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STANDARD ARTICLE

Evaluation of cell culture-grown *Bartonella* antigens in immunofluorescent antibody assays for the serological diagnosis of bartonellosis in dogs

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Background: Because of poor sensitivity and questionable specificity of immunofluorescent antibody assays (IFAs), serological diagnosis of *Bartonella* species infections in dogs remains challenging. Despite limitations, IFA testing is the historical "gold standard" for *Bartonella* serodiagnosis in animals and humans. Because most diagnostic laboratories test against only 1 or 2 *Bartonella* spp., testing against a broader panel of *Bartonella* antigens may enhance diagnostic sensitivity and specificity.

Objective: To evaluate the sensitivity and specificity of *Bartonella* IFA using 8 cell culture-grown *Bartonella* spp. isolates.

Animals: Archived serum samples from 34 *Bartonella* spp. naturally exposed, polymerase chain reaction (PCR)-positive dogs and from 26 PCR-negative and IFA-negative dogs.

Methods: *Bartonella* IFA sensitivity and specificity were assessed using cell culture-grown whole cell antigens derived from 3 *Bartonella henselae* (*Bh*) strains (*Bh* Houston 1, *Bh* San Antonio Type 2, *Bh* California 1), 3 *Bartonella vinsonii* subsp. *berkhoffii* genotypes (*Bvb* I, II, and III), *Bartonella koehlerae* (*Bk*), and *Bartonella quintana* (*Bq*).

Results: Only 62% of 34 *Bartonella* spp. PCR-positive dogs were seroreactive to any of the 8 *Bartonella* IFA antigens, indicating low IFA sensitivity. PCR-positive dogs were most often IFA seroreactive to *Bq* (n = 15), to *Bvb* II (n = 13), or to both (n = 9) antigens. Of the 26 previously IFA-negative/PCR-negative dogs, 4 (15%) were seroreactive using the expanded antigen panel.

Conclusion and Clinical Importance: Despite IFA testing of dogs against 8 different *Bartonella* isolates, IFA sensitivity remained poor, and specificity was only 85%. Development of a reliable serological assay is needed to facilitate the diagnosis of *Bartonella* infection in dogs.

KEYWORDS

bacteria, BAPGM, serology, vector-borne, zoonosis

Abbreviations: BAPGM, *Bartonella* alpha-proteobacteria growth medium; *Bcl*, *Bartonella clarridgeiae*; *Bh* CAL1, *Bartonella henselae* California 1; *Bh* H1, *Bartonella henselae* Houston 1; *Bh* SA2, *Bartonella henselae* San Antonio 2; Bh, *Bartonella henselae*; *Bk*, *Bartonella koehlerae*; *Bq*, *Bartonella quintana*; *Br*, *Bartonella rochalimae*; *Bvb*, *Bartonella vinsonii* subspecies *berkhoffii*; CVBD, canine vector-borne diseases; IFA, immunofluorescent antibody assay; ITS, intergenic transcribed spacer; NCSU-CVM-VBDDL, North Carolina State University, College of Veterinary Medicine, Vector Borne Diseases Diagnostic Laboratory; PBS, phosphate buffered saline; spp., species

1 | INTRODUCTION

Bartonellosis is an emerging, vector-borne zoonotic disease that affects dogs throughout much of the world. *Bartonella* spp. are transmitted to mammals by arthropod vectors, including ticks, fleas, keds, lice, mites, and sand flies.[1–3] Various arthropods transmit different *Bartonella* spp. among reservoir and incidental hosts, thereby

complicating and confounding clinical, diagnostic, and epidemiological analyses.[4–7] Currently, serology, as well as culture-based and polymerase chain reaction (PCR) assays, are relatively insensitive for the diagnosis of bartonellosis in dogs.[8–10]

