, as well as the presumed sensitivity of qPCR to amplify *Bartonella* spp. if concurrently growing in this cell line, since the DH82 cell line is also routinely used to grow *Bartonella* spp. intracellularly by the North Carolina State University College of Veterinary Medicine Vector Borne Diseases Diagnostic Laboratory (NCSU-VBDDL).

2.5 | Clinical monitoring

From arrival on December 19, 2017, through *Rr* inoculation on March 20, 2017 (PI phase), dogs were observed daily by LAR staff. After *Rr* inoculation and continuing for 35 days until April 28, 2017 (RM phase), dogs underwent daily observation as well as measurement of body temperature, pulse, and respiratory rate. Results were recorded by a veterinarian or veterinary staff member. After April 28, 2017 (EF phase), dogs were observed by LAR husbandry staff during the course of their daily care and examined by veterinary staff only if concerns were noted. A detailed timeline of diagnostic testing performed is included in the "Diagnostic sampling chronology" section. Complete blood counts were performed according to routine procedures by the NCSU Veterinary Hospital Clinical Pathology Laboratory.

2.6 | Diagnostic methods

Serum samples were tested using previously described indirect immunofluorescent antibody (IFA) assays, with results considered seroreactive at titer of 1:64 or greater.[30] Antibodies to 3 *Bartonella* species (*Bartonella henselae* San Antonio 2 strain [*Bh* SA2], *Bartonella vinsonii* subsp. *berkhoffii* genotype I [*Bvb I*], and *Bartonella koehlerae* [*Bk*]), as well as *Rr*, *Ehrlichia canis*, *Babesia canis*, *Babesia gibsoni*, and *Leishmania infantum* were assessed by IFA. *Rickettsia rickettsii* antibody titers were evaluated using 3 secondary antibodies: fluorescein isothiocyanate (FITC)-labeled goat anti-dog IgG (H + L), goat anti-dog IgG (gamma), and goat anti-dog IgM (mu) (KPL, Gaithersburg, Maryland).[30] This *Rr* IFA cannot distinguish between SFGR species due to the strong cross-reactivity within the group. *Bartonella* antibody titers were evaluated using 4 commercially available conjugates. The FITC-labeled Goat anti-Dog IgG (H + L) (Sigma-Aldrich, St. Louis, Missouri) conjugate is used for commercially available diagnostic testing of *Bartonella* spp. through the NCSU-VBDDL (referred to hereafter as "diagnostic IFA"). To elucidate serological response of different antibody isotypes over time, goat anti-dog IgG (gamma), and goat anti-dog IgM (mu) (KPL) were also used for IFA testing.[12,30] For manufacturer consistency with the isotype-specific conjugates, FITC-labeled goat anti-dog IgG (H + L) (KPL) was also used to test a subset of samples. Positive and negative control sera were tested concurrently with each IFA run. If results were equivocal or difficult to interpret, IFA was repeated on the same sample and the more conservative (lower) titer was reported.

A commercially available ELISA (4 DX Plus SNAP test, IDEXX Laboratories, Westbrook, Maine) was used to test for *Anaplasma phagocytophilum*, *Anaplasma platys*, *Borrelia burgdorferi*, *E canis*, and *Ehrlichia ewingii* antibodies and *Dirofilaria immitis* antigen.

Bartonella spp. bacteremia was assessed using enrichment blood culture with the *Bartonella* alpha proteobacteria growth medium (BAPGM) as previously described.[31] By using standard operating procedures, DNA was extracted from samples intended for *Bartonella* spp. PCR.[30] *Bartonella* spp. and strain classification was performed using primers designed to amplify 2 consensus sequences in the *Bartonella* 16S-23S internal transcribed spacer (ITS) region as described previously with minor modifications.[30,32-34] All amplicon products were commercially sequenced (Genewiz, Research Triangle Park, North Carolina) to determine the *Bartonella* sp. and strain type. DNA extracted from whole-blood and tissue samples was also used for *Rickettsia* genus-specific PCR as described previously.[35]

2.7 | Diagnostic sampling chronology

The diagnostic testing timeline is shown in Figure 2. Briefly, blood and serum specimens from all dogs were obtained at prespecified intervals during the PI and RM phases: 4 time points during the PI phase and 3 times weekly for 2 weeks then twice weekly for the subsequent 4 weeks during the RM phase. During the EF phase, samples were obtained at various time points for the dogs remaining in the study, at the discretion of the investigators.

Rr IFA serology was performed on serum from each dog at every time point blood was drawn during the PI and RM phases, and at 1 time point during the EF phase (July 20, 2017). One dog (*Kat*) also

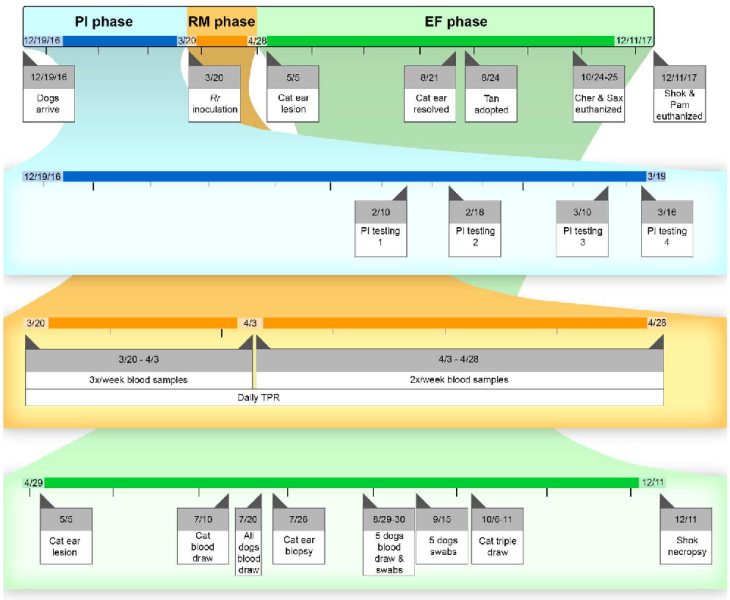

FIGURE 2 Study timeline. The overall timeline is shown on the top row, with the color indicating the study phase (blue = PI phase, orange = RM phase, green = EF phase). Black tick marks indicate 1 month, gray tick marks indicate 1 week. EF, extended follow up; PI, pre-inoculation; RM, *Rr* monitoring

had *Rr* IFA serology performed at multiple time points during the EF phase. *Rickettsia* spp. PCR was performed on whole blood from each dog at every time point blood was drawn during the PI and RM phases. One dog (*Kat*) also had *Rickettsia* spp. PCR performed once during the EF phase. *Bartonella* spp. IFA was performed on serum from each dog at every time point blood was drawn during all phases. *Bartonella* spp. PCR was performed on whole blood from all dogs at every time point blood was drawn during all phases. Bartonella alphaproteobacteria growth medium enrichment PCR (BAPGM ePCR) was performed on whole blood from all dogs at 1-5 time points during the EF phase only.

Immunofluorescent antibody assay serology for other canine vector-borne diseases (CVBDs) as described above was performed on serum from each dog at every time point blood was drawn during the PI phase only. The SNAP 4DX Plus was performed on serum from each dog at the first PI time point (10 February, 2017) and 1 EF time point (July 20, 2017). One dog (*Kat*) also had IFA serology for other CVBDs and SNAP 4DX Plus test performed multiple times during the EF phase. Complete blood counts were performed on whole blood from each dog at 1 time point during PI phase (March 16, 2017) and at each time point blood was drawn during RM phase.

During the EF phase, tissue samples were obtained from 2 dogs. One dog (*Kat*) had biopsies taken from skin lesions on the pinnae, as well as from normal skin on the abdomen. At the time of euthanasia, approximately 1 year after arrival (11 December, 2017), another dog (*Shok*) had postmortem samples taken from bone marrow, spleen, lung, and submandibular and mesenteric lymph nodes. To determine if body fluids could be a source of *Bartonella* spp. transmission in this setting, sterile swabs were used to collect samples from the periodontal surface, buccal mucus membranes, and vaginal vault from 5 dogs; oral swab samples were collected twice (August 30, 2017 and September 15, 2017), while vaginal swab samples were only collected on September 15, 2017. These tissues and swabs were all tested for *Rickettsia* and *Bartonella* spp. by PCR.

3 | RESULTS

All dogs were clinically healthy during the PI phase, with no reported problems from the LAR husbandry staff. All dogs had normal physical examinations and vital signs (body temperature, heart rate, respiration rate) on the day of *Rr* inoculation (March 20, 2017). After *Rr* inoculation, dogs exhibited a mild, short-duration systemic illness. All dogs had a mildly increased body temperature at various points between 2 and 7 days after inoculation (Figure 3A,B). One dog (*Pam*) also had an increased body temperature on April 10, 2017, and April 24, 2017 (3 and 5 weeks after inoculation). All dogs were clinically recovered based on physical examination and behavioral observation by 1 week after inoculation, without antibiotic administration.

During the EF phase, 5 of the 6 dogs remained clinically healthy. As reported,[36] about 1 week into the EF phase (May 5, 2017, 46 days after *Rr* inoculation), 1 dog (*Kat*) developed superficial skin lesions on the tip of the right pinna that resolved after about 1 week. Similar but more severe lesions recurred 3 times, and when the lesions persisted, biopsies were performed (July 20, 2017) and the dog was diagnosed with small vessel vasculitis and dermatitis. Photographs of these

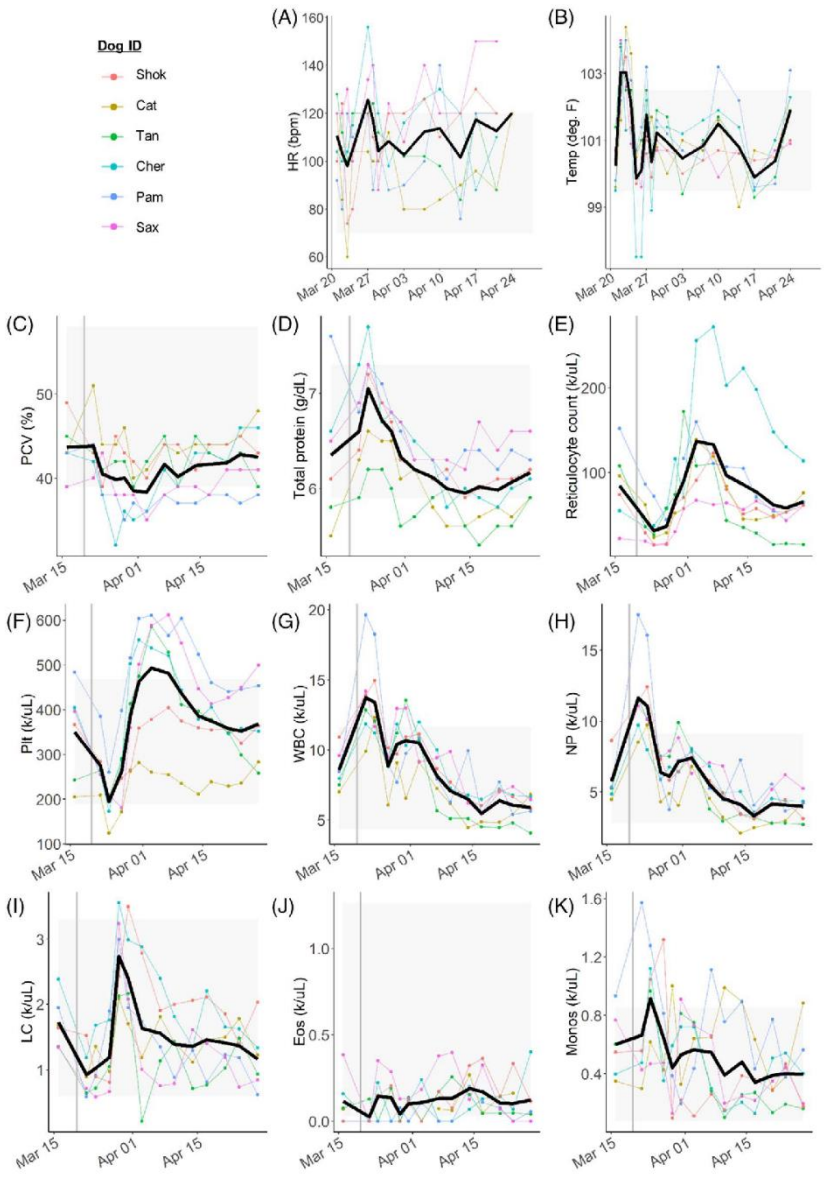

FIGURE 3 Spaghetti plots showing clinical and CBC results for all dogs. For all plots, the x-axis shows the testing date and the y-axis shows the value of each parameter. Vertical gray line represents the date of *Rr* inoculation. Gray boxes show reference ranges for each parameter. Black line represents the mean for all dogs. Colors correspond to each individual dog, shown in the top left. A, Heart rate. B, Body temperature. C-K, CBC parameters. Eos, absolute eosinophil count; HR, heart rate; LC, absolute lymphocyte count; Monos, absolute monocyte count; NP, absolute neutrophil count; PCV, packed cell volume; Plt, platelet count; Rr, *Rickettsia rickettsii*; Temp, body temperature; WBC, absolute white blood cell count

lesions and other pertinent details of the clinical case have been previously published.[36] *Bartonella henselae* DNA was amplified and sequenced, and *Bartonella* organisms were visualized by laser scanning confocal immunohistochemistry, from the aural margin biopsies.[36] After this diagnosis, the dog was treated with doxycycline 10 mg/kg PO every 12 hours and enrofloxacin 10 mg/kg PO every 24 hours for 6 weeks (August 22, 2017-October 3, 2017). The lesions improved and did not recur after this treatment, and the dog remains clinically healthy at the time of writing (approximately 2 years after onset of ear-tip vasculitis).

3.1 | Rickettsia infection

Rickettsia titers for each antibody isotype (IgG H + L, IgM, and IgG gamma) throughout the study (all phases) are shown in Figure 4. One dog was *Rr* seroreactive during the PI phase (*Kat*, February 10, 2017), with an IFA titer of 1:128; however, this dog was not *Rr* seroreactive at any of the 3 other PI phase time points or by IFA using IgM or IgG isotype-specific conjugates. The remaining 5 dogs were not *Rr* seroreactive during the PI phase using IgG H + L, IgM, or IgG gamma isotype-specific conjugates.

By using the diagnostic IgG H + L IFA, we found that all dogs seroconverted to *Rr* by post-inoculation day 11 (March 31, 2017, Table 1) on the IgG H + L IFA assay. Three dogs (*Kat*, *Tan*, *Pam*) became seroreactive by post-inoculation day 4 (March 24, 2017). All dogs remained seroreactive (IgG H + L) until the end of the RM phase (April 28, 2017, 39 days after inoculation). When next tested 4 months after *Rr* inoculation, only 1 of the 6 dogs (*Kat*) was *Rr* seroreactive (Figure 4). This dog remained *Rr*-seroreactive when tested again nearly 7 months after *Rr* inoculation and was the same dog that was *Rr* seroreactive when first tested during the PI phase.

Two dogs became *Rr* IgM seroreactive by 4 days after inoculation, and all dogs were IgM seroreactive at 7 days after inoculation. IgM seroreactivity persisted for a variable period of time; 4 of the 6 dogs were IgM non-seroreactive by 32 days after inoculation. However, IgM seroreactivity was detected in 1 dog (*Shok*) erratically, with low titers persisting until 39 days after inoculation. One dog was IgM seroreactive during the EF phase (*Kat*, July 20, 2017, 122 days after inoculation).

Two dogs became *Rr* IgG gamma seroreactive by 7 days after inoculation, and all dogs were IgG seroreactive by 18 days after inoculation. All dogs were IgG gamma seroreactive until the end of the RM phase (April 28, 2017, 39 days after inoculation). Only 1 dog was IgG gamma seroreactive during the EF phase (*Tan*, July 20, 2017, 122 days after inoculation).

Dogs were tested for rickettsemia by PCR of whole-blood DNA extractions. *Rickettsia rickettsii* DNA was not amplified from any dog's blood at any time point either before or after *Rr* experimental inoculation.

