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Analysis of Seroreactivity against Cell Culture–Derived *Bartonella* spp. Antigens in Dogs

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Background: Little is known about the specificity of *Bartonella* spp. immunofluorescent antibody (IFA) assays in dogs. Bacteremia in sick dogs most often has been associated with *Bartonella henselae* (*Bh*), *Bartonella vinsonii* subspecies *berkhoffii* (*Bvb*), and *Bartonella koehlerae* (*Bk*). Clarification of the diagnostic utility of IFA serology when testing against these organisms is needed.

Objective: To evaluate the specificity of *Bartonella* IFA assays utilizing 6 cell culture–grown antigen preparations.

Animals: Archived sera from SPF dogs (n = 29) and from dogs experimentally infected with *Bvb* (n = 10) and *Bh* (n = 3).

Methods: Antibodies (Abs) to *Bvb* genotypes I, II, and III, *Bh* serotype I, strains H-1 and SA2, and to *Bk* were determined by IFA testing.

Results: Serum from naïve SPF dogs shown to be negative for *Bartonella* bacteremia did not react with any of the 6 *Bartonella* antigens by IFA testing. Dogs experimentally infected with *Bvb* genotype I developed Abs against homologous antigens, with no cross-reactivity to heterologous *Bvb* genotypes, *Bh* H-1, SA2 strains, or to *Bk*. Dogs experimentally infected with *Bh* serotype I developed Abs against *Bh* H-1, but not to *Bh* SA2 strain with no cross-reactive Abs to *Bvb* genotypes I–III or to *Bk*.

Conclusions and Clinical Importance: *Bartonella* spp. Ab responses during acute experimental infections are species and type specific.

Key words: *Bartonella henselae*; *Bartonella koehlerae*; *Bartonella vinsonii*; Serodiagnostic limitations; Specificity.

Bartonella spp. constitute an environmentally diverse genus of vector-transmitted bacteria that appear to be the cause of an expanding spectrum of disease manifestations, and as such are considered important emerging pathogens in dogs worldwide.[1–3] Using a sensitive detection platform that combines *Bartonella* alpha-Proteobacteria growth medium (BAPGM) enrichment culture with polymerase chain reaction (PCR), *Bh, Bvb*, and *Bk* represent the most frequent species infecting dogs in North America.[2] When the BAPGM enrichment blood culture/PCR was used by Bai and colleagues to test dogs from Thailand, infection with additional rodent reservoir-adapted *Bartonella* spp. was identified.[4] Four *Bvb* genotypes have been implicated in bacteremic infections in cats, dogs,[2] horses, humans, and a red wolf (*Canis lupus rufus*).[5–7] Two serotypes of *Bh* including strains Houston-1 (serotype I), San Antonio-2 (serotype I), and California-1 (serotype II)[8] have been detected in cats, dogs, and humans. *Bvb, Bh,* and *Bk* also have been implicated in canine and human cases of endocarditis.[9]

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Abbreviations

Ab	antibody
Bh	*Bartonella henselae*
Bvb	*Bartonella vinsonii* subspecies *berkhoffii*
Bk	*Bartonella koehlerae*
Bartonella henselae strains	Houston-1 = H-1, San Antonio-2 = SA2, and California-1 = CAL-1
Bcl	*Bartonella clarridgeiae*
bp	base pair
BAPGM	*Bartonella* alpha-Proteobacteria growth medium
IFA	immunofluorescent antibody assay
ITS	intergenic transcribed spacer region
ELISA	enzyme-linked immunosorbent assay
PCR	polymerase chain reaction
PID	postinoculation day(s)
NCSU-CVM VBDDL	North Carolina State University-College of Veterinary Medicine Vector Borne Disease Diagnostic Laboratory
SPF	specific pathogen free purpose-bred dogs

Serosurveys involving convenience samples of canine sera using laboratory strains of *Bartonella*, primarily *Bh* (H-1), *Bvb* (genotype I), and *Bartonella clarridgeae* (*Bcl*), have identified a range of seroprevalence rates from 1 to 35% in dogs.[10–13] Among 1872 healthy working dogs tested against *Bvb* by ELISA, 162 (8.7%) were seroreactive.[10] A study that compared healthy (n = 99) and sick (n = 301) dogs by IFA using *Bh* and *Bvb* antigens showed that 10.1% of healthy dogs were seroreactive against *Bh*, whereas only 1% reacted with *Bvb*. The sick dog group had seroprevalence rates of 27.2 and 4.7% to *Bh* and *Bvb*, respectively.[11] Of 1920 dogs tested in a hospital setting and presumed sick, 69 (3.6%) were

