


## STANDARD ARTICLE

# *Babesia conradae* infection in coyote hunting dogs infected with multiple blood-borne pathogens

Jonathan D. Dear<sup>1</sup>  | Sean D. Owens<sup>2</sup> | LeAnn L. Lindsay<sup>1</sup> | Alex W. Biondo<sup>3</sup> | Bruno B. Chomel<sup>4</sup> | Mary Marcondes<sup>5</sup> | Jane E. Sykes<sup>1</sup>

<sup>1</sup>Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, California

<sup>2</sup>Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, California

<sup>3</sup>Department of Veterinary Medicine, Universidade Federal do Parana, Curitiba, Paraná, Brazil

<sup>4</sup>Department of Population Health and Reproduction, School of Veterinary Medicine, University of California, Davis, California

<sup>5</sup>School of Veterinary Medicine, São Paulo State University, Araçatuba, São Paulo, Brazil

**Correspondence**

Jonathan D. Dear, University of California, One Shields Avenue, Davis, CA 95616.  
Email: jddear@ucdavis.edu

**Funding information**

Center for Companion Animal Health; The Judith McBean Foundation

**Background:** *Babesia conradae* is an intraerythrocytic piroplasm infecting dogs in the southern United States. Ticks have been suspected, but unproven, as vectors. We identified *B. conradae* and other blood-borne pathogens in 2 kennels of sighthounds with a history of coyote fighting.

**Objectives:** To examine clinicopathologic abnormalities associated with *B. conradae* infection, risk factors for infection, and the prevalence of coinfections with other blood-borne pathogens.

**Animals:** Fifty-five Greyhounds and Greyhound mixes

**Methods:** Blood samples were collected from each dog for CBC, serum biochemistry panel, conventional and real-time PCR assays (*Babesia* spp., hemoplasmas, *Ehrlichia canis*, *Bartonella* spp., *Anaplasma* spp., and *Rickettsia* spp.), vector-borne pathogen ELISA, and immunofluorescent serology and culture for *Bartonella* spp and *Francisella tularensis* sero-agglutination test. Associations between *B. conradae* infection and coyote fighting, age and laboratory abnormalities were investigated.

**Results:** Twenty-nine dogs were PCR-positive for *B. conradae*. Of these, 16 were PCR-positive for other vector-borne organisms including *Mycoplasma haemocanis*, “*Candidatus* *Mycoplasma haematoparvum*,” *E. canis*, and a *Hepatozoon felis*-like organism. Twelve of the 20 dogs tested for seroreactivity to *Bartonella* spp. antigens were positive, but none were seropositive for tularmia. Infection with *B. conradae* was associated with a history of aggressive interactions with coyotes; lower hematocrit, leukocyte count, MCHC, platelet count and serum albumin concentration; and higher MCV, MPV, and serum globulin concentration.

**Conclusions and Clinical Importance:** *Babesia conradae* infection should be considered in dogs with anemia, leukopenia, thrombocytopenia, hypoalbuminemia and hyperglobulinemia. As with *B. gibsoni*, aggressive interactions with other canids may play a role in *B. conradae* transmission.

**KEYWORDS**

anemia, *Babesia*, *Bartonella*, mycoplasma, thrombocytopenia

## 1 | INTRODUCTION

*Babesia conradae* is a small intra-erythrocytic piroplasm initially discovered and described in shelter dogs in southern California in the early 1990s.<sup>1</sup> The first reports of canine babesiosis in California described

dogs with severe disease characterized by acute hemolytic anemia and profound thrombocytopenia. One dog that survived acute disease subsequently developed a protein losing nephropathy.<sup>1</sup> In a 1994 prospective study in southern California, the serologic prevalence to the infecting agent (*B. conradae*) ranged from 0 to 2.6% in shelter dogs and 7.7%–22% in coyotes.<sup>2</sup> At the time of these reports, the parasites were initially identified as *B. gibsoni* based on their cytologic appearance, as molecular detection and identification techniques were not

**Abbreviations:** ANOVA, analysis of variance; CT, cycle threshold; IFA, immunofluorescent antibody; VMTH, Veterinary Medical Teaching Hospital

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widely available, but later work showed them to be a distinct species, *B. conradae*.<sup>3</sup>

In 2006, PCR-sequencing of the 18S rRNA gene of this *Babesia* species revealed a distinct species within a clade of other *Babesia*-like protozoal parasites found in wild animals and humans throughout the western United States; it was re-named *B. conradae*.<sup>3</sup> Further work described diagnostic and epidemiologic characteristics of the infection and updated the piroplasmal phylogenetic position.<sup>3</sup> The epidemiology of *B. conradae* transmission is still not fully understood; although the organism was found in the salivary glands of *Rhipicephalus sanguineus* ticks, attempts to transmit infection using ticks were unsuccessful.<sup>4</sup> Vertical transmission has been proposed, as is seen in other canine babesial infections,<sup>5,6</sup> and a recent case series supported this as 8 dogs were descendants of a single infected dog.<sup>7</sup> In one study, hemolytic anemia was described in 12 affected companion dogs from Los Angeles County and dogs were successfully treated with atovaquone and azithromycin. Furthermore, *Rh. sanguineus* and *Ornithodoros* spp. ticks were found on infected dogs, suggesting a potential role of these ticks as vectors. More recently an unpublished abstract documented infected greyhounds with clinical babesiosis in Oklahoma.<sup>8</sup> While these reports have improved our understanding of some clinicopathological features and treatment of *B. conradae* infection, detailed clinicopathologic findings, definitive route of transmission and presence of coinfections were not described.