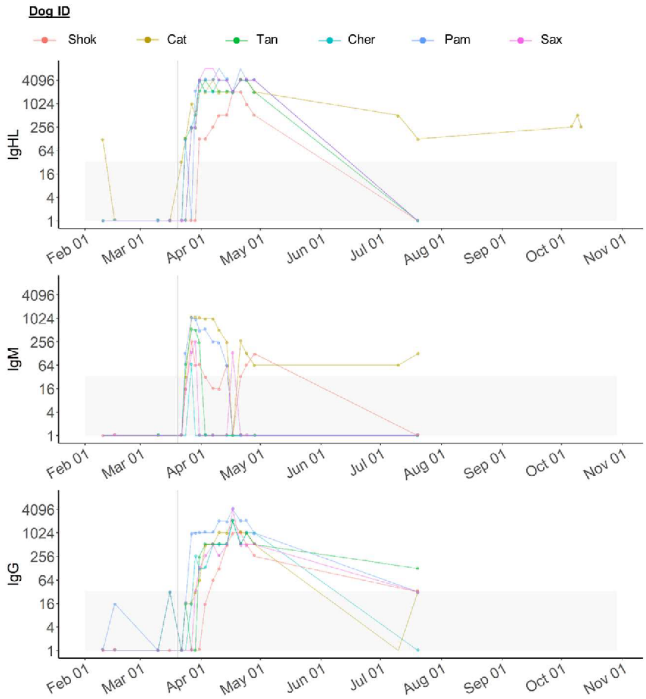

FIGURE 4 Spaghetti plots showing *Rickettsia* spp. Immunofluorescent antibody (IFA) titers before and after experimental *Rickettsia rickettsii* (*Rr*) inoculation. For all plots, the x-axis shows the testing date and the y-axis shows IFA titer of 1: value. Titers for *Rickettsia* spp. are shown for each specific antibody (IgHL top, IgM middle, IgG bottom). Vertical gray line represents date of *Rr* inoculation. Gray boxes show non-seroreactive titers. Colors correspond to each individual dog, shown in at the top of the figure. IgG, IgG gamma; IgHL, IgG heavy and light chains; IgM, IgM mu

3.2 | Bartonella serology

Bartonella spp. titers for diagnostic IgG H + L throughout the study (all phases) are shown in Figure 5. Based on the combined results using all 4 IFA isotype-specific conjugates and all 3 *Bartonella* sp. antigens, during the PI phase 3 dogs were *Bartonella* spp. seroreactive. One dog (*Shok*) was *Bh* seroreactive on February 10, 2017, and February 16, 2017, using the diagnostic IFA assay. Another dog (*Kat*) was *Bvb* seroreactive on February 10, 2017, and March 16, 2017, using the alternative IgG H + L isotype-specific conjugate but non-seroreactive using the diagnostic *Bvb* assay at all 4 PI phase time points. A third dog (*Tan*) was *Bvb* seroreactive using the IgM isotype-specific conjugate only, on February 10, 2017. The remaining 3 dogs (*Cher*, *Pam*, and *Sax*) were *Bh*, *Bvb*, and *Bk* non-seroreactive using any of the 4 isotype conjugates during the PI phase. All 6 dogs were *E canis*, *B canis*, *B gibsoni*, and *L infantum* IFA non-seroreactive, and *A phagocytophilum*, *A platys*, *B burgdorferi*, *E canis*, and *E ewingii* and *D immitis* ELISA negative during the PI phase.

Based on the combined results using all 4 IFA isotype-specific conjugates and all 3 *Bartonella* sp. antigens, during the RM phase all 6 dogs became *Bartonella* spp. seroreactive: all 6 dogs became *Bh* seroreactive, 2 dogs (*Tan* and *Pam*) became *Bvb* seroreactive, and 4 dogs (*Tan*, *Cher*, *Pam*, and *Sax*) became *Bk* seroreactive (Figure 5). When using the alternative IgG H + L isotype-specific conjugate, it was found that only 3 dogs were *Bartonella* spp. seroreactive during the RM phase: 2 dogs (*Tan* and *Cher*) were *Bh* seroreactive at a single time point each, 1 dog (*Tan*) was *Bvb* seroreactive at a single time point and 1 dog (*Pam*) was *Bk* seroreactive at multiple time points. Each dog that was seroreactive using the alternative IgG H+L conjugate was also seroreactive to the same Bartonella spp. antigen on the diagnostic IFA. When using the IgM and IgG gamma isotype-specific conjugates, it was found that only 1 dog (*Cher*) was *Bartonella* spp. seroreactive during the RM phase. This dog was *Bartonella* spp. seroreactive at 2 time points: On April 7, 2017, she was *Bk* IgM and *Bh* and *Bk* IgG gamma seroreactive, and on April 14, 2017, she was *Bh*

TABLE 1 *Rickettsia* spp. seroconversion following *Rickettsia rickettsii* inoculation

	IgHL	IgM	IgG
Shok	11	7	18
Cat	4	7	11
Tan	4	4	11
Cher	7	7	9
Pam	4	4	7
Sax	7	7	11

The number of days post-inoculation that each dog seroconverted based on each immunofluorescent antibody assay antibody is shown. Abbreviations: IgG, IgG gamma; IgHL, IgG heavy and light chains; IgM, IgM mu.

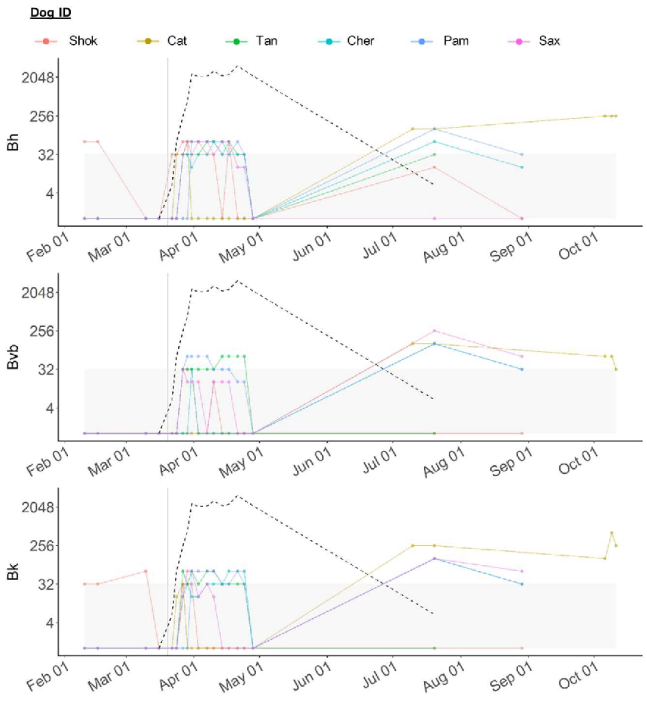

FIGURE 5 Spaghetti plots showing *Bartonella* spp. Immunofluorescent antibody (IFA) titers before and after experimental *Rickettsia rickettsii* (*Rr*) inoculation. For all plots, the x-axis shows testing date and the y-axis shows IFA titer of 1: value. Titers for diagnostic IFA (IgG H + L) are shown for each Bartonella species (*Bh* top, *Bvb* middle, *Bk* bottom). Vertical gray line represents date of *Rr* inoculation. Gray boxes show non-seroreactive titers. Black dotted line shows the mean *Rickettsia* spp. Immunofluorescent antibody (IFA) titer for all dogs to indicate the timeline of *Rickettsia* seroconversion. Colors correspond to each individual dog, shown at the top of the figure

TABLE 2 *Bartonella* testing of body fluids during EF phase

	7/20 *Bartonella* IFA	7/20 PCR & BAPGM blood	7/26 Pinnae & skin biopsies	8/29 *Bartonella* IFA	8/29 PCR & BAPGM blood	8/30 cheek	8/30 periodontal	9/15 cheek	9/15 periodontal	9/15 vaginal	10/6-25 PCR & BAPGM blood	12/11 Tissue biopsies[a]
Shok	–	–	NA	Bvb[b]	–	Bvb	–	–	–	–	NA	Bh[c]
Cat	Bh + Bvb + Bk	–	Bh[c]	NA	NA	Bvb	–	–	–	–	–x3[d]	NA
Tan	Bvb[b]	–	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Cher[e]	Bh + Bvb + Bk	–	NA	Bvb[f] + Bk[b]	–	–	–	–	–	–	–	NA
Pam	Bh + Bvb + Bk	–	NA	–	–	Bvb	Bvb	–	*Bartonella* spp.[g]	–	NA	NA
Sax[e]	Bvb + Bk	–	NA	Bvb + Bk	–	Bvb	Bvb	–	–	–	–	NA

Blood samples and swabs from the oral cavity and vaginal vault were taken on each date shown. Negative test results are indicated by the minus sign ("–"). Samples that were not available for testing are indicated by "NA."

Abbreviations: BAPGM, Bartonella alpha-proteobacteria growth medium; Bh, *Bartonella henselae*; Bk, *Bartonella koehlerae*; Bvb, *Bartonella vinsonii* subsp. *berkhoffii*; IFA, immunofluorescent antibody.

[a]Tissues included lung, bone marrow, lymph nodes.

[b]Detected with the alternative KPL secondary antibody only.

[c]Same Bartonella species and strain (Bh SA2).

[d]Three blood samples tested.

[e]Housed together in a single run.

[f]Detected with the IgM (mu) antibody only.

[g]Detected on real-time PCR using TaqMan probe, species unable to be determined.

IgG gamma seroreactive. She was *Bvb* IgM and IgG gamma non-seroreactive at all time points during the RM phase. The remaining 5 dogs were *Bh*, *Bvb*, and *Bk* IgM and IgG gamma non-seroreactive during the RM phase.

Based on the combined results using all 4 IFA isotype-specific conjugates and all 3 *Bartonella* sp. antigens, during the EF phase all 6 dogs were *Bartonella* spp. seroreactive. With the use of the diagnostic assay, 3 dogs (*Kat*, *Cher*, *Pam*) were seroreactive to all 3 *Bartonella* spp. and 1 dog (*Sax*) was seroreactive to *Bvb* and *Bk* (Figure 5). When using the alternative IgG H + L isotype-specific conjugate, it was found that only 3 dogs were seroreactive, each to a single species at a single time point (*Tan* to *Bvb* on July 20, 2017, *Shok* to *Bvb* on August 29, 2017, and *Cher* to *Bk* on August 29, 2017). One dog (*Cher*) was IgM seroreactive during the EF phase, with a titer of 1:128 to *Bvb* on August 29, 2017. The remaining 5 dogs were *Bh*, *Bvb*, and *Bk* IgM non-seroreactive during the EF phase. No dog was IgG gamma seroreactive to any *Bartonella* spp. during the EF phase.

3.3 | Bartonella PCR and DNA sequencing

Bartonella PCR on whole blood from all dogs was negative at every time point blood was drawn during all phases (Figure 2). Bartonella alpha-proteobacteria growth medium enrichment PCR on whole blood from all dogs was negative at every point tested (Figure 2, Table 2).

Bartonella PCR results during the EF phase are summarized in Table 2.On July 26, 2017, 1 dog (*Kat*) had biopsies obtained from skin lesions on the pinnae and from normal appearing skin on the abdomen. *Bartonella henselae* San Antonio 2 strain DNA was amplified and sequenced from the skin lesions on both pinnae and from the normal abdominal skin as previously reported.[36] *Rr* DNA was not PCR-amplified from any of the biopsies. Just before the biopsies (July 10, 2017, and July 20, 2017), this dog was *Rr* seroreactive (1:64 and 1:128), seroreactive to all 3 *Bartonella* spp. (*Bh* 1:128, *Bvb* 1:128, *Bk* 1:256), and *Babesia canis* seroreactive (1:64). Blood BAPGM ePCR was negative. She was not seroreactive to *B gibsoni*, *E canis*, or *L infantum*, and was negative on the SNAP 4DX Plus test. Additionally, this dog had blood samples taken on October 6, 2017, October 9, 2017, and October 11, 2017; she remained *Rr* and *Bartonella* spp. seroreactive (Figures 4 and 5) but was no longer *B canis* seroreactive. At each of those 3 time points, blood BAPGM ePCR was negative.

To determine if body fluids could be serving as a source of transmission among dogs in this confined vector-free setting, PCR was performed on oral and vaginal swabs on the 5 remaining dogs on August 30, 2017, and September 15, 2017. PCR results from these swabs are shown in Table 2. Four of the 5 dogs had *Bvb I* DNA amplified from 1 or more oral swabs on August 30, 2017 with 2 primer sets (100% DNA sequence identity, 399/399 and 563/563 bp, GenBank accession #AF167988.1). Vaginal swabs were not collected on this date. When sampling was repeated on September 15, 2017, only 1 dog (*Pam*) had *Bartonella* spp. DNA amplified from an oral swab (99.3% DNA identity, 138/139 bp *Bh* SA2, GenBank accession #AF369529). Due to the short DNA sequence obtained with the real-time PCR TaqMan probe, the *Bartonella* sp. could not be definitively determined; however, the sequence was not consistent with any of the 4 *Bvb* genotypes. DNA concentration of the swabs from September 15, 2017, were low (<2.5 ng/μL) compared to that of swabs obtained on August 30, 2017 (10-63 ng/μL). Also, during the EF phase, all dogs were tested 1-2 times for *Bartonella* bacteremia with BAPGM ePCR (all dogs on July 20, 2017, *Kat* on October 6, 2017, October 9, 2017, and October 11, 2017, *Sax* on October 24, 2017, and *Cher* on October 25, 2017): all were PCR-negative.

On December 11, 2017, at the time of euthanasia, tissues were collected from 1 dog (*Shok*). Gross postmortem examination findings were normal, and histopathology was not performed. Based on PCR and sequencing, *Bh* DNA was amplified from multiple tissues (lung, bone marrow, and lymph nodes). When multiple 16S-23S ITS primer sets were used, all amplified 16-23S rDNA sequences shared 100% identity with *Bh* SA2 (GenBank accession #AF369529): lung (363/363 bp), bone marrow (551/551 bp), and submandibular lymph node (527/527 bp). Mesenteric lymph node was also *Bartonella* genus positive by real-time PCR using a TaqMan probe, but the amplified sequence did not allow for discrimination among several *Bartonella* species. *Bartonella* spp. DNA was not amplified from the spleen. No *Bvb*, *Bk* or *Rr* DNA was amplified from this dog's tissues. This dog was *Bh* seroreactive before *Rr* inoculation, but *Bh* non-seroreactive during the EF phase. The *Bartonella* species and strain (*Bh* SA2) amplified from this dog's tissues were the same as that amplified from another dog's (*Kat*) tissues 5 months earlier.

The stored *Rr* inoculum was tested for *Bartonella* spp. by PCR: *Bartonella* spp. DNA was not amplified from stored inoculum.

3.4 | Complete blood count results

Complete blood count results for each dog during the PI and RM phases are shown in Figure 3C-K. Before *Rr* inoculation, most dogs had normal CBC findings, but 2 dogs (*Kat* and *Tan*) had mild hypoproteinemia and 1 dog (*Pam*) had multiple mild abnormalities including thrombocytosis, hyperproteinemia, monocytosis, and reticulocytosis. *Kat* was also the 1 dog *Rr* seroreactive during the PI phase, and *Kat* and *Tan* were 2 of the 3 dogs *Bartonella* spp. seroreactive during the PI phase. *Pam* was *Bartonella* spp. and *Rr* non-seroreactive during the PI phase.

After infection with *Rr*, there were multiple CBC changes. The PCV, hematocrit (HCT), or both decreased in all dogs; 2 dogs (*Cher* and *Pam*) developed anemia while the remaining dogs maintained PCV within reference range. All dogs developed reticulocytosis, but MCV and MCHC remained within reference range for all dogs. Platelet count initially decreased in all dogs and then increased above pre-*Rr* inoculation baseline; 2 dogs (*Kat* and *Tan*) developed thrombocytopenia and 4 dogs (*Cher*, *Tan*, *Pam*, *Sax*) developed rebound thrombocytosis. White blood cell count increased in all dogs, and all dogs developed mild neutrophilia and monocytosis. Two dogs (*Kat* and *Tan*) developed neutropenia 3-4 weeks after *Rr* infection.

4 | DISCUSSION

There were 2 unexpected findings during this investigation: after experimental *Rr* infection at a vector-free biocontainment facility, we found that all dogs developed *Bartonella* spp. seroreactivity and multiple dogs had DNA evidence of *Bartonella* spp. infection. Based on PCR from tissues, at least 2 of these 6 seroreactive dogs were actively infected with 1 or more *Bartonella* species at the time of testing: *Kat* had *Bh* amplified from skin biopsy specimens taken from lesions in July 2017, and *Shok* had *Bh* amplified from postmortem tissues in December 2017. In addition, 4 of 5 dogs had *Bvb I* amplified from oral swabs in August 2017, and 1 of those 4 dogs had another *Bartonella* sp. (most compatible with *Bh* SA2) amplified from an oral swab 2 weeks later. It is possible that these swab results represent active infection with salivary shedding of *Bvb* and *Bh*, occurring in 4 of 5 dogs. Despite detection of *Bartonella* spp. antibodies in all dogs at multiple time points, and amplification of *Bartonella* spp. DNA from tissues and oral swabs, *Bartonella* spp. DNA was not amplified from blood during the course of this investigation. While 5 of 6 dogs had *Bk* antibodies, *Bk* DNA was not amplified from either tissues or swabs.