Immunofluorescent antibody assays (IFAs) are the most frequently used serological testing modalities for the diagnosis of bartonellosis in dogs.[8,11,12] Studies involving dogs, humans, and other animals have reported inconsistent and variable sensitivities and specificities for *Bartonella* IFAs.[13–18] Genetically different *Bartonella* spp. and strains are widespread in humans and animals throughout the world.[19–21] Therefore, a possible explanation for variation among studies is exposure to *Bartonella* spp., subspecies, or strain that differs from the IFA antigen used for diagnostic testing.[12,13,16] Diagnostically important differences in *Bartonella* serological responses have been documented in animals and human patients depending on which *Bartonella* isolate/strain was used as an antigen.[13,16,22] Further complicating diagnoses, clinical signs, pathologic sequelae, and antibody kinetics can vary among individual animals infected with the same *Bartonella* strain.[23–25] Because members of the genus *Bartonella* can induce long-lasting bacteremia, the stage of infection (acute, subacute, or chronic) also contributes to variation in antibody detection.[23,24] Subjectivity associated with IFA interpretation and variability in technical variables among laboratories further contribute to differences in antibody detection or reported antibody titers. Thus, *Bartonella* spp. serodiagnosis is influenced by variations in bacterial, host, and laboratory variables.

Although 10 *Bartonella* spp. have been implicated in association with endocarditis, myocarditis, or other disease manifestations in dogs; *Bartonella henselae* (*Bh*), *Bartonella koehlerae* (*Bk*), and *Bartonella vinsonii* subsp. *berkhoffii* (*Bvb*) have been the most frequently documented species in North America.[20,21] Historically, *Bvb* represented the first *Bartonella* spp. isolated from dogs.[26] Therefore, initial IFA testing used *Bvb* as the sole antigen source for diagnostic and research purposes. Subsequently, 4 *Bvb* genotypes and several other *Bartonella* spp. were found to infect dogs, including *B. clarridgeiae (Bcl)*, *B. elizabethae*, *Bh*, *Bk*, *Bq*, *B. rochalimae (Br)*, *B. volans*, and *B. washoensis*.[27] After natural or experimental infection with a *Bartonella* spp., dogs develop a species-specific IFA antibody response.[12,28] However, bacteremic sick dogs frequently are seroreactive to multiple IFA antigens or alternatively they are not *Bartonella* spp. seroreactive despite extended illness durations.[8,15] Ideally, a serological assay used for epidemiological or diagnostic purposes should detect antibodies regardless of the infecting *Bartonella* spp., genotype, or strain. Currently, because there are at least 38 named and Candidatus *Bartonella* spp., with nearly half implicated in association with infections of dogs or humans, we posed the question: Would a broader panel of *Bartonella* spp. antigens increase the serodiagnostic sensitivity and specificity of IFAs? We hypothesized that a comprehensive panel of *Bartonella* spp. isolates would increase IFA serodiagnostic sensitivity, while optimizing specificity. Therefore, the purpose of our study was to evaluate the sensitivity and specificity of 8 IFAs using archived serum samples from *Bartonella* spp. naturally-exposed (PCR-positive) and presumptively non-exposed (seronegative/PCR-negative) dogs.

2 | MATERIALS AND METHODS

2.1 | Source of sera for immunofluorescent antibody assays testing

Sixty archived sera from dogs previously tested at the North Carolina State University, College of Veterinary Medicine, Vector Borne Diseases Diagnostic Laboratory (NCSU-CVM-VBDDL) were selected for comparative IFA testing against 8 cell culture-grown *Bartonella* spp. antigens. Serum samples were categorized into 2 groups to assess sensitivity and specificity. All sera were submitted to the NCSU-CVM-VBDDL for diagnostic testing between 2011 and 2016. After initial processing by the NCSU-CVM-VBDDL, sera were stored at −80 °C.

2.1.1 | Group I (polymerase chain reaction positive dogs)

Group I consisted of 34 stored frozen serum samples from *Bartonella* spp. naturally infected dogs (PCR-positive) for which the species, genotype, or strain was confirmed by DNA sequencing. We could only identify 34 *Bartonella* PCR-positive dogs with adequate sera to test against all 8 antigen preparations. Veterinarians often request only serology or only PCR, thus the number of matched specimens for inclusion in our study was limited. Group I sera were used to examine the sensitivity of each of the 8 IFAs. *Bartonella* 16S–23S intergenic transcribed spacer (ITS) region DNA was amplified from blood (n = 31) or *Bartonella* alpha-Proteobacteria growth medium (BAPGM) enrichment blood culture (n = 3), after which amplicons from each dog were sequenced for the confirmation of bacterial strain and species. Dogs were infected with *Bh* (n = 20), *Bvb* (n = 6), *B. vinsonii* (DNA sequence incomplete to determine subsp. n = 2), *Bcl* (1), *Br* (2), *Bk* (2), and *Bq* (1). For Group I dogs, *Bartonella* PCR-positive cases were included regardless of the tests requested by the attending clinician. Therefore, comprehensive serology and PCR testing was not available for most dogs in Group I.

