

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

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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 1*, *Bartonella vinsonii* subspecies *berkhoffii* genotype 1; *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.

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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.<sup>1,2</sup> Studies on *Bartonella* exposure in dogs have described an epidemiologic association between spotted fever group *Rickettsia* spp. (SFGR) and *Bartonella* species.<sup>3-7</sup> 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 coinfecting 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<sup>8-10</sup>—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.<sup>1,9-11</sup> 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.<sup>12-16</sup> There are also reports implicating transmission by bites or suggesting the possibility of viable *Bartonella* spp. bacteria in the mouth or saliva.<sup>17-19</sup> In Korea, *Bh* DNA was PCR-amplified from over 15% of pet canine saliva samples and almost 30% of toenail samples,<sup>20</sup> and in the United States 5 of 44 Golden Retrievers sampled had *Bartonella* spp. DNA on oral swabs.<sup>21</sup> *Bartonella henselae* DNA was found in the saliva of a man with angioedema of the tongue and in his healthy dog,<sup>22</sup> and in eastern China *Bartonella* exposure was associated with dog bites.<sup>23</sup> 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.<sup>24,25</sup> 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,<sup>26</sup> 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).

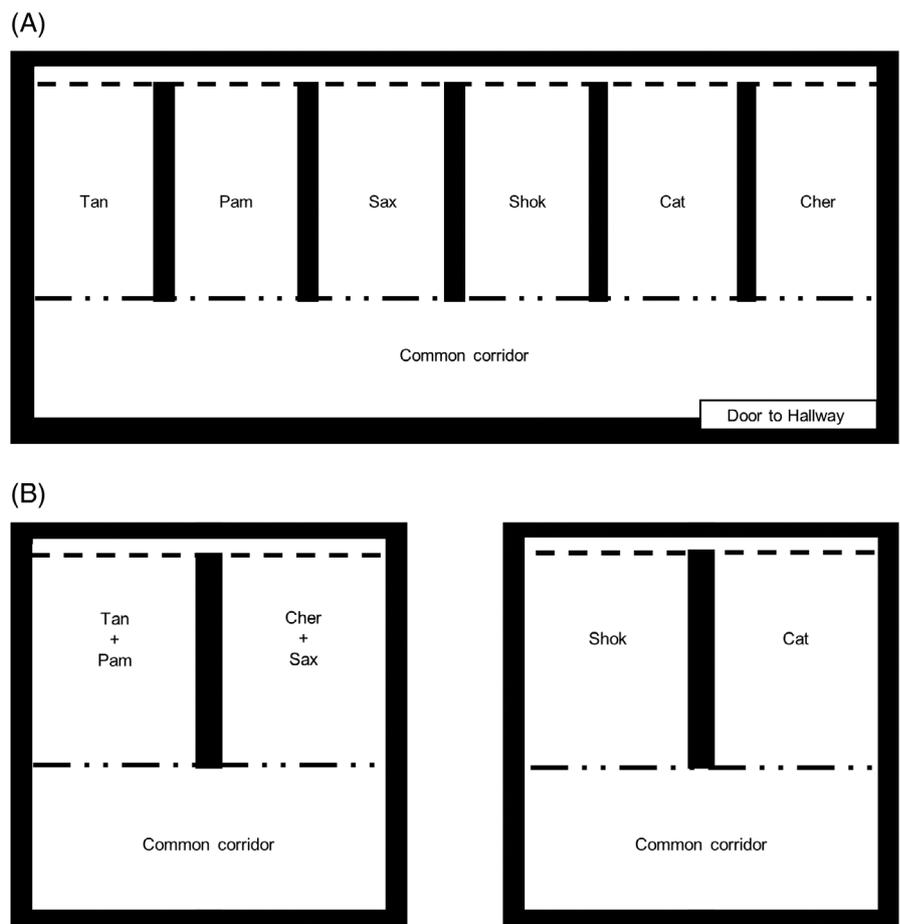
## 2.2 | Study timeline

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  $3 \times 10^5$  TCID<sub>50</sub> (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).<sup>27</sup> The inoculum was prepared in the NCSU College of Veterinary Medicine Biosafety Level III Laboratory and the dose determined from previous experiments.<sup>28,29</sup>

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.<sup>30</sup> 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).<sup>30</sup> 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.<sup>12,30</sup> 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.