We are only able to speculate on the origin of the *Bh* and putative *Bvb* infections. These dogs were either infected before arrival at NCSU LAR or newly infected while residing in the presumed vector-free housing at the NCSU LAR. If the dogs were infected before arrival at the NCSU, it is possible that the physiologic stress of experimental *Rr* infection caused reactivation of latent *Bartonella* infections. At the vendor's facility, the dogs were housed in indoor/outdoor runs and not treated with flea or tick preventatives, so it is possible that they were exposed during that time. The 3 dogs that were *Bartonella* spp. seroreactive at the first testing time point (February 10, 2017), and before *Rr* inoculation, support the possibility of preexisting chronic subclinical infection. The rapid development of *Bartonella* antibodies (by 9 days after *Rr* inoculation in 5 of 6 dogs), particularly those dogs that seroconverted to multiple *Bartonella* spp., also supports reactivation of latent infection. As these dogs had resided at the NCSU LAR for over 7 weeks at the time of this first blood collection, however, it is not possible to determine whether transmission occurred before or after shipment from the vendor. All dogs did receive 1 week of sulfamethoxazole for coccidiosis before shipping, but it is unlikely that would clear *Bartonella* spp. infections.[37] If the dogs were exposed at the vendor's facility, this highlights the need for researchers to specifically request dogs be treated with flea/tick preventatives and tested for infection prior to research studies, so that coinfection with these vector-borne diseases does not bias the results of their studies.

The possibility of reactivation of latent *Bartonella* infection in both humans and dogs has been raised in case reports previously. *Bartonella henselae* and Epstein-Barr virus were found simultaneously in a man with fever and lymphadenopathy after acute mononucleosis.[38] A dog presumed to have immune-mediated ineffective erythropoiesis was pharmacologically immunosuppressed; his HCT was improving or stable until he had an episode of presumptive kennel cough, at which time his HCT and regenerative response worsened with no change to his immunosuppressive medications.[39] *Bartonella henselae* was amplified from this dog's blood soon after, and once immunosuppression was discontinued and appropriate antibiotics administered, his ineffective erythropoiesis resolved. Therefore, it is possible that chronic *Bh* infection reactivated after infectious tracheobronchitis. In a second case,[4] an acutely ill *Rr* seroreactive dog failed to respond to antibiotic treatment directed against RMSF (doxycycline). When tested 10 days later, the dog had seroconverted to *Bh* and *Bk*. Unlike the dogs in this study, this dog developed very high *Bh*, *Bk*, and *Rr* titers simultaneously, supporting the theory that the dog was exposed to all 3 pathogens on or around the same time. However, it is also possible that that dog was chronically infected with 1 or more *Bartonella* spp. and experienced reactivation when infected with an SFGR species. Based on these previous reports and the results in this case series, it is likely that dogs can harbor chronic subclinical *Bartonella* infections that become recrudescent when exposed to the physiologic stress induced by coinfection.

However, it is instead possible that the dogs reported here were newly infected while residing at the NCSU LAR, which begs the question of transmission route. Because *Bartonella* species are considered to be primarily vector-borne, the animal housing areas at the NCSU LAR are maintained with strict vector-prevention methods. These dogs were checked daily for ectoparasites: none were reported throughout the duration of the study. Dogs were only allowed outdoor access during the EF phase (by which point all dogs had already seroconverted to *Bartonella* spp.), and all dogs that were given access to outdoor areas were treated monthly with a topical flea and tick preventative (Frontline Plus for Dogs, Merial Inc, Duluth, Georgia). There remains the unlikely possibility that a previously unknown vector remained undetected in the LAR facility and was able to facilitate transmission between dogs.

If we remove the possibility for vector transmission, then we must consider the possibility of direct transmission, via the contact allowed by the chain-link fence between individual runs during the PI and RM phase, comingling during the EF phase, or both. The amplification of *Bvb* from 4 of 5 dogs' oral swabs during the EF phase is supportive of the possibility for direct transmission via body fluids or oral mucus membranes. At that time the only 2 dogs remaining housed together (who had shared a run for approximately 4 months) had disparate results: *Sax* had *Bvb* DNA amplified from both cheek and periodontal swabs, but her kennel-mate *Cher* had no *Bartonella* spp. DNA amplified from any similar samples. However, at that time, *Cher* was seroreactive to *Bvb* IgM, which could be indicative of exposure due to direct transmission from *Sax*. While it is likely that the *Bvb* and other *Bartonella* spp. DNA found on oral/dental swabs in the dogs reported here were associated with active infection and shedding into the oral cavity, it is possible that this DNA represents environmental contamination from another source within LAR. If associated with active infection and shedding, the host and pathogen dynamics governing potential direct transmission should be urgently explored.

Regardless of the origin of *Bartonella* spp. exposure, all 6 dogs developed specific *Bh*, *Bvb*, and *Bk* antibody responses after *Rr* inoculation. Previous studies have documented that there is no cross-

reactivity between *Rr* and *Bh* or *Bvb* using the 3 respective IFA assays.[6,40] In the dogs reported here, the *Bartonella* spp. antibody response lagged behind the *Rr* seroconversion, and multiple dogs were *Bartonella* spp. seroreactive during the EF phase when *Rr* titers had waned. This further supports the conclusion that the *Bartonella* spp. seroreactivity was not cross-reactive with *Rr* cell culture grown antigens.

Our results also highlight the previously reported poor sensitivity of the diagnostic *Bartonella* spp. IFA,[26,41,42] as evidenced by the lack of *Bartonella* seroreactivity at various time points in dogs with positive tissue PCR (*Shok* and *Kat*) or oral swab PCR (*Pam*). Sensitivity and specificity for the IFAs performed in this study have not been previously explicitly evaluated; however, previous studies using similar IFA protocols have been done.[26,43,44] When 20 naturally infected *Bh* PCR-positive dogs had serology performed (on serum sampled concurrently with the PCR-positive blood samples), only 1 dog was *Bh* seroreactive for the antigen used in this study (*Bh* SA2).[43] In 3 dogs experimentally infected with *Bh* H1 and proven bacteremic, none were seroreactive against *Bh* SA2 (though all seroconverted to *Bh* H1).[44] Conversely, a single dog experimentally infected with *Bh* SA2 seroconverted to *Bh* SA2 2 weeks after infection and maintained seroreactivity for approximately 6 weeks, after which titers waxed and waned until finally remaining below 1:16 after 12 weeks.[26] The lack of sensitivity of *Bartonella* IFA overall could be due to immune-complexing of antibodies, antibodies below the level of detection, relapsing infection, genotype-specific antibody responses, or other as-yet undetermined mechanisms.[41,43] The specificity of IFA is higher than the sensitivity and was recently estimated to be at least 85% for an expanded panel of antigens but could be significantly higher given the previously described level of seroreactivity in the healthy/blood donor dog population.[43,45]

In addition to the poor sensitivity of IFA, there are other limitations to using IFA as a diagnostic test that impeded our ability to determine the origin or course of *Bartonella* spp. infection in this study. When evaluating IFA slides, interobserver differences in interpretation may include 1 dilution step (ie, 1:32 to 1:64, or 1:256 to 1:512), which is why as a general rule seroconversion is considered to occur only when antibody titers rise by 2 or more dilution steps (4-fold). A previous study of *Bartonella* IFA showed that even when evaluated by blinded, experienced scientists, disagreements on dilution can occur, highlighting the somewhat subjective nature of this diagnostic test.[46] Along with the low sensitivity, these inherent limitations of IFA may help to explain the differences in seroreactivity to each *Bartonella* spp. over time in the dogs reported here. However, studies of serological response to *Bh* infection in humans with classical Cat Scratch Disease have shown that even in that most straightforward, acute presentation, there is no "standard course" of anti-*Bh* IgG or IgM production: some patients produce high levels of both isotypes, some produce only high levels of IgM, and some produce only low levels of either isotype.[46-48] Though IgM and IgG isotype-specific serology is routinely used clinically in humans to attempt to distinguish acute infection from previous exposure, evaluation of *Bartonella* IgG and IgM isotype-specific antibody responses has not, to the authors knowledge, been previously reported in dogs (nor are these assays commercially available). Based on our results, the poor sensitivity of the IgG and IgM isotype-specific IFA precludes their routine use in clinical diagnosis of *Bartonella* spp. exposure or infection in dogs. Overall, since seroreactivity was detected only sporadically in all 6 dogs throughout the study and did not always match the species ultimately identified on PCR, failure to detect antibodies to any of 3 *Bartonella* spp. does not rule out the possibility of concurrent occult infection.

While previous studies have supported the diagnostic utility of BAPGM ePCR blood culture for confirmation of bloodstream infection in both healthy and sick dogs,[31,45,49,50] our efforts to PCR amplify or culture *Bartonella* spp. DNA from blood were not successful, even in dogs with PCR-positive saliva or tissue samples. Whether this is due to extremely low bloodstream levels, a relapsing time course of *Bartonella* spp. bacteremia, or sequestration of *Bartonella* organisms in cells outside the bloodstream (such as endothelial cells) is unknown. Additionally, we were unable to amplify *Bk* from any sample despite all but 1 dog seroconverting to *Bk* during either RM or EF phases. In a previous study,[26] when dogs naturally infected with *Bk* were challenged by intradermal inoculation with either *Bh* or *Bvb*, dogs seroconverted only to the inoculated species.

With regard to the *Rr* infection, we showed that the serologic response was consistent with previous experimental studies of RMSF in dogs.[28,29] Because *Rr* is an endotheliotropic pathogen infecting predominantly endothelial cells of small- and medium-size blood vessels, low numbers of *Rr* circulate in peripheral blood of most infected patients.[51,52] As such, *Rickettsia* genus-specific PCR using the 23S-5S intergenic region from blood specimens (as was performed in this study) was not expected to be positive in these dogs inoculated with low-dose *Rr* (although the use of more specific primers or nested PCR can enhance detection).[52-54] As expected, therefore, *Rr* DNA was not amplified at any time from these dogs, and IFA seroconversion was used to document infection. All dogs seroconverted to IgM by day 7, and all but 1 dog seroconverted to IgG gamma on days 7 through 11. This 1 dog (*Shok*) had an IgM response comparable to the other 5 dogs, but low IgG gamma titers and a slightly prolonged IgG gamma seroconversion (18 days after inoculation). While clinically healthy both before and after the mild RMSF, this dog was *Bh* seroreactive both pre- and post-*Rr* inoculation and had *Bh* DNA amplified from multiple tissues at the time of euthanasia approximately 8 months later. These findings may indicate delayed class shift in this dog associated with chronic subclinical *Bh* infection. We are not aware of other documented examples of a delayed class shift in association with occult infections in dogs that were challenged with a highly virulent organism such as *Rr*. Whether the delay in IgG gamma seroconversion in this dog was a cause or consequence of *Bh* coinfection is unknown.

Previous studies have shown that after experimental infection, repeat inoculation with *Rr* elicits no clinical illness, hematological changes, or recall IgM response.[28,29] Additionally, recrudescent infection with *Rr* is not thought to occur in dogs or humans.[55] When inoculated with *Rr*, the 1 dog in this study (*Kat*) that was *Rr* seroreactive became clinically ill and had a robust IgM response, making it unlikely

that she was previously infected with *Rr*. Because *Rr* IFA cross-reacts with multiple SFGR species, this dog likely had exposure to another SFG rickettsia (or a cross-reactive non-*Rickettsia* species) that did not provide cross-protection to *Rr* infection.

The major limitation of this study is the lack of sequential, prospective sampling of all dogs, particularly during the EF phase. For several reasons, samples were not obtained at predetermined time intervals after the RM phase. Prospective sampling to assess *Bartonella* spp. reactivation, transmission, or both possibilities was pursued only after documentation of intra-lesional *Bh* in the dog with ear-tip vasculitis.[36] Lack of sequential sample collection limits our ability to draw conclusions about when, where, and how the dogs in this study became infected. It is unfortunate that we did not obtain blood and tissue samples from the dogs immediately upon arrival at LAR, allowing us to determine more definitively the timing of infection. Additionally, there are inherent limitations in using IFA and PCR as previously discussed. Interpretation of IFA is subjective, and determining the end point titer is usually known to have a margin of error of 1-fold dilution above or below. This can result in misclassification bias, particularly when the IFA is performed unblinded (as ours were in this study). In few cases where IFA results differed upon repeat testing, we reported the more conservative (lower) titer, to limit the likelihood of false-positives.

Despite limitations, this study supports the possibility of chronic subclinical *Bh* infection with recrudescence after infection with *Rr*. Additionally, the possibility for nonvectorial direct transmission via saliva or other body fluids should be further investigated with controlled experimental studies. Occult infections in research animals could cause spurious conclusions in studies utilizing these animals for infectious disease or other biomedical research. Also, if direct transmission is able to occur for this pathogen that has been considered primarily vector-borne, there are wide-ranging biosafety and zoonotic disease implications of substantial veterinary and human medical importance.

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CONFLICT OF INTEREST DECLARATION

In conjunction with Dr. S. Sontakke and North Carolina State University, E. B. Breitschwerdt holds US Patent No. 7,115,385; Media and Methods for Cultivation of Microorganisms, which was issued on October 3, 2006. He is a co-founder, shareholder and Chief Scientific Officer for Galaxy Diagnostics, a company that provides advanced diagnostic testing for the detection of Bartonella spp. infections. Ramaswamy Chandrashekar is employed by IDEXX Laboratories. The remaining authors declare no conflicts of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

The study was approved by the North Carolina State University IACUC (Protocol #16-206).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

ORCID

Erin Lashnits https://orcid.org/0000-0003-0949-5698
Pradeep Neupane https://orcid.org/0000-0002-6775-6166
Ricardo G. Maggi https://orcid.org/0000-0001-7428-0138
Julie M. Bradley https://orcid.org/0000-0003-1812-5242
Edward B. Breitschwerdt https://orcid.org/0000-0002-3506-0279

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CHAPTER 5

Investigating clinical manifestations of *Bartonella* in dogs and humans

Lashnits E, Neupane P, Bradley JM, Richardson T, Thomas R, Linder KE, Breen M, Maggi RG, Breitschwerdt EB. Molecular prevalence of *Bartonella*, *Babesia*, and hemotropic *Mycoplasma* species in dogs with hemangiosarcoma from across the United States. PLoS One. 2020;15(1):e0227234. PMID: 31923195.

PLOS ONE

RESEARCH ARTICLE

Molecular prevalence of *Bartonella*, *Babesia*, and hemotropic *Mycoplasma* species in dogs with hemangiosarcoma from across the United States

Erin Lashnits[1], Pradeep Neupane[1], Julie M. Bradley[1], Toni Richardson[1], Rachael Thomas[2], Keith E. Linder[3], Matthew Breen[2], Ricardo G. Maggi[1,4], Edward B. Breitschwerdt[1,4]*

1 Intracellular Pathogens Research Laboratory, Comparative Medicine Institute, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina, United States of America, **2** Department of Molecular Biomedical Sciences, Comparative Genomics, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina, United States of America, **3** Department of Population Health and Pathobiology, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina, United States of America, **4** Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina, United States of America

* ebbreits@ncsu.edu

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Abstract

Hemangiosarcoma (HSA), a locally invasive and highly metastatic endothelial cell neoplasm, accounts for two-thirds of all cardiac and splenic neoplasms in dogs. *Bartonella* spp. infection has been reported in association with neoplastic and non-neoplastic vasoproliferative lesions in animals and humans. The objective of this study was to determine the prevalence of *Bartonella* spp. in conjunction with two other hemotropic pathogens, *Babesia* spp. and hemotropic *Mycoplasma* spp., in tissues and blood samples from 110 dogs with histopathologically diagnosed HSA from throughout the United States. This was a retrospective, observational study using clinical specimens from 110 dogs with HSA banked by the biospecimen repository of the Canine Comparative Oncology and Genomics Consortium. Samples provided for this study from each dog included: fresh frozen HSA tumor tissue (available from n = 100 of the 110 dogs), fresh frozen non-tumor tissue (n = 104), and whole blood and serum samples (n = 108 and 107 respectively). Blood and tissues were tested by qPCR for *Bartonella*, hemotropic *Mycoplasma*, and *Babesia* spp. DNA; serum was tested for *Bartonella* spp. antibodies. *Bartonella* spp. DNA was amplified and sequenced from 73% of dogs with HSA (80/110). In contrast, hemotropic *Mycoplasma* spp. DNA was amplified from a significantly smaller proportion (5%, $p<0.0001$) and *Babesia* spp. DNA was not amplified from any dog. Of the 100 HSA tumor samples submitted, 34% were *Bartonella* PCR positive (32% of splenic tumors, 57% of cardiac tumors, and 17% of other tumor locations). Of 104 non-tumor tissues, 63% were *Bartonella* PCR positive (56% of spleen samples, 93% of cardiac samples, and 63% of skin/subcutaneous samples). Of dogs with *Bartonella* positive HSA tumor, 76% were also positive in non-tumor tissue. *Bartonella* spp. DNA was not PCR amplified from whole blood. This study documented a high prevalence of *Bartonella* spp. DNA in dogs with HSA from geographically diverse regions of the United States. While 73%

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Competing interests: In conjunction with Dr. Sushama Sontakke and North Carolina State University, Edward B. Breitschwerdt, DVM holds U. S. Patent No. 7,115,385: "Media and Methods for Cultivation of Microorganisms", which was issued October 3, 2006. He is a co-founder, shareholder and Chief Scientific Officer for Galaxy Diagnostics, a company that provides advanced diagnostic testing for the detection of Bartonella species infections. This does not alter our adherence to PLOS ONE policies on sharing data and materials. All other authors declare no potential conflicts of interest.

of all tissue samples from these dogs were PCR positive for *Bartonella* DNA, none of the blood samples were, indicating that whole blood samples do not reflect tissue presence of this pathogen. Future studies are needed to further investigate the role of *Bartonella* spp. in the development of HSA.