2.1.2 | Group II (immunofluorescent antibody assay negative and polymerase chain reaction negative dogs)

Group II consisted of 26 dogs for which diagnostic testing was negative for evidence of exposure to canine vector-borne disease (CVBD) organisms that are routinely tested for in the NCSU-CVM-VBDDL. These sera were used to assess the specificity of the 8 IFAs. Sera from all 26 dogs were PCR-negative after whole blood DNA extraction for *Bartonella*, *Babesia*, *Ehrlichia*, *Anaplasma*, *Rickettsia*, hemotropic *Mycoplasma* and *Leishmania* spp. In addition, all sera were IFA nonreactive (titers <1:16) to the 3 *Bartonella* spp. (*Bh* SA2, *Bvb* I, and *Bk*), *Rickettsia rickettsii*, *Ehrlichia canis*, *Babesia canis*, *Babesia gibsoni* and were not seroreactive to *Anaplasma phagocytophilum*, *Anaplasma platys*, *Borrelia burgdorferi*, *Ehrlichia canis*, and *Ehrlichia ewingii* by ELISA (SNAP 4Dx PLUS ELISA, IDEXX Laboratories, Westbrook, Maine).

2.1.3 | Immunofluorescent antibody assay serology assays

Bartonella spp. antibody reactivity was determined by following traditional IFA practices with fluorescein-conjugated goat anti-dog IgG (Cappel, ICN, Costa Mesa, CA), as described previously.[12] *Bartonella* organisms derived from clinical isolates representative of common serotypes of *Bartonella* spp., specifically, *Bh* (strains *Bh* H1, *Bh* SA2,

and *Bh* CAL1), *Bvb* (genotypes I, II and III), *Bk*, and *Bq* each were grown in cell culture for antigen preparation. Previously frozen stocks of isolates were grown on blood agar plates. Once colonies were plentiful, bacterial cultures were passed into *Bartonella*-permissive cell lines, DH82 (a canine monocytoid cell line) for *Bvb* genotype I (NCSU 93CO-01, ATCC type strain #51672),[26] *Bvb* genotype II (NCSU 95CO-08),[29] *Bh* H1 (NCSU 93FO-23), *Bh* SA2 (NCSU 95FO-099), *Bk* (NCSU 09FO-01), and *Bq* (NCSU 11MO-01), FCR68 (a canine monocytoid cell line) for *Bh* CAL1 (NCSU 08HO-2424), and Vero cells (a monkey fibroblast cell line) for *Bvb* genotype III (NCSU 06CO-01).[30] *Bartonella* isolates used for antigen preparation in our study had not been passaged more than 2 times. For each isolate, cellular preparations were diluted to achieve a single layer of evenly-spaced cells that were 50% to 80% infected with bacteria when layered onto 30-well Teflon-coated slides. Acetone fixation was used for pathogen inactivation, to adhere cells to the slides and to permeate cellular membranes so that secondary anti-canine fluorescein-conjugated molecules could access antigens expressed within cells.

For IFA testing, serum was diluted in phosphate buffered saline (PBS) solution containing 1% normal goat serum, 0.05% Tween-20, and 0.5% powdered nonfat dry milk to block nonspecific antigen-binding sites. Sera were screened at 1:16 to 1:64 dilutions, after which reactive sera were titered out at 2-fold dilutions to 1:8192. To avoid interpretation issues induced by nonspecific background fluorescence found at low dilutions (1:16 or 1:32), a cutoff titer of 1:64 was used to define a seroreactive titer.