seroreactive to *Bvb* by IFA, with higher prevalence associated with outdoor lifestyle conditions.[12] A third study, again using ELISA assays with various *Bartonella* spp. antigens and a population of 3,417 sick dogs, demonstrated an overall *Bartonella* spp. seroreactivity in 102 (3.0%), with breed and outdoor lifestyle associated with seroreactivity. The antigens used indicated possible differences in specificity because 36/102 (35.3%) were reactive against *Bh*, 34/102 (33.3%) against *Bcl*, and only 2/102 (2%) against *Bvb*.[13] Cross-reactivity or exposure to multiple species was suggested by the 29.4% that showed reactivity to >1 antigen in that study. Without the aid of molecular or culture methods, definitive identification of more than 1 etiologic agent is difficult to achieve.

The purpose of this study was to evaluate *Bartonella* IFA serologic specificity by testing serum obtained from naïve SPF dogs and from dogs experimentally infected with *Bvb* or *Bh* against an expanded panel of *Bartonella* spp. antigens grown in tissue culture. In 1 previous study that tested dog sera by Western immunoblotting, no differences in seroreactivity were documented when agar culture–grown *Bvb* was compared with *Bvb* grown in DH82 cells.[14] The decision to grow diagnostic antigens in cell lines or to use agar-grown bacteria has been based largely upon laboratory preference or historical use patterns. For obligate intracellular pathogens, such as *Rickettsia* and *Ehrlichia* spp., the use of cell lines is mandatory to achieve organism growth while the cellular background provides an added benefit of providing a structural context for bacterial visualization. A number of mammalian cell lines (Vero, Hep-2, HeLa, and DH82) have been utilized successfully for the cultivation of *Bartonella* antigens for IFA testing.[15,16] In 2 previous studies, no serologic cross-reactivity was observed between *Bvb* genotype I and *Bh* when serum from dogs experimentally infected with *Bvb* or *Bh* was used in IFA tests utilizing tissue culture–grown antigens.[11,14] However, as a rapidly expanding number of *Bartonella* spp. have been found to infect dogs,[1–3] additional studies to characterize canine humoral immune responses are warranted to better understand potential serodiagnostic benefits and limitations.

Materials and Methods

Sources of Sera

Serum samples used in this study had been stored at −80°C. Dogs that were experimentally infected were in studies approved by the appropriate Institutional Animal Care and Use Committees (North Carolina State University or Colorado State University). Overall, serum samples came from the following sources:

Negative Controls. Archived serum samples from these 29 dogs were tested as a component of the present study to represent a set of negative control sera. These included sera from 13 SPF dogs that were *Bartonella* seronegative (<1 : 16) and agar culture negative before inoculation in 2 experimental *Bartonella* infection studies conducted at NCSU between 1995 and 1998[3,17] and sera from 16 SPF retired breeder beagles, also *Bartonella* seronegative as well as PCR and BAPGM enrichment culture negative before use in experimental *Bartonella* exposure studies conducted at CSU (Lappin, MR, unpublished data).

Bvb *Experimental Infection.* Archived serum samples collected from 10 dogs in the 1995 and 1998 experimental infection studies were chosen for testing against 6 *Bartonella* antigens. These 10 serum samples from purpose-bred dogs had been collected on days 28 or 34 after IV inoculation with 10^8–10^9 colony-forming units of agar-grown *Bvb* genotype I.[3,17] Based on blood agar culture using the lysis centrifugation method or *Bartonella* spp. 16-23S intergenic transcribed spacer region (ITS) PCR of DNA extracted from whole blood during the previous studies, bacteremia was confirmed in dogs selected from the 1995 and 1998 studies with evidence of persistence of infection of at least 1-month duration as shown by PCR or culture for all 10 dogs. Infection status also was assessed in these 2 previous experimental studies[3,17] by documentation of *Bvb* seroconversion in all dogs within the first 2 weeks after inoculation with reciprocal IgG antibody titers against *Bvb* I antigen rising from <16 to reciprocal titers ranging from 64 to 4,096.