Introduction

There are clear precedents for the involvement of bacterial infection in neoplastic development. Within the past 25 years, a considerable volume of research has been conducted on the oncogenic properties of infectious agents such as bacteria, mycoplasma, protozoa, and viruses.[1,2] Currently, infectious agents are accepted as a cause or co-factor in anywhere from 5–50% of human cancers worldwide, depending on the geographic region and its development status.[1–3] The involvement of infectious agents in the pathogenesis of some human cancers is therefore well established. The majority of infectious agents implicated in oncogenesis are viruses, such as Epstein Barr virus, human papillomaviruses, and Kaposi's sarcoma-associated herpesvirus.[1] These viruses have direct oncogenic properties through integration of viral genomes into host cells, or by secretion of gene products into healthy cells to create tumor cells. The extent to which other infectious agents, such as bacteria, lack the inherent oncogenic properties of their viral counterparts remains unclear. Bacteria most often promote cancer development indirectly through persistent replication, inflammation and chronic tissue damage.[4,5] *Helicobacter pylori*, for example, colonizes and replicates within the gastric mucosa, resulting in a chronic pro-inflammatory response that promotes cancer risk and oncogenesis of gastric cancer by altering epithelial cell proliferation and apoptosis.[6] With the difficulty of assessing causality, particularly for certain rare cancer types, there may be roles for other pathogenic bacteria in a range of different cancers that have not yet been discovered.[7]

Hemangiosarcoma (HSA) is a highly aggressive endothelial cell cancer that is associated with local invasiveness and a high metastatic potential in dogs (Fig 1).[8,9] The most common neoplasm of the spleen,[10] this cancer is found in up to 2% of all dogs with tissues submitted for autopsy.[11] While HSA may affect any dog breed, several of the most popular family owned pure breeds are highly predisposed, including the golden retriever, Labrador retriever and German Shepherd Dog.[12–14] Splenic HSA is among the most common canine cancers encountered in clinical practice, accounting for approximately two thirds of all splenic tumors (neoplasms of the spleen, benign or malignant, comprise approximately half of all splenic pathology in dogs).[9] Cardiac HSA is less common than splenic HSA (most studies reporting less than 1% of all canine neoplasms), but remains the most common cardiac tumor of dogs.[15,16] Other primary tumor locations such as the liver and skin are reported, but rare.

Splenic HSA is often difficult to diagnose without resorting to splenectomy, a major invasive abdominal surgery.[8,17] Moreover, as an indolent disease, malignant HSA masses that develop within the abdominal cavity often remain undetected until reaching an advanced stage, at which time there is a high risk of spontaneous rupture potentially leading to untreatable and ultimately fatal internal hemorrhage.[18] Because of this risk, as well as the lack of broadly effective chemotherapeutics, the prognosis is poor. The median survival after diagnosis ranges from less than 3 weeks with splenectomy surgery alone to six months with surgery plus cancer chemotherapy.[19,20] As a result there is a critical need for improved diagnostic modalities for earlier detection of HSA, as well as new treatments and preventative strategies to improve outcomes for this common tumor of family pets.

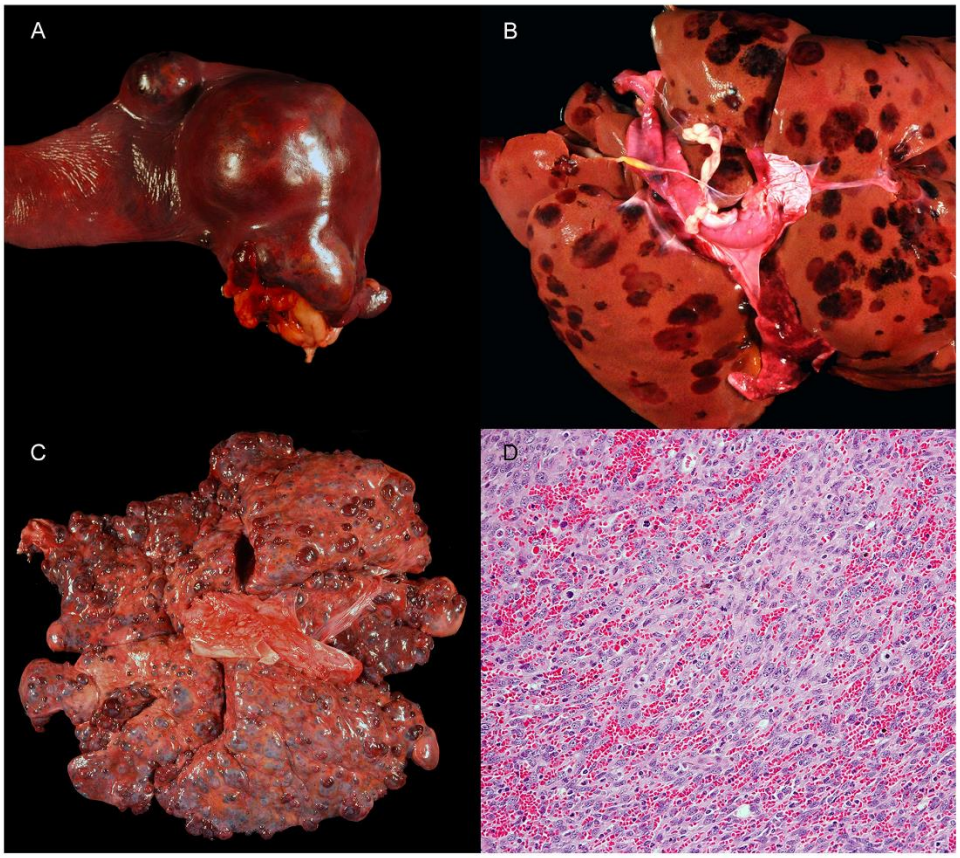

Fig 1. Images of hemangiosarcoma (HSA) tumors from dogs. A) Primary HSA mass in a spleen. B) Metastatic HSA in a liver with multifocal masses in all lobes. C) Metastatic hemangiosarcoma in the lungs with multifocal masses in all lobes. D) Photomicrograph of a splenic HSA from a dog in the current study that was PCR positive for *B. henselae*. 20X magnification. Hematoxylin and eosin. Credits: Talley A (image A), Sommer S (image B), Rasche B (image C) and Barnes J (image D).

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Despite substantial research, the etiology and pathogenesis of canine HSA remains unclear. As a malignant tumor of vascular endothelial cells, factors that have been hypothesized to contribute to the pathogenesis of HSA include chronic inflammation, macrophage activation, hypoxia, and angiogenesis.[21] *In vitro*, HSA cells produce growth factors promoting angiogenesis, including vascular endothelial growth factor-A (VEGF-A), platelet-derived growth factor-β (PDGF-β), and basic fibroblast growth factor (bFGF), and genes involved in inflammation, angiogenesis, and cellular adhesion and invasion can distinguish HSA cells from non-malignant endothelial cells.[9,21]

Because of established links between chronic intracellular infections, inflammation, and angiogenesis, efforts are being made to determine if chronic intravascular infection with bacteria or protozoa could contribute to HSA development in dogs. One previous study from our

laboratory examined the molecular prevalence of blood (erythrocyte)-borne pathogens in a small cohort of dogs from the southeastern United States with and without splenic pathology.[22] We found that *Bartonella* spp. were significantly more common than *Babesia* spp. or hemotropic *Mycoplasma* spp. in formalin-fixed, paraffin embedded biopsy samples from splenic HSA: 26% of dogs were positive for *Bartonella* spp. compared to 2% for *Babesia* spp. ($p < 0.001$) and 6% for hemotropic *Mycoplasma* spp. ($p = 0.006$). Moreover, *Bartonella* spp. were found more often in splenic HSA biopsy samples compared to samples from a non-neoplastic inflammatory disorder of the spleen (lymphoid nodular hyperplasia, LNH) and histologically normal splenic tissue from specific-pathogen-free dogs.[22] We have subsequently documented that *Bartonella* spp. DNA can be amplified from angioproliferative lesions in cats, cows, dogs and horses.[23] In addition, it has been demonstrated that multiple *Bartonella* spp. (*B. bacilliformis*, *B. quintana*, *B. henselae*, and three *Bartonella vinsonii* subsp. *berkhoffii* genotypes) can induce the *in vitro* production of VEGF.[23–25] *Bartonella* spp. can cause endothelial proliferative disorders, including bacillary angiomatosis and peliosis hepatis, in dogs and humans.[26–31] In combination, these observations suggest the potential for involvement of intra-erythrocytic and endotheliotropic *Bartonella* spp. in the initiation and/or progression of vascular endothelial neoplasia in dogs.

However, in our previous case control study demonstrating an association between *Bartonella* spp. infection and HSA,[22] samples were restricted to a single geographical region (North Carolina) and a single anatomical site (splenic HSA). Seroprevalence studies show that *Bartonella* spp. exposure in dogs can be seen throughout the United States, and there are relatively small but statistically significant regional differences in seroprevalence.[32,33] Additionally, the presence of *Bartonella* spp. DNA in splenic tissue could potentially by explained by the spleen's role in removal of hemotropic parasites from systemic circulation, or by *Bartonella* spp. bacteremia at the time of splenic specimen collection. To address these outstanding questions, this study sought to further clarify the potential involvement of *Bartonella* spp. in HSA in a broader anatomic and geographic context.

The objective of this study was to determine the prevalence of *Bartonella* spp. in conjunction with two other hemotropic pathogens, *Babesia* spp. and hemotropic *Mycoplasma* spp., in tissues and blood samples from dogs with histopathologically diagnosed HSA from throughout the United States. Our hypotheses were: 1) the prevalence of *Bartonella* spp. infection in dogs with HSA would be greater than the prevalence of *Babesia* or hemotropic *Mycoplasma* spp., and 2) the prevalence of *Bartonella* spp. infection in dogs with HSA in the spleen will be similar to prevalence in dogs with HSA in other anatomic locations, such as cardiac muscle.

Methods

Study design and sample sources

This was a retrospective, observational, descriptive study of 110 dogs with HSA. Specimens used for this study were previously collected and banked by the Canine Comparative Oncology and Genomics Consortium (CCOGC) based on previously published standard operating procedures.[34]. Briefly, the CCOGC collected samples from eight participating veterinary university teaching hospitals starting in 2006 with the goal of creating a repository of clinical samples from dogs with common naturally occurring cancers. Prior to sample submission to CCOGC, dogs were given a definitive diagnosis of neoplasia based on histopathology performed by a board-certified veterinary pathologist at the diagnostic laboratory of each participating university. Tissue samples for the CCOGC biospecimen repository were obtained from the primary tumor via surgical biopsy or post-mortem collection (HSA tumor tissue). As an internal control, tissue samples from each dog were also obtained from adjacent grossly normal tissue in

the same organ, or if no grossly normal tissue in the affected organ was apparent, skin biopsies were obtained (non-tumor tissue). Tissue submission (HSA tumor and non-tumor) from each dog required provision of both formalin-fixed (subsequently stored in ethanol) and non-fixed specimens snap-frozen in liquid nitrogen (subsequently stored at -80˚ C). Dogs also had serum and whole blood collected at the time of tissue sampling for submission to the CCOGC.

For this study, samples from dogs diagnosed with HSA were provided by the CCOGC repository to the investigators. Four sample types were provided for pathogen testing: fresh frozen HSA tumor tissue, fresh frozen non-tumor tissue, whole blood, and serum. Two sample types were provided for histopathological confirmation of tissue type and tumor presence included: formalin-fixed HSA tumor tissue and formalin-fixed non-tumor tissue. Any dog that had a diagnosis of HSA (as determined by the CCOGC SOP), and had an adequate amount of fresh frozen tissue stored in the biorepository at the time of the investigators' request for specimens, was included. This study, therefore, included 110 dogs with samples collected between May 2008 and November 2011.

Because of previous sample requests or lack of submission of all requested samples to the CCOGC, there were samples missing when provided to the investigators. Of the 110 dogs, 91 had a complete set of the 4 sample types for pathogen testing (fresh frozen HSA tumor tissue, fresh frozen non-tumor tissue, whole blood, serum) provided to the investigators. No dog was missing more than one sample type, and all dogs had at least one fresh-frozen tissue sample (HSA tumor, non-tumor tissue, or both) available for testing. For this reason, dogs with missing samples for pathogen testing were not excluded from the study. Similarly, of the 110 dogs only 37 had a complete set of the 2 sample types for histopathological confirmation (formalin-fixed HSA tumor tissue, formalin-fixed non-tumor tissue) provided to the investigators. Of the 110 dogs, 37 had formalin-fixed HSA tumor tissue and 93 had formalin-fixed non-tumor tissue provided. There were no formalin-fixed samples of HSA tumor or non-tumor tissue from cardiac tumors available for independent histopathologic review. Because a large proportion of dogs were missing formalin-fixed samples for independent histopathological confirmation, the subgroups of tissues with independent histopathologic confirmation of tissue type and tumor presence was analyzed separately.

The CCOGC provided demographic information for each dog, including age (years), breed, weight (kg) and sex and neuter status. The date and geographic location (university teaching hospital) of sample collection was also provided. The anatomic location of tumor and non-tumor tissue samples for each dog was also provided.

Independent confirmation of demographic and histologic data

When possible, information provided by the CCOGC was independently confirmed by the investigators for this study. Histopathology reports providing the diagnosis of hemangiosarcoma were provided for 102 dogs; these were reviewed by one author (EL) to confirm hemangiosarcoma diagnosis and tumor location.

For dogs with formalin-fixed biopsy samples provided (see above), biopsies were independently evaluated by a board-certified veterinary pathologist (KL) to confirm the tissue and tumor origin of the biopsy. Formalin fixed tissues were submitted to the NCSU College of Veterinary Medicine histology laboratory (Raleigh NC) and tissues were embedded in paraffin blocks (FFPE blocks). Slides containing 5 um sections were prepared from the FFPE blocks and stained with hematoxylin and eosin (H&E). Samples were categorized by organ type and HSA tumor or non-tumor tissue based on H&E staining. If the tissue of origin was not able to be determined from the biopsy (5 tumor samples and 2 non-tumor tissues), or if no formalin-fixed sample was provided (68 tumor samples and 14 non-tumor tissues), the tissue of origin

was categorized by the information provided by the CCOGC and the original diagnostic histopathology reports. Non-tumor tissues from skin or various subcutaneous tissues (including hair, skin, adipose, skeletal muscle, or mammary gland, or any combination of these tissues) were categorized as skin/SQ for analysis.

Pathogen detection methods

DNA was extracted from EDTA anti-coagulated blood and fresh frozen tissue samples using a Qiagen DNeasy® Blood and Tissue kit (Qiagen, Valencia, CA) following the manufacturer's protocols. For each tissue sample (HSA tumor and non-tumor), a 25 mg piece of tissue was excised from the entire sample using a new, prepackaged sterile scalpel. DNA yield and quality was assessed by spectrophotometry (Nanodrop, Wilmington, DE).

Each DNA sample was screened for the presence of *Bartonella* spp. DNA using conventional and qPCR, and *Babesia* spp. and hemotropic *Mycoplasma* spp. using qPCR. *Bartonella* qPCR was performed using primers targeting the 16S-23S intragenic transcribed spacer (ITS) region of *Bartonella* species as described previously[35], in conjunction with a BsppITS438 FAM-labeled hydrolysis probe (TaqMan, Applied Biosystems, Foster City, CA, USA). *Bartonella* qPCR was performed at two dilutions for each sample (using 1 uL and 5 uL of template DNA respectively). PCR screening for *Babesia* spp. and hemotropic *Mycoplasma* spp. were carried out as described previously.[22] Briefly, oligonucleotides Myco16S-322s (5′ GCCCA TATTCCTACGGGAAGCAGCAGT 3′) and Myco16S-938as (5′ CTCCACCACTTGTTCAGGT CCCCGTC 3′) were used as forward and reverse primers respectively for hemotropic *Mycoplasma* spp. DNA amplification. Oligonucleotides Piro18S-144s (5′ GATAACCGTGSTAATT STAGGGCTAATACATG 3′) and Piroplasma18S-722as (5′ GAATGCCCCCAACCGTTCCTA TTAAC 3′) were used as forward and reverse primers respectively for *Babesia* spp. DNA amplification.