2.1.4 | Positive and negative control sera

Serum from a naturally exposed dog (VB09–01611) as confirmed by prior serology testing (seroreactive to *Bh* H1 and *Bvb* I) at the NCSU-CVM-VBDDL was used as a positive IFA control. This dog's serum was reactive to all 8 *Bartonella* spp. antigens with endpoint titers that varied from 1:1024 to 1:8192. The negative control serum sample also was from a diagnostic accession (VB16–06078) and was nonseroreactive and PCR-negative to the same tick-borne pathogens used to screen Group II dogs. In addition, the negative control sera was not reactive to any of the 8 *Bartonella* spp. antigens at 1:16, 1:32 or 1:64 dilutions.

3 | RESULTS

3.1 | Group I (polymerase chain reaction positive dogs)

Seroreactivity to each of the 8 *Bartonella* spp. antigens is summarized in Table 1. Sixty-two percent of *Bartonella* spp. PCR-positive dogs were seroreactive to at least 1 of 8 *Bartonella* spp. antigens, of which 41% (14/34) were seroreactive to ≥2 *Bartonella* antigens, whereas 38% of the PCR-positive dogs were not seroreactive to any of the 8 test antigens (Table 1). Seven PCR-positive dogs were seroreactive to only 1 *Bartonella* spp. antigen, of which 4, 2, and 1 dog were seroreactive to *Bq*, *Bvb* II, and *Bvb* III, respectively.

Among the 8 selected antigen preparations, PCR-positive dogs most often were seroreactive to *Bq* (44%) and *Bvb* II (38%; Table 1). Although *Bq* antigen yielded the highest sensitivity compared to other selected *Bartonella* antigens, there was considerable background fluorescence compared to the other IFA preparations, making technician interpretation challenging at low serum dilutions. When individual results for *Bq* and *Bvb* II seroreactivity were combined, diagnostic use of these 2 antigens would have resulted in the identification of 19/21 IFA seroreactive/PCR-positive dogs, but would not have identified seroreactivity for the remaining 13 dogs. Historically, our laboratory used only *Bvb* I (the first dog isolate of a *Bartonella* spp. and the ATCC-type strain) for serodiagnostic testing.[26]

The IFA sensitivities for *Bvb* I (35%), *Bvb* II (38%), and *Bvb* III (35%) antigens were similar (Table 1). If all the 3 *Bvb* genotype antigens were used for diagnostic IFA testing, 50% (17/34) of *Bartonella* spp. PCR-positive dogs would have been seroreactive, of which 8/17 dogs were seroreactive to all 3 (*Bvb* I, *Bvb* II, and *Bvb* III) genotypes. Among the 3 *Bh* IFA antigens, use of *Bh* SA2 provided the highest sensitivity (24%) followed by *Bh* H1 (21%) and *Bh* CAL1 (15%). Sensitivity of IFA would have been only 26% (9/34) if all 3 *Bh* antigens were used for IFA testing. Only 18% (6) of the 34 PCR-positive dogs were *Bk* seroreactive.

To evaluate the potential association between PCR status and seroreactivity, PCR results of Group I dogs were compared with the corresponding IFA seroreactivity. Of the 21 IFA seroreactive dogs, 5 (3 *Bvb* II, 1 *Bcl*, and 1 *Br* PCR-positive) were seroreactive to all 8 *Bartonella* spp. antigens. Of the remaining 16 dogs, 7 were seroreactive to 1, 4 to 4, 3 to 2, 1 to 6, and 1 to 3 *Bartonella* antigens. Eleven (55%) of 20 *Bh* PCR-positive dogs were not seroreactive to any of the 8 *Bartonella* antigens and 18/20 were not seroreactive to any of the 3 *Bh* antigens. Of the 8 *Bvb* PCR-positive dogs (4 *Bvb* II, 1 *Bvb* I, 1 *Bvb* III, and 2 *Bvb* [genotype undetermined based upon limited DNA sequence]), 7 were seroreactive to at least 1 *Bartonella* antigen. The *Bvb* I PCR-positive dog was seroreactive to *Bvb* I, *Bvb* III, and *Bq*. One *Bvb* II PCR-positive dog was not reactive to any of the 8 *Bartonella* antigens, whereas the remaining 3 *Bvb* II PCR-positive dogs were seroreactive to all 8 *Bartonella* antigens. One dog each, infected with *Bvb* III and *Bvb* (genotype undetermined) was IFA seroreactive to all 3 *Bvb* genotypes and *Bq*. The other *Bvb* (genotype undetermined) infected dog was only *Bq* seroreactive. Among the subset of 8 *Bvb* PCR-positive dogs, 6 were seroreactive to at least 2 of the 3 *Bvb* antigens in the panel, whereas 2/8 were not seroreactive to any of the 3 *Bvb* antigens.