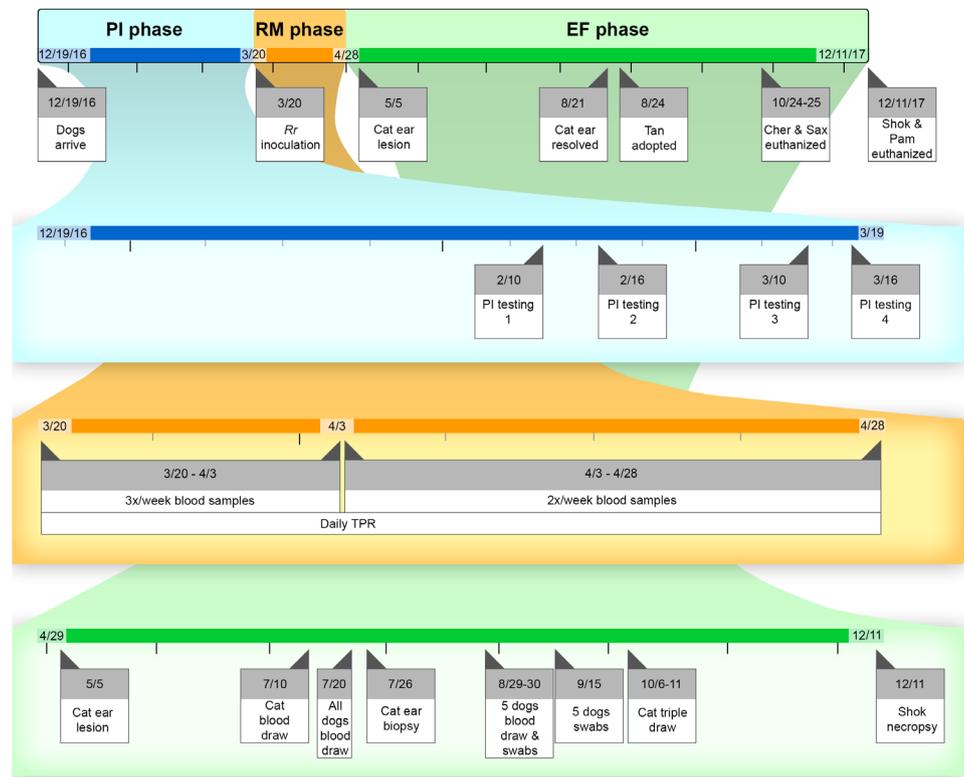
*Bartonella* spp. bacteremia was assessed using enrichment blood culture with the *Bartonella* alpha proteobacteria growth medium (BAPGM) as previously described.<sup>31</sup> By using standard operating procedures, DNA was extracted from samples intended for *Bartonella* spp. PCR.<sup>30</sup> *Bartonella* 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.<sup>30,32-34</sup> 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.<sup>35</sup>

## 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* alpha-proteobacteria 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.