Amplification was performed in a 25-μl final volume reaction containing 12.5 μl of MyTaq Premix (Bioline), 0.2 μl of 100 μM of each forward primer, reverse primer (IDT® DNA Technology), 7.1 μl of molecular–grade water, and 5 μl of DNA from each sample tested. PCR negative controls were prepared using 5 μl of DNA from blood of a healthy dog. Positive controls for PCR were prepared by using 5 μl of DNA from previously characterized positive dog (clinical cases). Conventional PCR was performed in an Eppendorf Mastercycler EPgradient® under the following conditions: a single hot-start cycle at 95˚C for 2 minutes followed by 55 cycles of denaturing at 94˚C for 15 seconds, annealing at 68˚C for 15 seconds, and extension at 72˚C for 18 seconds. Amplification was completed by an additional cycle at 72˚C for 1 minute, and products were analyzed by 2% agarose gel electrophoresis with detection using ethidium bromide under ultraviolet light.

Validation of positive results was performed by Sanger sequencing of amplicons followed by chromatogram evaluation and sequence alignment using Contig-Express and Align X software (Vector NTI Suite 10.1, Invitrogen Corp, CA, USA). For bacterial species identification, DNA sequences were analyzed for nucleotide sequence homology at NCBI nucleotide database using BLAST version 2.0. A sample was considered *Bartonella* spp. PCR positive if one or more PCR tests (qPCR or conventional PCR) were positive (tests run in parallel). Stringent processing methods were used to avoid DNA carryover during tissue processing.[36] Specifically, tissue samples were processed independently using manual DNA extraction. For all batches of DNA extractions, between 2 and 4 blanks samples (water) were used as negative controls. All negative controls for DNA extractions rendered negative results on all PCR assays. DNA carryover after PCR amplification was avoided by processing each sample in three separate laboratory rooms (one for sample sorting and DNA extraction, a second for

PCR processing, and a third for PCR analysis post amplification), and strict use of personal protective equipment for sample handling by laboratory personnel.

For IFA testing, *Bartonella* antibodies were determined using 3 cell culture grown *Bartonella* spp. (*Bartonella henselae*, *Bartonella vinsonii* subsp. *berkhoffii*, and *Bartonella koehlerae*) as antigens and following standard immunofluorescent antibody assay (IFA) techniques. [37,38] Briefly, bacterial colony isolates were passed from agar plate grown cultures into permissive cell lines. For each antigen, heavily infected cell cultures were spotted onto 30-well Teflon-coated slides (Cel-Line/Thermo Scientific), air-dried, acetone-fixed, and stored frozen. Fluorescein conjugated goat anti-dog IgG (KPL, SeraCare, Milford MA) was used to detect bacteria within cells using a fluorescent microscope (Carl Zeiss Microscopy, LLC, Thornwood NY). Serum samples were diluted in phosphate-buffered saline (PBS) solution containing normal goat serum, Tween-20, and powdered nonfat dry milk to block nonspecific antigen binding sites. Sera were first screened at dilutions of 1:16 to 1:64. All sera that are reactive at 1:64 were further tested with two-fold dilutions to 1:8192.

Study size

An initial power calculation was based on a request for samples from 150 HSA cases from the CCOGC. With 150 HSA cases, assuming the prevalence of *Bartonella* PCR positive cases reported previously in dogs with HSA,[22] using an alpha of 0.05 this study would have 80% power to detect a difference in the prevalence of *Bartonella* spp. DNA in canine cardiac vs. splenic HSA (assuming samples were split evenly between the two tumor locations) if 49% or more, or 7% or less of the cardiac samples were *Bartonella* PCR positive. In addition, assuming the prevalence of each hemotropic pathogen reported previously in dogs with HSA,[22] using an alpha of 0.05 this study would have 80% power to detect similar size differences with 124 cases for hemotropic *Mycoplasma* and 80 cases for *Babesia* spp.

Statistical methods

Descriptive statistics were obtained for demographic factors (age, weight, sex, breed, season of sample collection, geographic location). We assessed statistical differences in the proportion of dogs PCR positive for each hemotropic pathogen using the Chi-squared test of independence (or Fisher's exact tests for small sample numbers). We also assessed statistical differences in the proportion of samples PCR positive from each anatomic location using the Chi-squared test of independence (or Fisher's exact tests for small sample numbers). Differences between demographic factors for *Bartonella* PCR positive and negative dogs were determined using the Wilcoxon Rank-Sum test for continuous variables (age, weight), and Fisher's exact tests for categorical variables. Multivariable logistic regression was used to identify associations between *Bartonella* infection and anatomic location of tissue sample, with potential demographic confounders included as explanatory variables (age, weight, sex, breed, season of sample collection, and geographic location of sample collection). Odds ratios (adjusted ORs) and 95% confidence intervals (95% CIs) were calculated. To determine agreement between tests on two different samples within the same dog, the kappa statistic was calculated.[39] Data analysis was performed using R 3.6.0 (https://www.R-project.org/).

Results

Demographic characteristics of the included dogs are shown in Table 1. There were 58 female dogs (93% spayed) and 52 male dogs (85% neutered). The most common breeds were mixed breed dogs (n = 31), Labrador retrievers (18), and golden retrievers (13); breeds with 3 or fewer individuals were grouped into the Other breed category (34). The median age was 10

Table 1. Demographic and clinicopathologic characteristics of study population. Median and range shown for continuous data. The * indicates a proportion significantly different from baseline (breed baseline = mixed breed).

	All		*Bartonella* +		*Bartonella* -		*p-value*
	Median	Range	Median	Range	Median	Range	
Age (yrs)	10	4–20	10	4–20	10	6–13	0.438
Weight (kg)	33.2	6.1–68.0	34.0	8.5–68.0	30.3	6.1–48.8	0.800
	All dogs Number in each category (%)		**Number *Bartonella* + (%)**				**p-value**
Sex							0.31
FI	4 (4)		3 (75)				
FS	54 (49)		37 (69)				
MI	8 (7)		8 (100)				
MN	44 (40)		32 (73)				
Breed							0.046
Mix	31 (28)		26 (84)				
Lab	18 (16)		10 (56)*				
Golden	13 (12)		9 (69)				
GSD	6 (5)		2 (33)*				
Boxer	4 (4)		4 (100)				
Bichon Frise	4 (4)		2 (50)				
Other	34 (31)		27 (79)				
Season							0.778
Autumn	38 (35)		27 (71)				
Winter	23 (21)		18 (78)				
Spring	20 (18)		13 (65)				
Summer	29 (26)		22 (76)				
Location							0.658
MA	65		44 (71)				
WI	19		16 (84)				
CA	9		6 (86)				
MI	6		3 (50)				
MO	3		2 (67)				
OH	3		2 (67)				
TN	3		3 (100)				
CO	2		2 (100)				

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years old, and the median weight was 33.2 kg. No weight was provided for 14 dogs. HSA was diagnosed in all four seasons. Most samples were collected at Tufts University (n = 65) and the University of Wisconsin (19), with between 2–9 dogs included from six other participating veterinary schools (Fig 2).

The number of samples of each type (fresh frozen HSA tumor tissue, fresh frozen non-tumor tissue, whole blood, serum) provided by the CCOGC is shown in Table 2. Of the 100 dogs with fresh frozen HSA tumor samples submitted, 74 were splenic, 14 were cardiac, and 12 were from other sites (5 liver, 2 SQ, 1 lung, 1 undetermined retroperitoneal, 1 undetermined intrathoracic, and 2 undetermined). Of the 104 dogs with fresh frozen non-tumor tissue samples submitted, 39 were splenic, 14 were cardiac, 49 were skin or various subcutaneous tissues (skin/SQ), and 2 were from other sites (1 liver, 1 kidney). The anatomic location of HSA tumor and non-tumor tissue samples is summarized in Table 2. For dogs with splenic HSA tumors, 43% (32/74) had adjacent non-tumor splenic tissue submitted and 49% (36/74) had

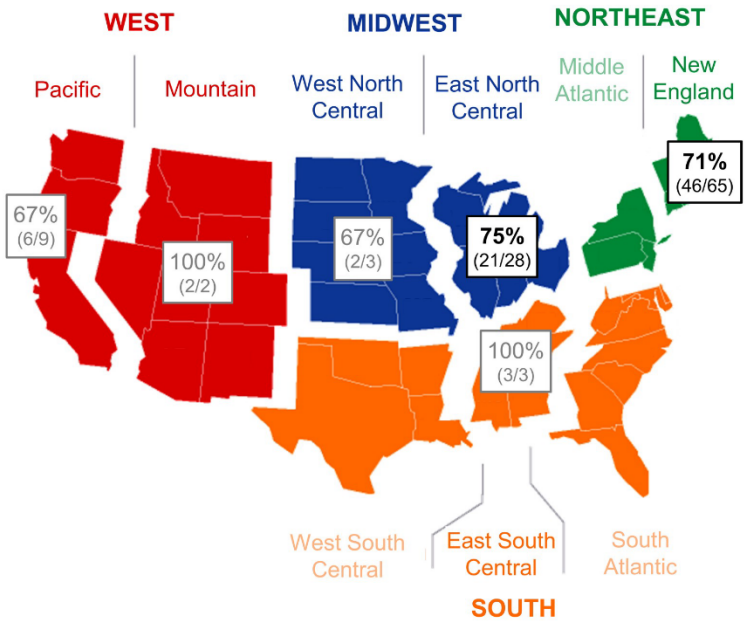

Fig 2. Geographic distribution of dogs with HSA. Shows the percentage of dogs positive for *Bartonella* DNA on PCR of HSA tumor tissue and/or non-tumor tissue biopsy. Color indicates region. Black text indicates regions from which 10 or more samples were received, and gray text indicates regions from < 10 samples were received.

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skin/SQ non-tumor tissue submitted (the remaining 6 dogs with splenic HSA tumors did not have non-tumor tissue submitted). For dogs with HSA tumors from other anatomic locations (including cardiac), the non-tumor tissues submitted were all from the affected organ.

Hemotropic pathogen testing results for all samples are summarized in Table 2. Of the 110 HSA dogs, 80 (73%) were *Bartonella* spp. PCR positive in at least one fresh frozen tissue sample. No dog was *Bartonella* spp. PCR positive on whole blood and only 6 (6%) were IFA seroreactive to *B. henselae*, *B. vinsonii* subsp. *berkhoffii*, or *B. koehlerae* antigens (Fig 3A). In these 6 dogs, 3 were only seroreactive to *B. henselae*, 2 were *B. henselae* and *B. koehlerae* seroreactive, and 1 was only *B. koehlerae* seroreactive.

With the exception of breed, there was no difference in the proportion of dogs *Bartonella* spp. PCR positive based on any of the demographic factors considered (Table 1). The proportion of German Shepherd Dogs (OR 0.60, 95% CI 0.41–0.88) and Labrador retrievers (OR 0.75, 95% CI 0.59–0.97) positive for *Bartonella* spp. DNA was significantly lower than that of mixed breed dogs. When adjusted for other potentially confounding demographic variables using multivariable logistic regression, only Labrador retrievers had a lower proportion positive for *Bartonella* spp. DNA compared to mixed breed dogs (OR 0.69, 95% CI 0.52–0.92). The proportion of *Bartonella* spp. PCR positive dogs from each geographic location, based on the location of the submitting veterinary college, is shown in Fig 2. Tthere were no statistically significant differences in *Bartonella* spp. between geographic locations (Table 1), with *Bartonella* positive dogs distributed broadly throughout the United States.

Table 2. Anatomic location of samples. Number of samples tested from each anatomic location, and number of each sample positive for each pathogen tested. Serum was tested for *Bartonella* spp. antibodies using IFA; serology for hemotropic *Mycoplasma* and *Babesia* spp. was not performed. All other samples were tested by PCR for DNA of each pathogen.

	Number of samples	Number of samples (%) *Bartonella* spp. positive	Subgroup of independently confirmed samples: # *Bartonella* spp positive/# tested (% *Bartonella* spp. positive)	Number of samples hemotropic *Mycoplasma* spp. positive	Number of samples *Babesia* spp. positive
HSA tumors					
Spleen	74	24 (32)	5/18 (28%)	1	0
Cardiac	14	8 (57)	0/0 (N/A)	0	0
Other	12	2 (17)	0/3 (0%)	0	0
TOTAL	100	34 (34)		1	0
Non-tumor tissue					
Spleen	39	22 (56)	19/35 (54%)	0	0
Cardiac	14	13 (93)	0/0 (N/A)	1	0
Skin/SQ	49	31 (63)	31/49 (63%)	1	0
Other	2	0 (0)	0/2 (0%)	0	0
TOTAL	104	66 (63)		2	0
Blood					
Whole blood	108	0	NA	2	0
Serum	107	6	NA	N/A	N/A

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Fresh frozen tissues from only 3 dogs were hemotropic *Mycoplasma* spp. PCR positive, a significantly smaller proportion (3%, $p < 0.0001$) compared to *Bartonella* spp. (Fig 4). Hemotropic *Mycoplasma* spp. DNA was amplified from two non-tumor tissues (one skin/SQ and one cardiac), and one HSA tumor (spleen). The 2 hemotropic *Mycoplasma* spp. positive non-tumor tissues were both also *Bartonella* spp. PCR positive; the hemotropic *Mycoplasma* positive tumor tissues was *Bartonella* spp. PCR negative. Hemotropic *Mycoplasma* spp. DNA was amplified from whole blood of two dogs, neither of which had hemotropic *Mycoplasma* spp. DNA in their tissues. *Babesia* spp. DNA was not amplified from any whole blood or tissue specimen (Fig 4).

The proportion of HSA tumor and non-tumor tissues *Bartonella* spp. PCR positive for each anatomic location is summarized in Fig 3B. The proportion of non-tumor tissues that were *Bartonella* spp. PCR positive (63%) was significantly higher than the proportion of HSA tumors that were *Bartonella* spp. PCR positive 34%, ($p < 0.001$). The proportion of *Bartonella* spp. PCR positive HSA tumor samples did not differ significantly between anatomic location ($p = 0.083$). Based on the multivariable logistic regression model, the anatomic location of the HSA tumor was not associated with *Bartonella* spp. PCR positivity. The proportions of non-tumor tissues *Bartonella* spp. PCR positive were significantly different based on anatomic location ($p < 0.0001$), with a higher proportion of non-tumor cardiac samples positive for *Bartonella* spp. PCR compared to non-tumor spleen samples when adjusted for possible demographic confounders (adjusted OR 1.75, 95% CI 1.25–2.47).

When comparing the *Bartonella* spp. PCR results for HSA tumor and non-tumor tissues, there was only slight agreement between the two samples (kappa = 0.14). Only 36% of dogs with a *Bartonella* spp. PCR positive non-tumor tissue sample had a positive HSA tumor, whereas 76% of dogs with a positive HSA tumor sample were positive in non-tumor tissue.

In both tumor and non-tumor samples, the most common *Bartonella* species identified was *B. henselae*. Homologies ranged from 99.3% to 100% (of 138 bp analyzed) with *B. henselae*

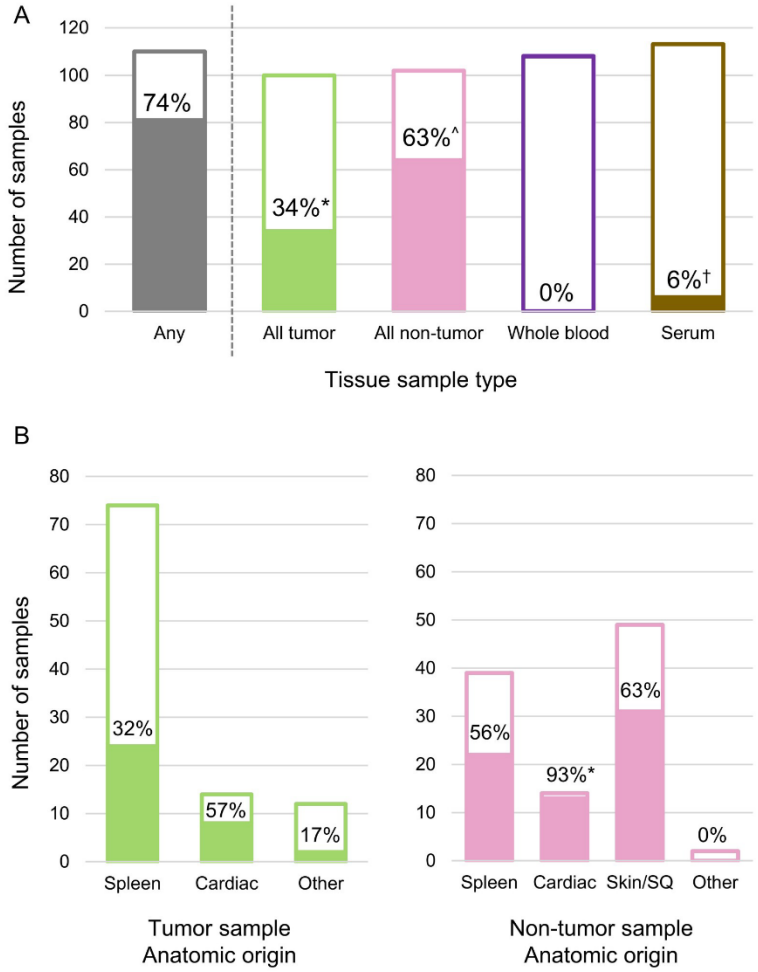

Fig 3. Proportion of samples positive for *Bartonella* spp. Color indicates sample type (HSA tumors, green; non-tumor tissue, pink; whole blood, purple; serum, brown; any sample, grey). A) Blood, serum, HSA tumors, and non-tumor tissues (all anatomic locations combined). Blood and tissue were tested by PCR for *Bartonella* spp. DNA, serum was tested by IFA for *Bartonella* spp. antibodies. Different superscripts indicate significantly different proportions ($p < 0.05$, Chi-squared test) B) Left panel shows HSA tumors by anatomic location, right panel shows non-tumor tissues by anatomic location. The * indicates a statistically significant difference in proportion from spleen samples ($p < 0.05$, Chi-squared or Fisher's exact test).