3.2 | Group II (immunofluorescent antibody assay negative/polymerase chain reaction negative dogs)

Of the 26 PCR-negative nonseroreactive dogs, 22 dogs (85%) were not seroreactive to any of the 8 *Bartonella* IFA antigens (Table 1). One dog was seroreactive to *Bvb* II, *Bvb* III, *Bh* H1, *Bh* SA2, and *Bh* CAL1, and 1 dog each was seroreactive to either *Bvb* II, *Bvb* III, or *Bq* antigens. No Group II dog was seroreactive to *Bvb* I or *Bk* antigens.

4 | DISCUSSION

In our study, sera from *Bartonella* PCR-positive dogs varied from nonseroreactive to strongly seroreactive to all 8 IFA antigen preparations.

TABLE 1 Comparison of dog sera IFA seroreactivity with PCR assay

Groups	PCR results (*N* = PCR positive)	Number of *Bartonella* spp. seroreactors								Number of dogs nonseroreactive to all 8 antigens	Dogs sero reactive to any *Bh* Ag	Dogs sero reactive to any *Bvb* Ag	Total dogs sero reactive to any Bartonella spp.
		Bh H1	*Bh* SA2	*Bh* CAL1	*Bvb* I	*Bvb* II	*Bvb* III	*Bk*	*Bq*				
Group I	*Bh* (20)	2	1	0	2	4	3	0	3	11			
	Bvb I (1)	0	0	0	1	0	1	0	1	0			
	Bvb II (4)	3	3	3	3	3	3	3	3	1			
	Bvb III (1)	0	0	0	1	1	1	0	1	0			
	Bvb (2)[a]	0	0	0	1	1	1	0	2	0	9 (26%)	17 (50%)	21 (62%)
	Bk (2)	0	1	0	1	1	0	0	2	0			
	Bq (1)	0	0	0	0	0	0	0	0	1			
	Bcl (1)	1	1	1	1	1	1	1	1	0			
	Br (2)	1	2	1	2	2	2	2	2	0			
		7 (21%)	8 (24%)	5 (15%)	12 (35%)	13 (38%, 2*)	12 (35%, 1*)	6 (18%)	15 (44%, 4*)	13			
Group II	PCR-negative	1 (4%)	1 (4%)	1 (4%)	None	2 (7%, 1*)	1 (4%)	None	1* (4%)	22	1 (4%)	3 (12%)	4 (15%)

Comparison of IFA seroreactivity results among dogs infected with various *Bartonella* species, subspecies or strains. Serology results for each of the eight IFA antigens were compared with *Bartonella* spp. PCR/DNA sequencing status. Results are reported as the number of dogs that were seroreactive to each antigen (antibody titers ≥1:64) or were not seroreactive to any of the 8 antigens (IFA antibody titers ≤1:32). **Group I** (*Bartonella* spp. PCR+) and **Group II** (*Bartonella* spp. IFA negative and PCR negative) dogs are reported separately. Results for each group are reported as the number and percentage of dogs that were seroreactive (antibody titers ≥1:64). * represents number of dogs that were seroreactive only to that *Bartonella* antigen by IFA testing.

[a]ITS DNA sequence inadequate to genotype.

Abbreviations: Ag: Antigen; ***Bcl:*** *Bartonella clarridgeiae*; ***Bh* CAL1**: *Bartonella henselae* California 1; ***Bh* H1:** *Bartonella henselae* Houston 1; ***Bh* SA2:** *Bartonella henselae* San Antonio 2; ***Bh:*** *Bartonella henselae*; ***Bk:*** *Bartonella koehlerae*; ***Br:*** *Bartonella rochalimae*; ***Bvb:*** *Bartonella vinsonii* subspecies *berkhoffii*; ***Bvb* I**: *Bartonella vinsonii* subspecies *berkhoffii* genotype I; ***Bvb* II:** *Bartonella vinsonii* subspecies *berkhoffii* genotype II; ***Bvb* III:** *Bartonella vinsonii* subspecies *berkhoffii* genotype III; ***Bq:*** *Bartonella quintana*, **spp:** species; **IFA:** immunofluorescent antibody assay; **ITS:** intergenic transcribed spacer; **PCR:** polymerase chain reaction.