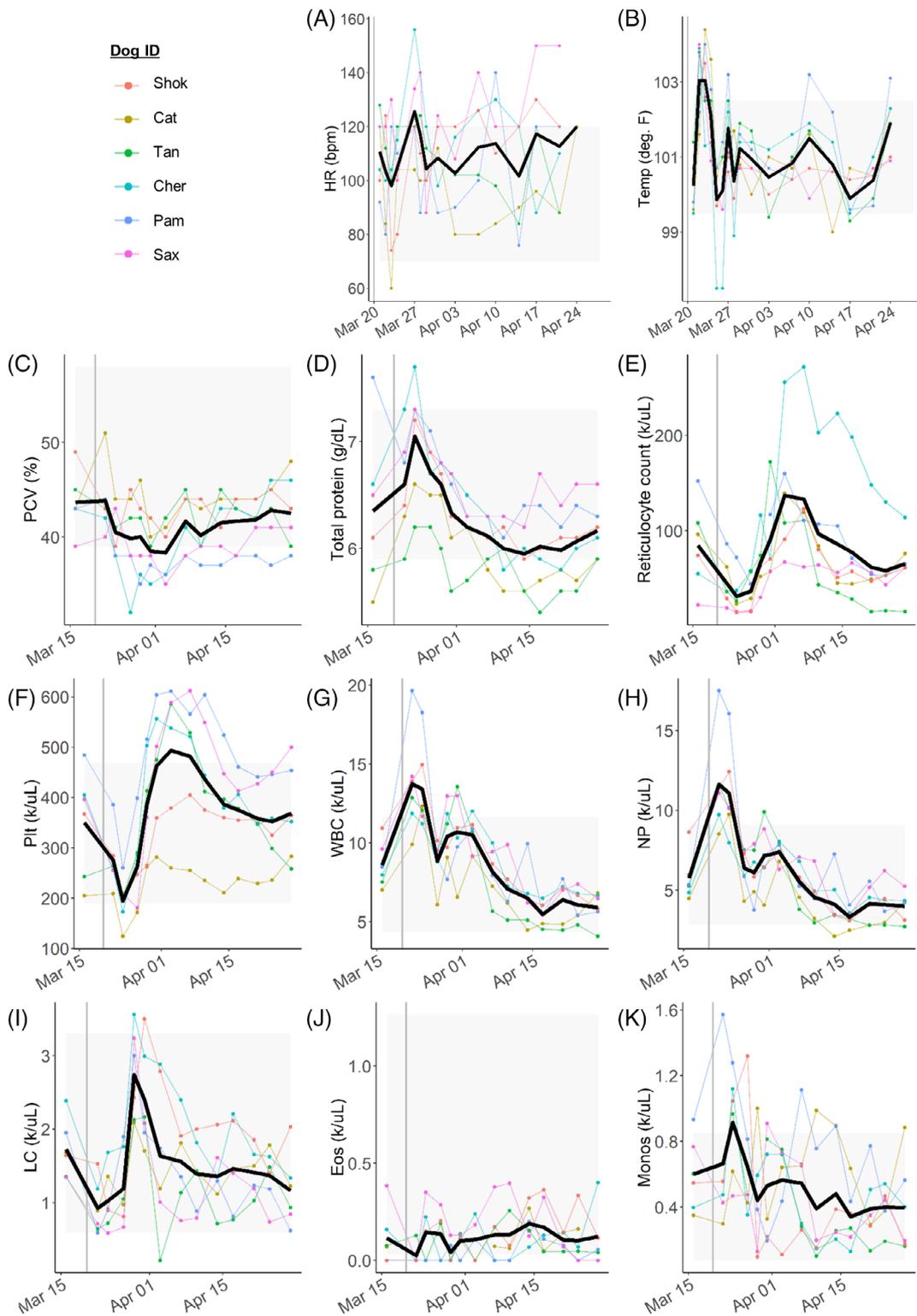
During the EF phase, tissue samples were obtained from 2 dogs. One dog (*Kat*) had biopsies taken from skin lesions on the pinnae, as well as from normal skin on the abdomen. At the time of euthanasia, approximately 1 year after arrival (11 December, 2017), another dog (*Shok*) had postmortem samples 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 mucous 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,<sup>36</sup> 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.<sup>36</sup> *Bartonella henselae* DNA was amplified and sequenced, and *Bartonella* organisms were visualized by laser scanning confocal immunohistochemistry, from the aural margin biopsies.<sup>36</sup> 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*)