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CAL1 and SA2 (Genbank accessions AF369527and AF369529, respectively). Of the HSA tumors, 27 contained *B. henselae* (including 2 co-infected with *B. henselae* and *B. koehlerae*), 1 contained only *B. koehlerae* (140/140 bp, 100% homology with Genbank accession AF312490), 1 contained *Bartonella apis* (94/94 bp, 100% homology with Genbank accession CP015625)

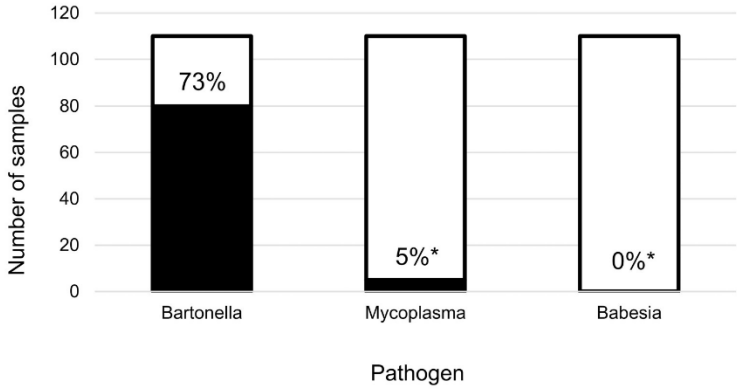

Fig 4. Proportion of dogs with HSA positive for hemotropic pathogen DNA on any sample. * indicates statistically significant difference in proportion from *Bartonella* spp. ($p < 0.05$, Chi-squared test).

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and 3 had a *Bartonella* species that was most closely related to *B. henselae* (with homology ranging from 95% to 98% with either *B. henselae* CAL1 or SA2). In non-tumor tissues, 58 contained *B. henselae*, 1 contained *B. koehelerae* (140/140 bp, 100% homology with Genbank accession AF312490), 6 contained a *Bartonella* species that was most closely related to *B. henselae* (with homology ranging from 95% to 98% with either *B. henselae* CAL1 or SA2), and 1 contained a *Bartonella* species that was unable to be identified to the species level.

When considering only those tissue samples that were able to be independently assigned an anatomic location and tumor presence based on histopathology of formalin-fixed samples performed by the investigators, the proportions of each tissue type positive for *Bartonella* spp. DNA by PCR (Table 2) was similar to that of the entire sample set. For HSA tumors in the spleen, the independently confirmed subgroup had 5 of 18 positive (28%), compared to the entire set with 32% positive ($p = 0.515$). For non-tumor tissues from the spleen, the independently confirmed subgroup had 19 of 35 positive (56%), compared to the entire set with 54% positive ($p = 0.960$).

Discussion

Overall, in this study 73% of dogs with HSA were *Bartonella* spp. PCR positive on HSA tumor tissue, non-tumor tissue, or bothtissue samples. Consistent with our previous study,[22] the proportion of *Bartonella* spp. infection in dogs with HSA was significantly greater than the proportion of *Babesia* or hemotropic *Mycoplasma* spp. Somewhat surprisingly, *Bartonella* spp. DNA was amplified more often from non-tumor tissues (63%) compared to HSA tumors tissues (34%).

There are several potential explanations for the difference in *Bartonella* prevalence between HSA tumor and non-tumor tissues. It is possible that in large vascular splenic tumors there are necrotic or infarcted regions that contain fewer organisms, placing these samples below the level of PCR detection. This possibility is supported by amplification of *Bartonella* spp. DNA from 75% of non-tumor tissues obtained from the 32 dogs that were PCR positive from their HSA tumors. It is also possible that the high prevalence of *Bartonella* DNA in non-tumor tissues reflects asymptomatic carriage and is not associated with HSA. However since all 110 dogs had HSA, the higher prevalence in non-tumor tissue does not necessarily contradict the

hypothesis that *Bartonella* infection is associated with HSA–rather, in the absence of a control group of dogs without HSA, we cannot conclude whether this high prevalence is seen in the absence of HSA as well. Further studies using a case-control or cohort design to compare *Bartonella* spp. PCR prevalence between dogs with HSA and without HSA will be needed to determine if this is the case.

While the proportion of dogs with *Bartonella* spp. DNA in one or more tissue samples was surprisingly high, no dog had *Bartonella* DNA amplified from a blood sample. This suggests that *Bartonella* DNA found in any given tissue is not due to blood contamination of the tissue, but rather that the *Bartonella* may be intracellularly localized within that tissue. Similar results have been reported previously: in one case dogs experimentally infected with *Bartonella* spp. failed to become detectably bacteremic, despite *Bartonella* spp. being isolated from tissues (bone marrow and lung) post-mortem; in another, a dog had *B. henselae* DNA amplified from biopsies of vasculitis lesions and normal skin, but no *Bartonella* DNA on multiple sequential blood samples.[40,41] Reasons that *Bartonella* DNA is not found in blood samples from dogs with *Bartonella* present in tissue could include intermittent bacteremia, rapid clearance of bacteremia by the dogs' immune system, or low levels of bacteremia that are below the current limit of PCR detection. Regardless, *Bartonella* blood PCR does not effectively predict whether a dog is infected with a *Bartonella* spp. Similarly, *Bartonella* spp. serology was also positive in only a small fraction of those dogs with positive *Bartonella* spp. PCR in tissue. Previous results documenting poor agreement between *Bartonella* spp. exposure based on IFA, and *Bartonella* spp. bacteremia in dogs have been reported.[42,43] Finally, in comparison with FFPE splenic HSA tumors tested for *Bartonella* in our previous study, *Bartonella* testing on fresh-frozen splenic HSA tumors yielded very slightly higher levels of detection (26% of fixed tissue vs. 28–32% of fresh frozen tissue). Based upon this study, we recommend that when possible, fresh frozen tissue biopsies from either the affected organ, or unaffected skin, be collected and submitted for PCR testing to maximize the potential for detection of *Bartonella* spp. Future studies will be needed to guide medical decision making when *Bartonella* spp. infection is documented in a dog with HSA.

To address the possibility that *Bartonella* spp. DNA is found in high proportions of dogs' spleens due to the spleen's role in immunologic clearance of bacteria or infected erythrocytes from circulation, we tested multiple other organs for the presence of *Bartonella* DNA. Rather than seeing the highest proportions of splenic tissue positive for *Bartonella* DNA, in both tumor and non-tumor tissues we found cardiac tissues to have a higher proportion positive. This may reflect a tissue tropism of *Bartonella* for cardiac tissue, which would be compatible with the known ability of *Bartonella* to infect heart valves as a cause of endocarditis (or less commonly, myocarditis).[44–47] Additionally, when examining non-tumor tissues, we found a similar proportion of skin/SQ samples were positive for *Bartonella* compared to spleen samples. This may reflect an alternative tissue tropism for the bacteria, which is vector-borne and relies on the bite of an arthropod vector for transmission. Establishing latent infection in the skin could be an evolutionary response to enhance the likelihood of uptake during blood feeding by arthropod vectors. Based on the results presented here, it is unlikely that the high proportion of dog spleen samples positive for *Bartonella* spp. DNA on PCR reflect solely splenic clearance of bacteria or infected erythrocytes.

Because of the surprisingly high proportion of dogs with HSA with *Bartonella* DNA in tissue samples in this study, and the well-established ability of *Bartonella* spp. to induce angiogenesis and chronic inflammation *in vivo* and *in vitro*, we speculate that *B. henselae* could be a cause or cofactor in the development of HSA in dogs. Angiogenesis is a fundamental component of primary tumor cell proliferation and metastasis, particularly in HSA.[21,48–52] Specifically, dogs with HSA have increased amounts of plasma VEGF compared to healthy dogs,[48]

VEGF and its receptors are present in tumors (when evaluated with IHC)[52] and upregulated in HSA compared to benign hemangioma[49] (and in xenograft tumors using a mouse model) [50]. *In vitro*, *B. henselae* induces angiogenesis and proliferation of endothelial cells in part by stimulating production of VEGF.[23–25,31,53,54] It is well established that *Bartonella* spp. cause the non-neoplastic endothelial proliferative disorders bacillary angiomatosis and peliosis hepatis in humans, and there have also been rare reports of these conditions in dogs infected with *Bartonella* spp.[26–31,53,55,56] In addition, there have been case reports documenting neoplastic endothelial cell tumors in humans and dogs infected with *Bartonella* spp.: *B. vinsonii* subsp. *berkhoffii* was isolated from a dog with hemangiopericytoma, and from humans residing on three continents with epithelioid hemangioendothelioma.[57,58] The high prevalence of *Bartonella* DNA in tissues from dogs with HSA supports the need for further studies on the mechanistic basis of a potential link between *Bartonella* spp. infection and vascular endothelial cancers like HSA.

Limitations of this study include the use of archived specimens, which precluded systematic sampling from particular organs of interest. This study was designed to be descriptive, and as such there was no control group consisting of dogs without HSA. Because of the lack of control group in this study, we cannot determine whether *Bartonella* spp. infection is epidemiologically associated with HSA. Additionally, only approximately one third of tumor tissue samples were able to be independently reviewed to confirm their anatomic tissue of origin and that they contained neoplastic cells. However, the overall pattern of lower Bartonella DNA prevalence in HSA tumor tissue compared to non-tumor tissue that was evident in both the spleen and cardiac tissues was also present in the subgroup of splenic tissues that did have independent histopathologic confirmation of anatomic location and tumor cell presence. There was not fixed tissue provided for independent histopathologic review from any of the cardiac samples (tumor or non-tumor), so diagnosis relied on the histopathologic reports from the submitting veterinary colleges. This may have led to misclassification of cardiac tissues as tumor or non-tumor. However due to the biological behavior of HSA in the heart, with most tumors presenting as a mass involving the right atrium (even in dogs with concurrent splenic HSA), correct classification of tissue as HSA (mass in right atrium) or non-tumor (other unaffected cardiac tissue) at the time of sample collection was assumed to be accurate. This study was also limited by using IFA to detect *Bartonella* spp. antibodies. IFA is known to have low sensitivity and may underestimate the true seroprevalence of *Bartonella* spp. in dogs.[42,59–62] The use of other serological assays currently under development, such as Western Blot or ELISA, may improve sensitivity of detection of seroreactivity. Finally, the initial power calculations for determining a statistically significant difference in *Bartonella* infection of tumors from different anatomic locations (splenic vs. cardiac) was based on 75 splenic and 75 cardiac cases. In fact, we received many fewer cardiac samples than expected, which decreased the power of this aim of the study. With the sample size used (69 splenic and 13 cardiac), the study was underpowered to detect the empiric difference that was found (33% *Bartonella* PCR positive for splenic and 54% for cardiac). In contrast, because all 105 dogs were able to be tested for each pathogen, despite the slightly smaller sample size than expected this aim was adequately powered to detect the empiric differences found (74% *Bartonella* spp. PCR positive, 3% hemotropic *Mycoplasma* PCR positive, 0% *Babesia* spp. PCR positive).

We conclude that our findings strengthen the need to further investigate the role of *Bartonella* in the development of HSA, particularly with well controlled epidemiologic studies and mechanistic research to identify how this genus may contribute to tumor development. Ultimately, the development of a vaccine to protect dogs against *Bartonella* infection could potentially decrease the prevalence of this highly malignant neoplasm.

Author Contributions

Conceptualization: Keith E. Linder, Matthew Breen, Ricardo G. Maggi, Edward B. Breitschwerdt.

Data curation: Erin Lashnits, Pradeep Neupane, Keith E. Linder, Matthew Breen.

Formal analysis: Erin Lashnits, Keith E. Linder, Ricardo G. Maggi, Edward B. Breitschwerdt.

Funding acquisition: Keith E. Linder, Matthew Breen, Ricardo G. Maggi, Edward B. Breitschwerdt.

Investigation: Pradeep Neupane, Julie M. Bradley, Toni Richardson, Keith E. Linder, Ricardo G. Maggi.

Methodology: Pradeep Neupane, Rachael Thomas, Keith E. Linder, Matthew Breen, Ricardo G. Maggi, Edward B. Breitschwerdt.

Project administration: Julie M. Bradley, Matthew Breen, Edward B. Breitschwerdt.

Resources: Rachael Thomas, Keith E. Linder, Matthew Breen, Ricardo G. Maggi, Edward B. Breitschwerdt.

Supervision: Matthew Breen, Edward B. Breitschwerdt.

Validation: Matthew Breen, Ricardo G. Maggi, Edward B. Breitschwerdt.

Visualization: Erin Lashnits, Keith E. Linder.

Writing – original draft: Erin Lashnits, Toni Richardson, Edward B. Breitschwerdt.

Writing – review & editing: Erin Lashnits, Pradeep Neupane, Julie M. Bradley, Rachael Thomas, Ricardo G. Maggi, Edward B. Breitschwerdt.

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Schizophrenia and *Bartonella* spp. infection: a pilot case-control study

Erin Lashnits, Ricardo Maggi, Fred Jarskog, Julie Bradley, Edward Breitschwerdt, Flavio Frohlich

Abstract

Recently, infections with the emerging zoonotic bacterial pathogens of the genus *Bartonella* have been reported in association with a range of CNS symptoms, some of which resolved with aggressive antibiotic treatment. Yet it remains unknown if infection with *Bartonella* spp. is associated with symptoms of schizophrenia. The objective of this study was to determine if there was an association between *Bartonella* species infection and schizophrenia. A secondary objective was to determine if schizophrenia symptoms were more severe amongst schizophrenic people with *Bartonella* spp. infection.

Using a case-control study design, 17 cases and 13 controls were evaluated with a series of clinical and cognitive assessments. Blood samples were collected and tested for *Bartonella* spp. using serological (IFA), microbiological (BAPGM) and molecular techniques (qPCR and ddPCR).

People with schizophrenia were more likely than healthy volunteers to have *Bartonella* spp. DNA in their bloodstream, with 11 of 17 cases (65%) positive by *Bartonella* spp. ddPCR. In comparison, only one healthy volunteer was positive by *Bartonella* spp. ddPCR (8%, $p = 0.0024$). Based on serology, the healthy volunteers had and unexpectedly high *Bartonella* spp. antibody prevalence, with 12 of 13 controls seroreactive to one or more *Bartonella* spp. antigen. While *Bartonella* spp. seroreactivity was also common amongst people with schizophrenia (12 of 17), because of the unexpectedly high level of exposure in the control group there was no statistically significant association between *Bartonella* spp. seroreactivity and schizophrenia ($p = 0.1961$). Among cases, there was no significant difference in schizophrenia symptom severity scores between ddPCR positive and ddPCR negative individuals.

This study provides preliminary evidence in support future investigations of the potential contribution of *Bartonella* spp. infection to the neuropsychological manifestations of schizophrenia. Future research should focus on strengthening this preliminary epidemiological association. If further substantiated, studies establishing the mechanistic underpinning of *Bartonella* spp. infection in schizophrenia, and considering treatment of *Bartonella* infection as an adjunctive therapy for schizophrenia, would be warranted.

Background

Exposure to infectious agents can cause neuropsychiatric symptoms. For some infectious diseases such as neurosyphilis, the neuropsychiatric symptoms are well known. For schizophrenia, there is substantial evidence for an association with infection by *Toxoplasma gondii*; however, the demonstration of a causal link between toxoplasmosis and symptoms of schizophrenia has remained elusive.[17–19] Given that cat ownership during childhood is robustly associated with an increased risk of developing schizophrenia, it is reasonable to consider a potential contribution of cat-transmitted infectious agents other than *T. gondii* in the etiology of schizophrenia.[20–22]

Recently, infections with the genus *Bartonella*, emerging zoonotic bacterial pathogens, have been reported to cause a range of CNS symptoms such as hallucinations and depressive symptoms that resolved with aggressive antibiotic treatment.[6,16,23–27] The domestic cat is the reservoir host for multiple species of zoonotic *Bartonella*, the most well-studied of which is *Bartonella henselae. B. henselae* is a relatively recently identified pathogen, first described in 1990.[1] Over the following decade the role of *B. henselae* as a zoonotic pathogen was further defined, as the etiologic agent of a syndrome termed “cat scratch disease” (CSD).[4,5] Bartonellosis

was therefore historically defined by CSD, an acute-onset illness that includes a triad of a cat scratch or bite, fever, and regional lymphadenopathy. However, with the advent of increasingly sensitive molecular and microbiological tools with which to diagnose *Bartonella* spp. infection, there is an increasing number of reports of atypical manifestations of bartonellosis (historically termed "atypical CSD"), including severe chronic neurological and neuropsychological manifestations and Pediatric Acute-onset Neuropsychiatric Syndrome (PANS).[6,24,26,27] Yet, to our knowledge no previous controlled epidemiologic study has directly investigated the role of *Bartonella* in a sample of people with a serious mental illness such as schizophrenia

The objective of this study was to determine if *Bartonella* spp. infection is associated with schizophrenia. A secondary objective was to compare symptom severity between people with schizophrenia who were infected with *Bartonella* to those who had no comparable evidence of infection. Our hypothesis was that infection with *Bartonella* spp. would be more common in people with schizophrenia compared to healthy controls. Secondarily, we expected *Bartonella* infected cases to exhibit more severe symptomatology.