In 2011, an outbreak with high case fatality of an undefined illness was reported to the UC Davis VMTH in a kennel of 20 sight-hounds used for coyote-hunting from south-central California. Later found to be associated with *B. conradae* infection, these dogs provided an ideal population to study this disease. Accordingly, the goal of our study was to better characterize physical examination and clinicopathologic features associated with the disease. Our hypothesis was that dogs infected with *B. conradae* would be more likely to have clinical and laboratory evidence of disease than uninfected dogs. In addition, presence of coinfections was also investigated to verify that it was *B. conradae* infection that was associated with lower hematocrit, lower platelet count, and that age and aggressive interactions are risk factors associated with *B. conradae* infection.

## 2 | MATERIALS AND METHODS

### 2.1 | Index case

In April 2011, 1 of the authors (JES) was contacted by a client from south-central California that owned a kennel with 20 greyhounds and greyhound mixes. Over the last 10 years, 15 of the dogs had died at <6 years of age in association with anemia and a bleeding diathesis. Clinical signs of pallor, decreased appetite, and weight loss had occurred in the hounds. The colony had been established using 3 coyote-hunting dogs from southwestern Oklahoma in 2000, with subsequent introduction of dogs from Idaho and Georgia. Because of unsuccessful breeding attempts in some of these dogs and death because of anemia in 2 of the 3 original dogs by 2004, new dogs were

obtained in 2006 from Kansas and New Mexico and died with similar clinical signs. In 2008, 1 dog was evaluated by the referring veterinarian and was seropositive to *Babesia vogeli* antigens. Four dogs were treated with imidocarb dipropionate with transient clinical improvement, but 2 of these dogs later died. Treatment with doxycycline also did not result in clinical improvement.

In 2011, 21 dogs were tested for *Leishmania* antibodies (*Leishmania* indirect IFAT, Centers for Disease Control and Prevention, Atlanta, Georgia) and all dogs tested negative. Subsequently, 1 of the dogs, dog 1, was brought to the VMTH for evaluation. Physical examination of this dog revealed pallor, mild pyrexia (39.5°C/103.0°F), and moderate splenomegaly. A CBC showed a hematocrit of 36.2% (reference range, 40%-55%), with an MCV of 72.8 fL (reference range, 65–75 fL), an MCHC of 30.9 g/dL (reference range, 30–36 g/dL), a red cell distribution width of 17.2% (reference range, 11%-14%), reticulocytosis (296 700/μL, reference range 7000–65 000/μL), 2 nucleated RBC/HPF, thrombocytopenia (83 000 platelets/μL, reference range 150 000–400 000 platelets/μL), and mild leukopenia (5200 WBC/μL, reference range 6000–13 000 WBC/μL) but the differential cell count was unremarkable. The MPV was increased at 23.3 fL (reference range, 7–13 fL) and a few macroplatelets were noted.

DNA was extracted from 50 μL of the dog's blood using an automated nucleic acid extraction system, following the manufacturer's protocol (QIAxtractor, Qiagen, Venlo, Netherlands). Real-time PCR assays for *Anaplasma phagocytophilum*, *Anaplasma platys*, *Bartonella* spp., *Leishmania* spp., *Ehrlichia canis*, *Rickettsia rickettsii*, *Mycoplasma haemocanis*, "*Candidatus* *Mycoplasma haematoparvum*," and *Babesia* spp. were performed at a commercial diagnostic laboratory (University of California, Davis Real-Time PCR Research and Diagnostics Core Facility, Davis, California). The *Babesia* spp. assay was a broad-spectrum real-time PCR assay designed for detection but not differentiation of *Babesia gibsoni*, *Babesia vogeli* and *Babesia divergens* (Unpublished assay, designed between 530 and 650 base pair region of GenBank accession KX857477.1). A PCR assay for the host 18S rRNA gene (internal sample control) was used to determine DNA quantity and quality in addition to routine use of negative and positive controls. Results were negative for all organisms tested.