Bh *Experimental Infection Study*. Cats inoculated with the CSU-1 strain of *B. henselae* (serotype I) were infested with *Ctenocephalides felis* fleas that then were allowed to migrate through mesh enclosure barriers to 2 adjacent cages housing dogs. Sera from 3 of the 16 SPF dogs in this study were selected based upon documentation of *Bh* DNA in peripheral blood using *Bartonella* ITS PCR of BAPGM-enriched blood cultures. Serum samples from these dogs were used for further testing against 6 *Bartonella* antigens in the present study. Evidence of infection also had been assessed during the CSU study (unpublished data) by documentation of reciprocal IgG antibody titers to *Bh* H1 antigen rising in all 3 dogs from <16 to reciprocal titers of 64 after 5 weeks of flea exposure.

Bartonella *IFA Serologic Testing*

Antibody titers to *Bvb, Bh,* and *Bk* antigens were determined in all 42 sera by IFA assays in the Intracellular Pathogens Research Laboratory, the research component of the NCSU-CVM VBDDL. *Bartonella* strains were isolated from naturally infected cats or dogs with species characterizations made by PCR amplification and DNA sequencing. Canine isolates of *Bvb* genotype I (NCSU 93CO-01, ATCC type strain #51672),[18] *Bvb* genotype II (NCSU 95CO-08)[19], and *Bvb* genotype III (NCSU 06CO-01)[20] and feline isolates of *Bh* H-1 strain (NCSU 93FO-23), *Bh* SA2 strain (NCSU 95FO-099), both considered serotype I[8], and *Bk* (NCSU 09FO-01) colonies were passed from agar plate–grown cultures into *Bartonella*-permissive cell lines, DH82 cells (a canine monocytoid cell line) for *Bh* strains H-1 and SA2, *Bvb* I and *Bk* and Vero cells (a mammalian fibroblast cell line) for *Bvb* II and III to obtain antigens for IFA testing. For each antigen, heavily infected cell cultures were spotted onto 30-well Teflon-coated slides (Cel-Line[a]), air dried, acetone-fixed, and stored frozen. Serum samples diluted in phosphate-buffered saline containing normal goat serum, Tween-20, and powdered nonfat dry milk to block nonspecific antigen binding sites[21] 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 (ICN[a]) was used to visualize bacteria within cells using a fluorescent microscope.[b] To avoid confusion with possible nonspecific binding found at low dilutions, a cutoff of 1 : 64 was used to define a seroreactive titer.

Results

SPF Dogs

Sera from 29 blood culture–negative SPF dogs were not reactive (<1 : 16) to any of the 6 *Bartonella* antigens tested.

Bvb *Experimental Infection Study*

Infection previously had been confirmed in all 10 *Bvb* genotype I-inoculated dogs by bacterial isolation on agar or PCR from postmortem tissues. During the course of the earlier study, *Bvb* genotype I IFA titers progressively ranged from 1 : 64 to 1 : 4,096. In the serum samples examined for this study from PID 28 or 34, IgG titers against the homologous Bvb antigen were between 1 : 256 and 1 : 1,024, whereas titers against *Bvb* genotypes II and III, *Bh,* and *Bk* were consistently negative at 1 : 16.

Bh *Experimental Infection Study*

Three dogs experimentally infected with *Bh* by flea exposure and proven *Bh*-bacteremic by culture in BAPGM with detection of *Bartonella* DNA by PCR developed Ab response against *Bh* H-1 (titers of 1 : 128), but not to *Bh* SA2. In addition, there was no seroreactivity against *Bvb* genotypes I, II, and III or to *Bk* antigens.

Discussion

Based upon the results obtained from this study, naïve SPF dogs were not seroreactive to the panel of *Bartonella* spp. antigens. Serum samples from experimentally infected dogs (seronegative and PCR-negative) with *Bvb* genotype I or *Bh* serotype I developed a specific antibody response to the infecting organism. For example, dogs experimentally infected with *Bvb* genotype I reacted to the homologous antigen with no cross-reactivity to heterologous *Bvb* genotypes, *Bh* or *Bk* antigens. Serum samples obtained from dogs experimentally infected with *Bh* by fleas reacted to *Bh* H-1, but not to *Bh* SA2, *Bk,* or *Bvb* genotypes I, II, or III. If infection with other *Bartonella* spp. results in similar antigenic specificity in dogs, serology will have major diagnostic limitations, unless large panels of antigens are used to test each patient's serum. There are now over 30 named or Candidatus *Bartonella* spp. and at least 14 *Bartonella* spp. have been associated with human infections[22] whereas 8 *Bartonella* species have been associated with infections in dogs.[23]