Materials and Methods

Study Design and Setting

This was a prospective case-control study conducted at University of North Carolina-Chapel Hill and approved by the UNC-Chapel Hill Biomedical Institutional Review Board. Participants were enrolled between March 1 and October 31, 2019. Cases were recruited from the local community using fliers and newspaper advertisements. Controls were healthy volunteers recruited from the surrounding local community via targeted online and email advertising. Recruitment material did not specify the purpose of the study, thus minimizing

selection bias by not recruiting participants with concerns about potential *Bartonella* spp. exposure. Participants were paid $80 to compensate them for their time. The STROBE checklist is included as supplementary material.

Participants

Inclusion criteria for cases was a diagnosis of schizophrenia or schizoaffective disorder (confirmed by the Structured Clinical Interview for DSM-V); clinical stability as demonstrated by no psychiatric hospitalizations for the past 3 months; stable dosing of antipsychotic medications (no changes in medication or dose for 1 month prior to enrollment); ability to provide written informed consent; and at least one set of blood samples collected for microbiological and molecular testing.

Controls were considered healthy based on self-reported health status, and were excluded if reporting a previous diagnosis of schizophrenia or schizoaffective disorder.

For this pilot study, controls were not age or sex matched, due to the difficulty of predicting the age/sex structure of the case group and challenges associated with enrolling age/sex matched controls. Rather, controls were selected from a local volunteer population, expected to have similar exposures for potentially acquiring *Bartonella* infection.

Variables, Data Sources, and Measurement

Whole blood and serum were collected from each participant. To maximize the likelihood for detection of intermittent bacteremia, blood samples were collected twice within a one-week period.[28]

As described in previous studies,[29–31] each participant was tested using six indirect fluorescent antibody (IFA) assays, each representing a unique *Bartonella* species or subspecies.

A sample was considered seroreactive if *Bartonella* spp. seroreactive at an IFA titer of ≥1:64 for any one or more antigen

Bartonella alpha proteobacteria growth medium (BAPGM) enrichment blood culture and qPCR was performed as previously described.[31] Briefly, qPCR was performed on each whole blood sample; whole blood was then culture enriched in BAPGM, and DNA was extracted from the growth media and tested by qPCR at 7, 14, and 21 days of culture. All qPCR amplifications were performed by targeting the *Bartonella* intergenic 16S-23S rRNA (ITS) region. A sample was considered BAPGM/qPCR positive if any one or more of these four qPCR tests was positive on any one or more whole blood sample.

In addition to BAPGM/qPCR, all blood and BAPGM enrichment blood culture DNA extractions were tested by droplet digital PCR (ddPCR) for *Bartonella* DNA using the QX200 Droplet Digital PCR (Bio-Rad, Hercules, CA) system. Digital PCR amplification of the *Bartonella* ITS region, and the human hydroxymethylbilane synthase (HMBS) as house-keeping human reference gene, was conducted as previously described.[32] Bio-Rad QuantaSoft Analysis Pro software was utilized to track and analyze the fluorescent drop distribution and to define the positive detection threshold readings for each channel (FAM channel 1 for *Bartonella,* and HEX channel 2 for house-keeping gene amplification). A sample was considered BAPGM/ddPCR positive if any one or more of these four ddPCR tests was positive on any one or more whole blood sample. Due to technical limitations associated with ddPCR instrument design, *Bartonella* species identity could not be determined by DNA sequencing for ddPCR positive samples.

For the primary aim, study participants were considered to have *Bartonella* spp. exposure if they were *Bartonella* spp. seroreactive at an IFA titer of ≥1:64 for any one or more antigen. Participants were considered to have *Bartonella* spp. infection if they were positive on any one

or more BAPGM/qPCR or BAPGM/ddPCR assay. All *Bartonella* testing was performed by researchers blinded to participant identity and group assignment.

For the secondary aim, schizophrenia severity was measured with a series of clinical and cognitive assessments including the Positive and Negative Syndrome Scale (PANSS),[33] Brief Assessment of Cognition in Schizophrenia (BACS),[34] and Quality of Life Enjoyment and Satisfaction Questionnaire (Q-LES).[35] PANSS results were analyzed using the positive, negative, and general symptom subscores, and a total PANSS score. BACS results for each primary measure were first converted to Z scores, then the Z scores from each primary measure were averaged to create a BACS composite score. For the Q-LES, the results were normalized to the possible total score and reported as a percentage.

Data on possible confounders or effect modifiers was collected with a questionnaire (health history questionnaire, Supplementary material 2) assessing employment, geographic location, health history, and animal and insect vector contact.

Bias

To address bias, researchers performing *Bartonella* spp. diagnostic tests were blinded to sample group. Recruiting healthy volunteers as a control group may introduce bias if the volunteers have different exposures than the enrolled cases. Because of the small sample size, potential confounding was not able to be addressed in statistical analysis. Misclassification bias due to poor sensitivity or specificity of *Bartonella* spp. diagnostic testing was possible, but expected to be non-differential and would have a similar effect in both case and control groups.

Study Size

During the 8-month recruitment period, 17 cases and 13 controls were enrolled. Based on previous studies of healthy volunteers, we estimated that 15% or less of the control group would

have *Bartonella* spp. exposure.[30,36] This sample size therefore gave us 80% power at alpha <0.05 to detect a statistically significant difference in *Bartonella* infection between the cases and controls if less than 15% of controls and more than 70% of the cases had infection.

Statistical Methods

Summary statistics for demographics, *Bartonella* test results, schizophrenia symptom measures, and health history questionnaire results were calculated for cases and controls. For continuous variables, groups were compared using the Wilcoxon rank sum test for non-parametric data. For categorical variables, cases and controls were compared using chi-squared test (or Fisher's exact test for small group sizes). Correction for multiple comparisons for the primary and secondary outcome was performed using Bonferroni correction. For the remaining comparisons, including demographics, animal and insect exposure, and self-reported symptoms from the health history questionnaire, statistical significance was set at $p < 0.05$. This manuscript was prepared in accordance with STROBE guidelines for case-control studies.[37] All statistical analysis was performed in R v. 4.0.2.[38]

Results

Demographic and geographic characteristics of the cases and controls are shown in table 1. Cases were significantly older than controls. Race and employment status were both significantly different between cases and controls. Cases reported living in North Carolina for significantly longer than controls. Gender and urban/suburban/rural living environment did not differ significantly between cases and controls.

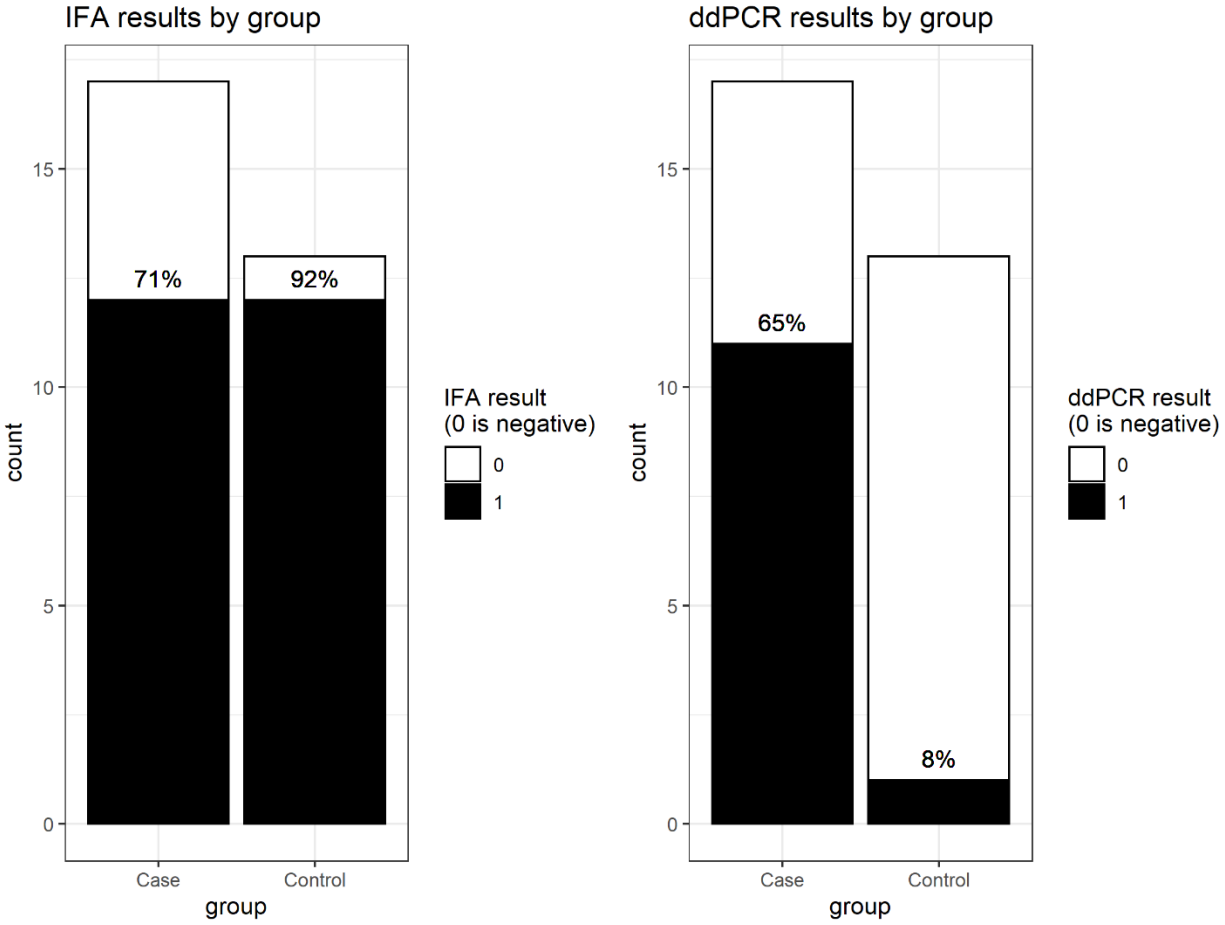
Table 1: Demographics and geography. Demographic and geographic information for study participants. P values for comparison between cases and controls, using chi-squared tests (or Fisher's Exact tests for small group numbers). Statistical significant considered at $p < 0.0083$ to correct for multiple comparisons (indicated with *).

	Control ***n = 13***	**Case** ***n = 17***	**p-value**
Age: Median (range)	20.9 (18.1, 45.4)	43.3 (29.4, 63.3)	< 0.0001 *
Sex:			0.0271
Male	3	12	
Female	10	5	
Race:			0.0004 *
Caucasian	6	9	
Asian	7	0	
African-American	0	7	
Other	0	1	
Employment:			< 0.0001 *
Student	9	0	
Medically Disabled	0	14	
Part-Time	3	2	
Full-Time	1	0	
Retired	0	1	
Years in NC: Median (range)	14 (1, 24)	28.5 (3, 49)	0.0003 *
Living Area:			
Urban	2	7	0.3105
Suburban	9	7	
Rural	2	3	

For the primary aim, *Bartonella* testing results are shown in figure 1. Based on BAPGM/ddPCR testing, *Bartonella* spp. infection was significantly more common in cases (65%, 11/17) than controls (8%, 1/13; $p = 0.0024$). Based on IFA seroreactivity, *Bartonella* spp. exposure was not significantly different between cases and controls: 71% of cases and 92% of controls were seroreactive to one or more *Bartonella* species antigens ($p = 0.1961$). BAPGM/qPCR failed to amplify *Bartonella* spp. DNA from any participant blood or enrichment

culture sample; by this enrichment blood culture/qPCR method no participant had evidence of *Bartonella* spp. infection.

Figure 1: Bartonella testing results. Left panel shows IFA results, right panel shows ddPCR results. No participant was positive on qPCR/BAPGM (not shown). Black = positive test results, white = negative test result.



Of the 12 BAPGM/ddPCR positive participants, 5 were positive only directly from whole blood and 7 were positive only after BAPGM enrichment blood culture. No participants were ddPCR positive both directly from whole blood and from BAPGM enrichment blood culture. One participant was ddPCR positive on BAPGM blood culture of their first blood sample at all three culture time points (7, 14, and 21 days of incubation); that participant's second blood sample and BAPGM enrichment blood culture from the second blood sample were all ddPCR

negative. Another participant was only ddPCR positive on BAPGM enrichment blood culture day 14 from both the first and second blood samples; whole blood and other culture time points were ddPCR negative.

Based on serology, among both cases and controls, seroreactivity against *Bartonella henselae* was the most common species (65% of cases, 92% of controls). However, most seroreactive participants had reactivity against more than one *Bartonella* species/strain. *Bartonella* spp. IFA results are shown in table 2.

Table 2: Bartonella spp. IFA results. Number (and percentage) of participants in each group that were seroreactive against each *Bartonella* species/genotype antigen tested. Bh = *Bartonella henselae*, Bk = *Bartonella koehlerae*, Bq = *Bartonella quintana*, Bvb I = *Bartonella vinsonii* subsp. *berkhoffii* genotype I, Bvb II = *Bartonella vinsonii* subsp. *berkhoffii* genotype II, Bvb III = *Bartonella vinsonii* subsp. *berkhoffii* genotype III.

	Control ***n = 13***	**Case** ***n = 17***
Bh	12 (92%)	11 (65%)
Bk	7 (54%)	7 (41%)
Bq	6 (46%)	7 (41%)
Bvb I	2 (15%)	3 (18%)
Bvb II	7 (54%)	4 (24%)
Bvb III	9 (69%)	7 (41%)

Clinical assessment test results for cases and controls are shown in table 3. As expected, cases had significantly lower BACS composite scores, and significantly higher PANSS scores in each of the three primary categories (positive, negative, general). There was no significant difference in quality of life between cases and controls as measured by the Q-LES. Among cases, there was no significant difference in any of the severity scores between BAPGM/ddPCR positive and BAPGM/ddPCR negative individuals. There were two participants with schizophrenia receiving medications previously reported to have activity against *Toxoplasma*

gondii (fluphenazine and loxapine)[39,40]: both participants were *Bartonella* spp. seroreactive, and one was positive by *Bartonella* spp. BAPGM/ddPCR.

Table 3: Schizophrenia severity scores. Median and range of severity scores based on three assessment tools for cases and controls. Statistical significant considered at $p < 0.01$ to correct for multiple comparisons (indicated with *).

	Control ***n = 13***	**Case** ***n = 17***	**p-value**
Q-LES			
Percentage	78.6 (57.1, 96.4)	69.6 (44.6, 92.9)	0.1799
PANSS			
Positive	8.5 (7, 18)	18.5 (10, 29)	< 0.0001 *
Negative	9.5 (7, 15)	14 (10, 23)	0.0019 *
General	21 (16, 29)	33.5 (20, 46)	0.0003 *
BACS			
Composite	0.739 (0.113, 1.12)	-0.390 (-1.31, 0.676)	< 0.0001 *

Animal and insect exposures are shown in table 4. There were no significant differences in dog or cat ownership, or reports of dog or cat bites, between cases and controls. A significantly higher proportion of cases (41%) reported exposure to bedbugs compared to controls (0, $p = 0.0273$). A higher proportion of cases (41%) reported exposure to fleas compared to controls (15%), but this difference was not statistically significant. There were no other significant differences in reported exposure to potential insect vectors between cases and controls. When all participants were considered together, there were no significant differences in dog or cat ownership, reports of dog or cat bites, or exposure to potential insect vectors between seropositive or BAPGM/ddPCR positive participants and those who were seronegative or BAPGM/ddPCR negative.

Table 4: Reported exposures. Number of participants reporting each type of exposure. Years of ownership reported as median and range, otherwise number of participants reported. Statistical significant considered at $p < 0.0036$ to correct for multiple comparisons (indicated with *).