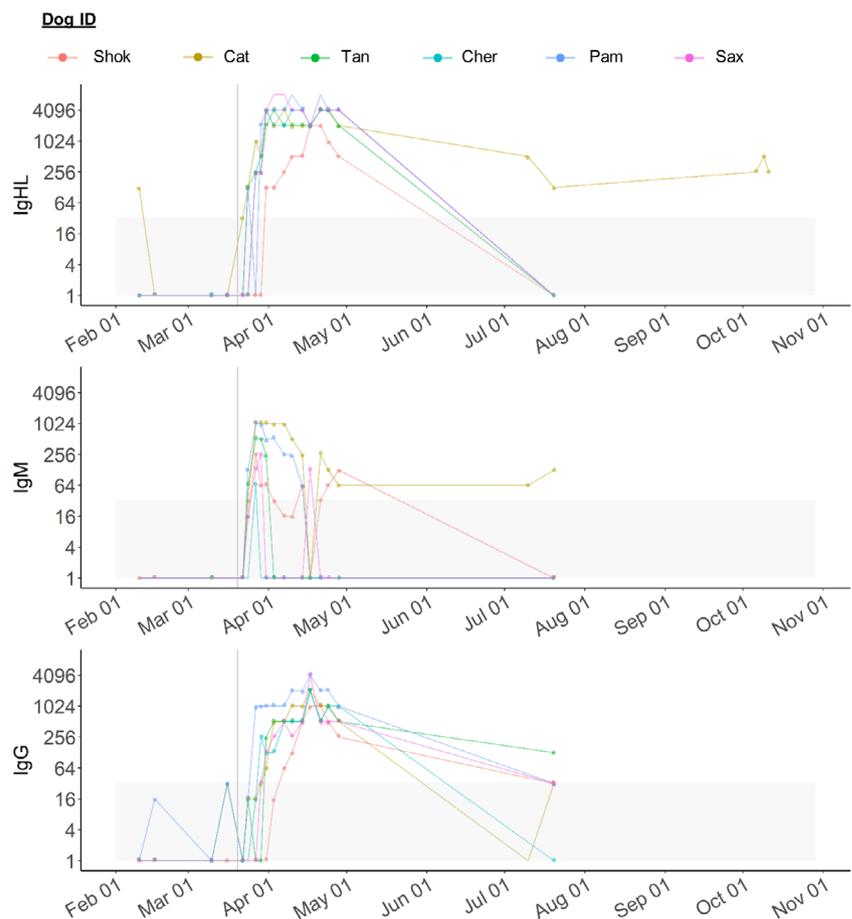
became seroreactive by post-inoculation day 4 (March 24, 2017). All dogs remained seroreactive (IgG H + L) until the end of the RM phase (April 28, 2017, 39 days after inoculation). When next tested 4 months after *Rr* inoculation, only 1 of the 6 dogs (*Kat*) was *Rr* seroreactive (Figure 4). This dog 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

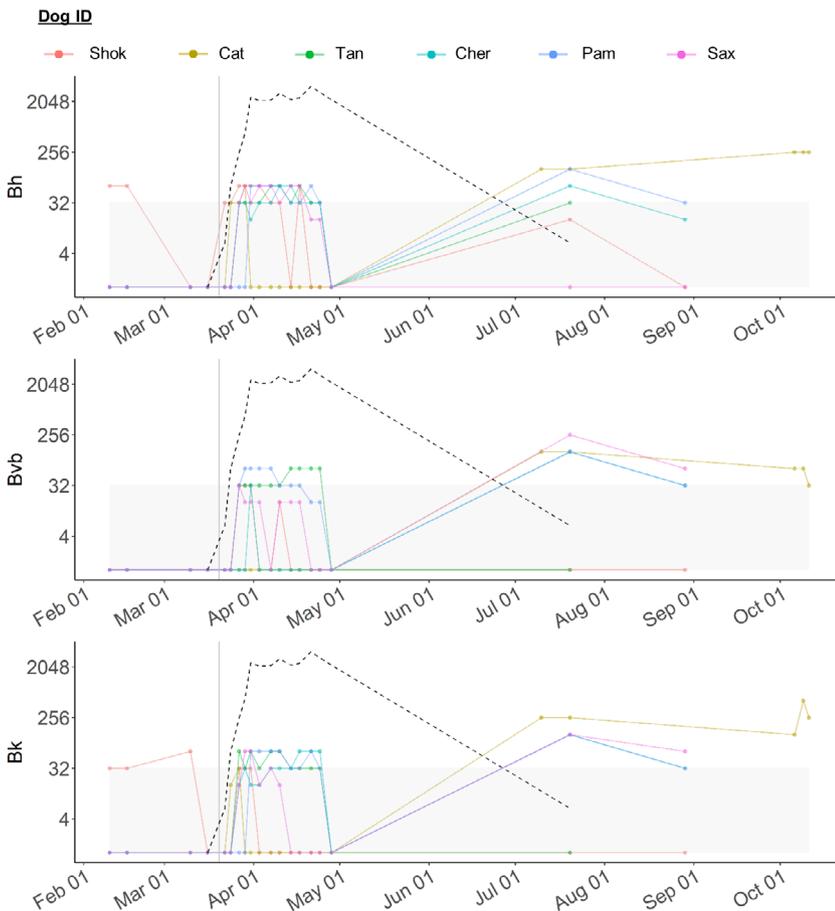
**TABLE 1** *Rickettsia* spp. seroconversion following *Rickettsia rickettsii* inoculation