Despite testing against 8 *Bartonella* spp. or strains, only 62% of *Bartonella* spp. PCR-positive dogs were seroreactive to any test antigen. Based on our results, increasing the number of *Bartonella* spp. IFA antigens is not a practical approach to increase IFA sensitivity for the serodiagnosis of bartonellosis. Increasing the number of antigens would substantially increase diagnostic testing costs associated with the time and materials required for antigen growth in cell culture followed by IFA slide preparation, increased IFA conjugate requirements, and substantially increased technician time for interpretation of each antigen preparation. Of the 8 IFA assays examined, seroreactivity most often was documented to *Bq* (44%), followed by *Bvb* II (38%), *Bvb* I (35%), *Bvb* III (35%), and *Bh* SA2 (24%). In contrast to poor antigenic sensitivity, IFA specificity to the panel of 8 antigens was at least 85% in 26 *Bartonella* spp. PCR-negative dogs. Similar to the findings reported in previous studies,[4,8] our study documented a lack of agreement between infection (PCR or BAPGM culture/PCR) and IFA seroreactivity (infection or exposure).

Among the subset of PCR-positive/IFA seroreactive dogs, 90% (19/21) would have been identified if tested using only the *Bvb* II and *Bq* IFA assays, but 13 Group I dogs were not *Bvb* II or *Bq* seroreactive, further illustrating IFA sensitivity limitations. Clinically, *Bq* and all 4 *Bvb* genotypes have been isolated or detected in dogs or humans with endocarditis, myocarditis, and neurological diseases.[26,29–32] Although *Bvb* I was the first *Bartonella* spp. isolated from a dog, *Bvb* II was subsequently found to be a more frequent infection in dogs and humans tested in our diagnostic (dogs) or research laboratory (humans).[29,32] *Bq* has been isolated from 2 dogs with endocarditis,[33] from *Ctenocephalides felis*, the common cat and dog flea,[34] and from a woman and subsequently from the feral cat that bit her.[35] Collectively, these and other observations suggest that the epidemiology of *Bq* in dogs, a historically important *Bartonella* spp. that caused Trench Fever in WWI, should be reassessed, particularly in light of the possibility that cat fleas and head lice, as well as the human body louse may transmit this organism.[36,37]

Despite testing dogs against 3 different *Bh* strains, only 10% of the 20 *Bh* PCR-positive dogs were *Bh* IFA seroreactive, which is even less than noted in a previous study[8] in which only 25% of *Bh*-infected dogs were *Bh* IFA seroreactive to a single *Bh* strain. In contrast to the *Bh* IFA results, 9 of 20 *Bh* bacteremic dogs were seroreactive (IFA titers ≥1:64) to at least 1 of the 3 *Bvb* genotypes, *Bq* antigens, or both. Whether these disparate IFA results in *Bh* PCR-positive dogs reflect serological cross-reactivity or prior exposure to other *Bartonella* spp. is unknown. In contrast to the overall lack of seroreactivity among *Bh*-infected dogs, most dogs that were PCR-positive for *Br*, *Bcl*, and *Bvb* II were seroreactive to all 8 *Bartonella* antigens, including reactivity (*Bh* titer ≥1:64) to all 3 *Bh* strains. Collectively, our results indicate that seroreactivity patterns in PCR-positive dogs are highly variable and often do not correlate with the organism that was PCR amplified and sequenced from their blood.