In a 1997 study conducted at NCSU, *Bvb* antibody reactivity was tested by IFA in SPF dogs experimentally infected with *Ehrlichia canis* (n = 10) and *Rickettsia rickettsii* (n = 10). Neither of these organisms induced cross-reactions against *Bvb* I antigens.[12] In a subsequent study, there was no cross-reactivity against *Bh* antigens in sera from 6 dogs experimentally infected with *R. rickettsii* and 7 dogs infected with *E.canis*.[11] Although *Bartonella, Ehrlichia,* and *Rickettsia* all are genera within the class alpha Proteobacteria, cross-reactivity across genera was not detected in these 2 experimental infection studies. In human *Bh* and *B. quintana* endocarditis patients, serologic cross-reactivity has been reported to *Chlamydophila psittaci* and *Coxiella burnetii*.[24] Based upon the results of this study and previously published results, it seems more likely that reactivity to multiple genera is the result of prior exposure to, or concurrent infection with, other organisms rather than cross-reactivity among phylogenetically related genera. As some dogs and human patients are seroreactive to multiple *Bartonella* sp. antigens, chronic infection with a specific *Bartonella* sp. could result in repeated exposure of the host to bacterial antigens and potentially different antigens over time, which might result in cross-reactivity among *Bartonella* species or strains. In addition, sequential or concurrent transmission of >1 vector-borne *Bartonella* sp. may further complicate the clinical and microbiologic interpretation of serology results in naturally exposed dogs.

Failure to detect antibodies by IFA testing in dogs infected with *Bartonella* spp. has been previously documented.[2] For reasons that remain unclear, while using the same IFA assays as described in this study, seroreactivity was not detected in the majority of *Bh*-infected dogs (75% seronegative) or in approximately half of *Bvb*-infected dogs tested in a previous NCSU study involving 61 actively infected (based upon enrichment culture PCR and DNA sequencing) sick dogs.[2] Serotypes and genotypes of the *Bh*- and *Bvb*-infected dogs, respectively, were not specified in the previous 61-dog study; however, the same 3 *Bvb* genotypes were used for IFA testing in that study as were used in the current study. Inasmuch as *Bh* H-1 and *Bh* SA2 (both serotype I strains) are the predominant 16S-23 ITS strain types identified in dogs tested by the NCSU-VBDDL, a serotype II isolate, ie, the Marseille serotype, was not included in the previous[2] or the current study. Therefore, some IFA-negative serology results may have been attributable to the test strain selected by the laboratory for IFA testing.[8] Thus, IFA serology appears to have low diagnostic sensitivity, and negative IFA results should be interpreted with caution in seroepidemiologic studies, when testing individual dogs, or when screening healthy dogs as potential blood donors. Similarly, a subset of 69 of 92 *Bartonella*-infected humans did not have IFA antibodies to the infecting *Bartonella* species or genotype in another NCSU study using the same antigen sources described in this study.[25]

We conclude that dogs develop a *Bartonella* species– or *Bvb* genotype–specific antibody response after experimental IV inoculation or flea-associated infection with a defined *Bartonella* sp. As illustrated by a recent study that examined Bartonella infections in a multi-dog and human household,[26] there is greater ecologic and serodiagnostic complexity associated with exposure to, and infection with, ≥1 *Bartonella* spp. than is currently appreciated in veterinary or human medicine. Also, with added sensitivity in PCR and culture modalities, the evidence of blood-borne infections caused by a diversity of *Bartonella* spp. as illustrated in pet dogs in the United States[2] or stray dogs in Thailand[4] is increasing. Because of the escalating number of *Bartonella* spp. that are being described around the world, the use of IFA to diagnose acute *Bartonella*

infections may be problematic unless patient sera are tested against an extensive panel of antigens.

Footnotes

[a] Thermo Fisher Scientific, Waltham, MA
[b] Carl Zeiss Microscopy, LLC, Thornwood, NY

Acknowledgments

Conflict of Interest: In conjunction with Dr Sushama Sontakke and North Carolina State University, Dr Breitschwerdt holds U.S. Patent No. 7,115,385; Media and Methods for cultivation of microorganisms, which was issued October 3, 2006. He is the chief scientific officer for Galaxy Diagnostics, a company that provides diagnostic testing for the detection of *Bartonella* species infection in animals and human patients. Dr Ricardo Maggi has led research efforts to optimize the BAPGM platform and is the Scientific Technical Advisor for Galaxy Diagnostics. All other authors have no potential conflicts.

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