	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.

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*



**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	7/20	7/26	8/29	8/29	8/29	8/30	8/30	8/30	9/15	9/15	9/15	10/6-25	12/11
	<i>Bartonella</i> IFA	PCR & BAPGM blood	Pinnae & skin biopsies	<i>Bartonella</i> IFA	PCR & BAPGM blood	8/30 cheek	8/30 periodontal	8/30 cheek	9/15 periodontal	9/15 vaginal	10/6-25 PCR & BAPGM blood	12/11 Tissue biopsies <sup>a</sup>		
Shok	–	–	NA	Bvb <sup>b</sup>	–	Bvb	–	–	–	–	NA	Bh <sup>c</sup>	–	–
Cat	Bh + Bvb + Bk	–	Bh <sup>c</sup>	NA	NA	Bvb	–	–	–	–	–x <sup>3d</sup>	NA	–	–
Tan	Bvb <sup>b</sup>	–	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	–	–
Cher <sup>e</sup>	Bh + Bvb + Bk	–	NA	Bvb <sup>f</sup> + Bk <sup>b</sup>	–	–	–	–	–	–	–	NA	–	–
Pam	Bh + Bvb + Bk	–	NA	–	–	Bvb	Bvb	–	<i>Bartonella</i> spp. <sup>g</sup>	–	NA	NA	–	–
Sax <sup>e</sup>	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.

<sup>a</sup>Tissues included lung, bone marrow, lymph nodes.

<sup>b</sup>Detected with the alternative KPL secondary antibody only.

<sup>c</sup>Same *Bartonella* species and strain (Bh SA2).

<sup>d</sup>Three blood samples tested.

<sup>e</sup>Housed together in a single run.

<sup>f</sup>Detected with the IgM (mu) antibody only.

<sup>g</sup>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.<sup>36</sup> *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* 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/ $\mu$ L) compared to that of swabs obtained on August 30, 2017 (10-63 ng/ $\mu$ L). 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.<sup>37</sup> 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.<sup>38</sup> 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.<sup>39</sup> *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,<sup>4</sup> 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 recrudescence 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.<sup>6,40</sup> 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,<sup>26,41,42</sup> 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.<sup>26,43,44</sup> 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).<sup>43</sup> In 3 dogs experimentally infected with *Bh* H1 and proven bacteremic, none were seroreactive against *Bh* SA2 (though all seroconverted to *Bh* H1).<sup>44</sup> 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.<sup>26</sup> 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.<sup>41,43</sup> 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.<sup>43,45</sup>

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.<sup>46</sup> 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.<sup>46-48</sup> 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,<sup>31,45,49,50</sup> 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,<sup>26</sup> 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.<sup>28,29</sup> 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.<sup>51,52</sup> 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).<sup>52-54</sup> 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.<sup>28,29</sup> Additionally, recrudescence of infection with *Rr* is not thought to occur in dogs or humans.<sup>55</sup> 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.<sup>36</sup> 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.

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