Previously, we documented a lack of cross-reactivity to *Bartonella* spp. antigens when testing sera from dogs experimentally infected with *Rickettsia rickettsii* or *Ehrlichia canis*, 2 organisms that are alpha proteobacteria phylogenetically closely related to *Bartonella* spp. in the evolutionary microbial tree of life.[12,15,38]Also, cross-reactivity among *Bh*, *Bvb*, and *Bk* antigens did not occur in 2 dogs infected with each of these 3 *Bartonella* spp.[28] Canine vector-borne disease (CVBD) serology results (15 451 diagnostic submissions) generated between January 1, 2008 and December 31, 2014 at the NCSU-CVM-VBDDL recently were retrospectively reviewed.[39] *Bh* (2.13%), *Bk* (2.39%), and *Bvb* I (1.42%, $P < 0.0001$) seroreactivities among dogs tested because of suspicion of a vector-borne disease were low, further supporting the specificity of *Bartonella* spp. IFA testing.

In an effort to further examine IFA specificity, we assessed sera from 26 dogs that were PCR-negative/IFA-negative to our panel of vector-borne pathogens and IFA nonseroreactive to the 3 *Bartonella* spp. (*Bh* SA2, *Bvb* I, and *Bk*) used diagnostically in the NCSU-CVM-VBDDL. When tested against the 8 *Bartonella* antigens, 85% (22/26) were not seroreactive to any antigen and no dog was seroreactive to *Bvb* I or *Bk* IFA antigens routinely used for serodiagnostic testing of dogs in our laboratory. Collectively, our research to date supports low sensitivity, but relatively good specificity for the historical panel of *Bartonella* antigens used in the NCSU-CVM-VBDDL. Interestingly, 3 Group II dogs were only *Bq*, *Bvb* II, or *Bvb* III seroreactive, respectively. If *Bq*, *Bvb* II, or *Bvb* III seroreactivity reflects exposure solely to *Bq*, *Bvb* II, or *Bvb* III, rather than nonspecific IFA immunofluorescence, our historical antigen panel has most likely underestimated *Bartonella* seroprevalence in dogs.[15,39] The remaining PCR-negative/IFA-negative dog was seroreactive to *Bvb* II and III, *Bh* H1, and *Bh* SA2. This dog was previously reported IFA-negative using the NCSU-CVM-VBDDL antigen preparations. This finding illustrates that IFA antibody titers should be interpreted with caution because of variability among antigen preparations (ie, the species or strain used for testing), immunoglobulin conjugates, and the diagnostic technician's interpretation of specific versus nonspecific immunofluorescence.

Although serology, culture, and PCR remain the mainstays for diagnosing bartonellosis in dogs, clinicians and diagnosticians should recognize that each of these assays lacks sensitivity, specificity, or both.[5,40,41] These limitations adversely influence seroepidemiological conclusions; potentially result in the transfusion of *Bartonella* spp. bacteremic donor blood into sick dogs; impede attainment of an accurate microbiological diagnosis upon which to base-directed therapy; and, may adversely influence patient outcomes. Although an effective antibiotic regimen for the treatment of bartonellosis in dogs has not been established, treatment failures have been associated with both short-term and long-term antibiotic administration.[27,42] Thus, an accurate diagnosis of bartonellosis in dogs is critical to facilitate directed antimicrobial treatment, and to avoid the unnecessary use of antibiotics that may contribute to antimicrobial resistance.

In conclusion, we report comparative data for 8 cell culture-grown *Bartonella* isolates used in IFAs for serodiagnosis of bartonellosis. With the rapid expansion of characterized *Bartonella* spp., many of which appear to be pathogenic for dogs, a serological assay that confirms exposure to any *Bartonella* spp. would be optimal. Currently, there are no highly sensitive serological assays to determine if a dog has been exposed to or is infected with a *Bartonella* spp. Determining which *Bartonella* spp., genotype or strain a dog has been exposed to may have important epidemiological or zoonotic implications. Based on existing knowledge, however, these phylogenetic classifications currently do not influence antibiotic selection, treatment duration, or prognosis. Increasing the number of *Bartonella* spp., genotypes or

strains used in IFA panels is technically time consuming, increases the cost of diagnostic testing and, based upon the results of our study, does not substantially enhance overall diagnostic sensitivity. Our findings will be used in future research efforts aimed at improving the serodiagnosis of *Bartonella* spp. infections in dogs.

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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.

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

Samples were obtained from the diagnostic sample back at the Vector Borne Disease Diagnostic Laboratory at North Carolina State University, College of Veterinary Medicine so no IACUC was required.

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