	Control ***n = 13***	**Case** ***n = 17***	**p value**
Dog Contact:			
Number of participants who currently own a dog	6	4	0.3619
Years participant has owned a dog (median, range)	4 yrs (1, 11)	8 yrs (1, 41)	0.2887
Number participants bitten/scratched by dog	7	6	0.5193
Cat Contact:			
Number participants who currently own a cat	4	3	0.6844
Years participant has owned a cat (median, range)	8.5 yrs (1, 22)	20 yrs (2.5, 48)	0.1325
Number participants bitten/scratched by cat	9	7	0.2473
Insect Bites:			
Mosquitoes	12	14	0.8003
Bees	8	11	1
Ticks	6	6	0.8215
Fleas	2	7	0.2603
Spiders	3	4	1
Biting Flies	4	4	0.9778
Bedbugs	0	7	0.0273
Lice	3	1	0.406

Based on the health history questionnaire, the most commonly reported symptoms were different between cases and controls (table 5). Cases also reported a significantly higher number of symptoms (median 8, range 1-24) compared to controls (median 0, range 0-3; $p < 0.0001$).

Table 5: Reported health symptoms. Most common symptoms reported on the health history questionnaire, cases and controls.

Control ***n = 13***		**Case** ***n = 17***	
Sleepiness	3	Hallucinations	13
Fatigue	1	Difficulty remembering	10
Headache	1	Mental confusion	10
Difficulty sleeping	1	Weight Gain	9
n/a		Depression	8

Paired *Bartonella* testing results were missing for 6 cases for IFA: 4 cases did not return for the second blood draw, 1 case returned but the blood sample was unable to be drawn due to difficulty obtaining venous access, and for 1 case the sample hemolyzed during storage and

serum was unable to be separated. Paired *Bartonella* testing results were missing for 7 cases for BAPGM/ddPCR and BAPGM/qPCR: 4 cases did not return for the second blood draw, and 3 cases returned but whole blood was unable to be drawn due to difficulty in obtaining venous access. All controls had paired blood testing results available. Comparisons of *Bartonella* test results between paired samples are shown in table 6. For IFA, paired (first and second timepoint for same participant) results showed substantial agreement (kappa = 0.71). In contrast, paired results for BAPGM/ddPCR showed agreement slightly less than chance alone (kappa = -0.01). When comparing IFA to BAPGM/ddPCR results for the same participant, agreement was also less than chance alone (kappa = -0.31).

Table 6: Paired Bartonella test results. Agreement between paired samples from all participants (drawn between 2-8 days apart) for IFA and ddPCR, and agreement between IFA and ddPCR for each participant. Cohen's kappa score indicates agreement between tests.

	All participants	**Kappa**
IFA Test/Retest:	***n = 24***	0.71
Pos/Pos	15	
Pos/Neg	3	
Neg/Pos	0	
Neg/Neg	6	
ddPCR Test/Retest:	***n = 23***	- 0.01
Pos/Pos	1	
Pos/Neg	5	
Neg/Pos	3	
Neg/Neg	14	
ddPCR/IFA:	***n = 30***	-0.31
Pos/Pos	7	
Pos/Neg	5	
Neg/Pos	17	
Neg/Neg	1	

Discussion

This study found that people with schizophrenia were more likely than healthy volunteers to have evidence of *Bartonella* spp. infection, with 11 of 17 cases (65%) *Bartonella* spp.

BAPGM/ddPCR positive. In comparison, only one healthy volunteer (8%) was *Bartonella* spp. BAPGM/ddPCR positive. A surprisingly high proportion of healthy volunteers had *Bartonella* spp. antibodies, with 12 of 13 controls seroreactive to one or more *Bartonella* species. *Bartonella* spp. seroreactivity was similarly common among people with schizophrenia (12 of 17), with no statistically significant difference between the groups.

The use of ddPCR allows absolute quantification of target DNA sequences with increased efficiency and sensitivity compared to qPCR. This is possible by massively partitioning samples (15,000 to 20,000 1ηL sized droplets per sample).[41,42] One of the most significant advantages of ddPCR over its real-time qPCR counterpart is that by sequestering target DNA among individual droplets, ddPCR reduces the impact of inhibitory substances (including hemoglobin, heparin, and high concentrations of host DNA) that would typically compete with low-concentration pathogen DNA during amplification. In this way, the analytical sensitivity of ddPCR is improved compared to qPCR for detection of low-concentration pathogen DNA.[32,43]

The 5 participants who were ddPCR positive directly from whole blood samples but negative following enrichment culture of those same blood samples suggest that viable bacteria was not present in blood, or that bacterial growth occurred only transiently or below the limit of ddPCR detection sensitivity. In contrast, the 7 participants who were ddPCR positive only after BAPGM enrichment culture of their blood suggest that viable bacteria were present in their blood at the time of blood collection. Two unique participants were presented in the results – one was ddPCR positive on BAPGM blood culture of their first blood sample at all three culture time points (days 7, 14, and 21), but negative from blood and all BAPGM enrichment blood cultures from the second blood sample. In addition to highlighting the microbiological complexity

associated with detecting *Bartonella* spp. infection in immunocompetent people, this result also supports the possibility that *Bartonella* spp. induce a relapsing bacteremia.

The reason for such high prevalence of *Bartonella* spp. antibodies in the healthy volunteers is unknown. Previous studies of healthy control populations have found a wide range of seroreactivity, seemingly depending on the number of *Bartonella* spp. antigens tested, the cutoff titer utilized to define a seropositive result, and the population under study. At the high end, *Bartonella* spp. seroreactivity was documented in 83% of 100 participants, using a six-antigen panel (the same panel as used in this study) when enrolling healthy volunteers from among physicians, nurses, researchers, medical students, and administrative personnel who worked at the Center of Biomedical Research (including the Center of Rickettsiosis and Arthropod-borne Diseases) and San Pedro's University Hospital at La Rioja, in northern Spain.[44] In contrast, early studies (now over 20 years old) using one or two antigens and blood from blood donors have shown seroreactivity as low as 3-7%.[5,45] More recently, sampling from diverse populations have shown seroreactivity anywhere from 3%, in a convenience sample of adult students and employees at Duke University Medical Center (Durham, NC),[30] to 32% of 500 blood donors in Campinas, a large city in southeastern Brazil, using serology performed at the Centers for Disease Control and Prevention (Atlanta, GA).[36] The differences in seroreactivity can be partially explained by differences in exposures, further highlighting the importance of sampling from an appropriate control population with similar exposures to the case population in a case-control study. The study from Duke University showing 3% seroreactivity in healthy volunteers[30] highlights the need to control for other confounders than simply geography, since that study and this one used the same panel of 6 *Bartonella* spp. antigens and recruited healthy volunteers from locations within the same geographic area of North Carolina.

Among participants with schizophrenia, *Bartonella* spp. infection was not associated with more severe symptoms. However, this study included a small sample size (17 people with schizophrenia), so it was likely underpowered to detect such differences. With a larger sample size, it would be possible to better understand whether *Bartonella* infection is associated with positive or negative symptoms, or differences in more specific domains of cognition and overall functional capacity.

The case and control samples in this study had multiple significant differences, including age, race, employment status, and years living in North Carolina. These demographic and lifestyle factors are possible confounders, and could not be adjusted for in analysis due to the small sample size for this initial study. While there may prove to be an association between *Bartonella* spp. infection and schizophrenia, an epidemiological association does not imply a causal link. Future epidemiological studies investigating a possible association between *Bartonella* spp. infection and schizophrenia should either use a matched design, or have a sample size large enough to control for these confounders in the statistical analysis.

There were no significant differences in animal exposures between cases and controls. While cat ownership has been previously implicated as a risk factor for schizophrenia, in this small sample cat ownership was not associated with schizophrenia. In fact, cat ownership was more common among healthy volunteers (31%) than people with schizophrenia (18%), though this difference was not statistically significant. Of relevance to *B. henselae* transmission, bites or scratches from a cat, as well as exposure to fleas, was commonly reported among both cases and controls. Exposure to bedbugs was more common among cases than controls: over 40% of cases reported exposure to bedbugs, while bedbug exposure was not reported by any healthy volunteers. Bedbugs have been shown to be competent vectors for *Bartonella quintana*,[46] a

human-adapted *Bartonella* species, but in a survey of bedbugs from across the United States, *Bartonella* DNA was not amplified from any of the >300 bedbugs screened.[47]

Paired *Bartonella* spp. IFA tests showed substantial agreement for both cases and controls, indicating that most seropositive participants likely had not been acutely exposed to *Bartonella* spp. but rather had previous exposures leading to relatively stable antibody titers. In contrast, paired BAPGM/ddPCR results had very poor agreement. This may be due to relapsing or intermittent bacteremia in people with chronic *Bartonella* infection, as has been documented in *Bartonella quintana* infection in people,[48–51] and other *Bartonella* spp. in cats[52] and rodents.[53,54] Additionally, IFA and BAPGM/ddPCR results often did not agree, indicating the possibility that seroreactivity may be providing some degree of protection, or that *Bartonella* antibodies effectively suppress the number of circulating bacteria below the level of BAPGM/qPCR or even BAPGM/ddPCR detection.

There are several limitations to this study. The specificity of *Bartonella* spp. IFA has been questioned due to the potential for cross-reactivity with other bacterial organisms – mainly *Coxiella* spp., but also other Rickettsia-like pathogens including *Ehrlichia* and *Chlamydia* species.[55–58] However epidemiologic studies do not consistently support the existence of cross-reactivity, but rather suggest that at least in some cases presumed "cross-reactivity" is due to exposure to multiple bacterial pathogens and that IFA specificity is high.[30,59,60] Sensitivity of IFA, however, is often poor: patients with demonstrated *Bartonella* spp. infection are often seronegative, particularly in patients with chronic infections.[61–63] When positive, serology can only provide evidence for previous exposure. Low sensitivity is also a concern for BAPGM/qPCR, as evidenced by none of the participants having a positive BAPGM/qPCR despite multiple participants being positive by BAPGM/ddPCR.[59] Unfortunately the droplets

generated during ddPCR cannot be obtained for DNA sequencing, so it is currently impossible to determine the *Bartonella* species detected in these participants by ddPCR. To increase the likelihood of detection of *Bartonella,* two blood samples were collected from each participant. However, paired samples were unable to be obtained from 7 of the 17 cases because they either did not return for their second visit, or because of difficult blood draws; in contrast all controls had both blood samples available. This difference could lead to bias, since fewer samples for cases were tested than controls, but this would bias the results toward the null hypthesis and lead to an underestimate of the difference between cases and controls.

Given the limitations of this study, no conclusions regarding a causal role for *Bartonella* in schizophrenia should be inferred. However, the results of this pilot study do support the need for a more in-depth examination of the epidemiology of *Bartonella* infection in people with schizophrenia. Such a larger follow-up study would control for confounding variables and would lay the groundwork for subsequent studies to investigate if any potentially observed effects are replicable across sample populations. Finally, if the epidemiological association between *Bartonella* infection and schizophrenia is replicated, studies could be designed both to test whether *Bartonella*-targeted antimicrobial therapy can ameliorate one or more domains of schizophrenia symptoms, as well to test the hypothesis that *Bartonella* infection may contribute to the pathogenesis of schizophrenia for affected individuals.

CHAPTER 6

Conclusions

The research presented here aimed to describe the epidemiologic landscape of *Bartonella* spp. exposure in dogs, characterize potential risk factors for exposure and describe possible transmission scenarios. Additionally, the clinical research studies included here evaluated one potential disease manifestation in dogs, and another in people, among the many currently proposed for investigation.

In Chapter 2, the seroepidemiologic study[64] found that *Bartonella* exposure was most common in male intact dogs, with no seasonal trend to exposure risk (unlike in humans)[65], and associated with exposure to a number of other vector-borne pathogens. *Bartonella* exposure was also possible broadly across the United States and Canada, with wide geographic variation. Chapter 3 provided a detailed follow-up investigation of possible drivers of that variation in exposure, specifically within North Carolina.[66] That study reported a number of demographic, ecological, and socioeconomic associations with *Bartonella henselae* exposure in dogs; these potential drivers were thought more likely to be associated with ticks as vectors of *B. henselae* transmission, particularly because fleas can live for multiple generations entirely indoors thus circumventing the role of many of the ecological and climatological drivers that would otherwise affect their abundance. Whether ticks truly play an ecologically important role in *Bartonella* spp. transmission to/among dogs remains unclear, and is worthy of further study in naturally infected dogs.

Next, Chapter 4 reported the unexpected circumstances surrounding an apparent cluster of *Bartonella* spp. infections in laboratory beagles, which allowed for an individual-level investigation of possible transmission in dogs.[67] Testing of blood samples previously collected

for the purposes of another study, followed by prospective collection and testing of blood, saliva, and tissue, provided evidence for possible non-vectorial transmission of *B. henselae* between dogs in this highly controlled laboratory setting. Non-vectorial routes of transmission of *Bartonella* spp. – including through bites or saliva – have been previously proposed,[15,16,68,69] but this outbreak investigation provided the first laboratory-animal evidence for direct transmission among dogs. The exact route(s) of potential direct transmission, and the extent to which saliva might be infectious, warrant further investigation. Further ecological and laboratory research will be needed to determine whether non-vectorial transmission is possible, and whether it is an epidemiologically relevant source of exposure for dogs.

Together these studies highlight the gaps that remain in evaluating *Bartonella* exposure in dogs, namely that the source of exposure for most dogs (like for most infected people) remains unknown. Much of the variation in exposure risk appears to be at an individual or household scale – whether a dog is routinely treated with flea and tick preventatives, or has regular contact with potentially infected cats or other reservoir hosts, for example. In the past, case reports (many from the Breitschwerdt laboratory) have documented infection with the same *Bartonella* spp. in people and their pets,[15,16,68] but only in rare or unusual circumstances have investigations included people, pets, and potential vectors.[26,70] Previous studies have instead focused solely on infection of potential vectors, infection or exposure of pets or dogs/cats in shelters, or infection/exposure in various human populations with or without animal or vector contact.[23,30,63,71] These gaps will need to be addressed by prospective studies targeting this hyper-local level of data collection, and investigating humans, their companion animals, and the surrounding environments to effectively define exposure risks. Ultimately, from a One Health perspective, the techniques developed and used to investigate and define exposure risk in dogs

can be adapted to human studies to more definitively determine risks to human health associated with *Bartonella* spp. exposure.

The current state of knowledge of canine bartonellosis is mainly anecdotal, with most evidence of disease associated with *Bartonella* spp. infection in dogs – other than endocarditis – from case reports or case series. The observational study of *Bartonella* in dogs with HSA presented in Chapter 5 reported that an intriguingly high proportion of dogs with HSA – nearly 75% – have *Bartonella* DNA in their tissues.[72] However, in the absence of a control group (due to that study's design) an epidemiologic association could not be made. A case-control study will be needed in the future to determine whether there is such an association, and if confirmed, further research into a potential mechanistic basis for *Bartonella* infection to cause HSA would be warranted. From the clinical research side, if evidence for this association is found, a treatment trial could also be considered – both as a way to help improve survival in dogs with this uniformly fatal diagnosis, but also to investigate potential causation.

Finally, in Chapter 5, a small pilot case-control study reported that a significantly higher proportion of people with schizophrenia had *Bartonella* spp. DNA in their blood (65%), when compared to healthy volunteers (8%). While this was a small sample, including only 17 schizophrenic people, this study provides justification to investigate this potential association with a larger case-control study that can effectively control for possible demographic confounding. If the association is borne out, a randomized controlled trial treating *Bartonella* infection in schizophrenic patients could provide evidence for a novel treatment modality for the medical management of this difficult disease.

While studies of potential clinical manifestations of *Bartonella* spp. in dogs and people are needed to begin to elucidate specific syndromes of bartonellosis, the true impact of

bartonellosis – on both a patient and population level in both human and veterinary medicine – remains unknown. This is largely because of the difficulty obtaining a definitive clinical diagnosis – a challenge in humans that is, unsurprisingly, recapitulated in veterinary medicine. The range of non-specific and variable symptoms currently reported with *Bartonella* spp. infection causes uncertainty for clinicians treating all species, placing more importance on the role of an accurate gold standard diagnostic test. However, current reference standard diagnostic tests for *Bartonella* infection in both human and veterinary medicine remain far from the perfect gold standard.[73,74] While not explicitly addressed by the studies here, this remains a significant gap in knowledge that limits understanding of *Bartonella* from a clinical, epidemiological, and ecological perspective, and should therefore be the focus of future research. One method by which to address this shortcoming is through the use of a statistical modeling technique known as latent class modeling (LCM) to create estimates of sensitivity and specificity of these diagnostic tests in the context of imperfect reference standards, the results of which would serve to inform testing strategies for not only clinical patients, but also future ecological and epidemiological studies.

Further understanding of *Bartonella* infection will most effectively been gained with a true One Health approach. While investigating *Bartonella* infection in cats helps to elucidate transmission risk in ecological and epidemiological studies, as well as for individual human patients, accurately diagnosing dogs may help by providing a natural model system in which to study zoonotic *Bartonella* species. As has been seen with Lyme Disease, the dog may have similar exposure risks and biological/immunological response to *Bartonella* spp. infection as humans, so that both transmission risks and disease manifestations may be similar. Investigating *Bartonella* spp. infection in both cats and dogs is, therefore, of paramount importance to

informing our understanding of human *Bartonella* spp. transmission and infection and ultimately protecting human health.

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