Three separate endpoint (conventional) PCR assays were used on DNA extracted for the previous real-time PCR assay, designed to detect the 16S rRNA gene of hemotropic mycoplasmas, the 18S rRNA gene of *Babesia* spp., and the *ITS-2* region of *Babesia* spp. The assays were performed as previously described.<sup>3,9,10</sup> PCR products were visualized after electrophoresis in a 2.5% agarose gel after staining (GelStar nucleic acid gel stain, Lonza, Rockland, Maine) and visualization by UV transillumination. Negative controls (ultrapure water) were included for each assay. Both *Babesia* PCR assays yielded products, but the hemoplasma PCR assay was negative. PCR products were directly sequenced in both directions (College of Biological Sciences DNA Sequencing Facility, University of California, Davis, California). Sequence homology was 99.43% for *B. conradae* comparing both 18S rRNA (AF158702.1) and *ITS-2* gene sequences (AY965739.1). PCR for the *ITS-2* gene and sequencing was performed for a total of 9 dogs (including the index case) and the results were the same as for the index case. On retrospective review of blood smears from the index

dog using light microscopy by a board-certified clinical pathologist (SDO), small (2–3  $\mu\text{m}$ ) piroplasms were identified occurring singly within infected erythrocytes on careful slide review. The degree of parasitemia was not otherwise quantified.

Subsequently, a specific *B. conradae* real-time PCR assay was developed. PCR was performed using the forward primer 5'-CGAATC AACAGACCTCTGAACGT-3' and the reverse primer 5'-AGTTCAC TGAAATGGGAGTACCG-3', and the probe FAM-5'-ACACCGCTCT GCTT-3'. Each real-time PCR reaction contained 20 $\times$  primer and probes for the respective TaqMan system with a final concentration of 400 nM for each primer and 80 nM for the TaqMan probe and commercially available PCR mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems, Waltham, Massachusetts) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl<sub>2</sub>, 2.5 mM deoxynucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction. One microliter of genomic DNA and 4  $\mu\text{L}$  of water were added to the qPCR to a final volume of 12  $\mu\text{L}$ . The samples were placed in 384 well plates and amplified in an automated fluorometer (Prism 7900HT Fast Real-Time PCR System, Applied Biosystems, Waltham, Massachusetts). The manufacturer's standard amplification conditions were used: 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C, and 60 seconds at 60°C (TaqMan Universal PCR Mastermix, Applied Biosystems, Waltham, Massachusetts). Fluorescent signals were collected during the annealing temperature and CT values extracted with a threshold of 0.04 and baseline values of 3–12. The real-time PCR assay was validated using established protocols.<sup>11</sup> Amplification efficiency was calculated using the linear equation  $E = 10^{1/\text{slope}} - 1$  (s: slope) and was determined to be 99.3%. The sensitivity of the assay as determined using serial dilution of a plasmid was 1 gene copy. The standard curve was run in triplicate.

## 2.2 | Case-control study

Six weeks after dog 1 was examined, all dogs in the kennel ( $n = 20$ ) were examined on the owner's property. Information collected included age, date of birth, medical history (including blood transfusions), identity of the dam and sire (when born on premises), past breeding history, tick exposure, and history of travel and aggressive interactions either with coyotes or other dogs. The owner maintained detailed written records of breeding history, travel, and interactions with coyotes for each dog. Complete physical examinations were performed by 1 of 2 board-certified internal medicine specialists (JDD or JES), with attention paid to evidence of trauma or scars, splenomegaly, lymphadenomegaly, cardiac abnormalities, and evidence of ectoparasites. A limited echocardiogram was performed. Nine milliliter of blood was collected from the saphenous vein of each dog. Of this, 4 mL was placed in EDTA tubes for a CBC and nucleic acid detection assays for *B. conradae*, other *Babesia* spp., *M. haemocanis*, "*Candidatus M. haematoparvum*," *A. phagocytophilum*, *A. platys*, *Bartonella* spp., *Leishmania* spp., *E. canis*, and *R. rickettsii*. Five milliliter was placed in a serum tube for a serum biochemistry panel and serology for blood-borne pathogens. Serologic assays consisted of an in-clinic ELISA assay that detects the antigen of *Dirofilaria immitis*, and antibodies to *Borrelia burgdorferi*, *Ehrlichia* spp., and *Anaplasma* spp (4Dx SNAP Plus,

IDEXX Laboratories, Westbrook, Maine). Additional infectious disease testing performed included *Francisella tularensis* serology, *Bartonella* culture and IFA serology for *Bartonella henselae*, *B. clarridgeiae*, and *B. vinsonii* subsp. *berkhoffii*, as previously described.<sup>12,14</sup> Urine was collected by cystocentesis for a urinalysis and urine protein to creatinine ratio, when indicated (presence of proteinuria in absence of an active sediment). Specimens were transported over several hours in a pre-cooled transport container (Igloo Coolers, Katy, Texas) to the diagnostic laboratories at the VMTH, where serum was immediately separated, and all specimens were refrigerated at 4°C. All assays were performed within 48 hours of collection, and any remaining serum and whole blood was stored at -80°C for additional testing up to 1 month later.

The kennel was monitored with at least an annual property visit through January 2017. Two dogs from this kennel were not examined by the authors but blood was drawn by the local veterinarian and shipped to the VMTH for analysis because of suspicion of *Babesia* infection. Additionally, a second kennel with 25 dogs in south-central California was identified as having dogs infected with *B. conradae* in November 2015 and was visited annually through January 2017. Assessments on these dogs included a complete physical examination by 1 author (JDD), CBC, serum biochemistry panel, nucleic acid detection assays for blood-borne pathogens as described above, and serology for blood-borne pathogens (4Dx SNAP Plus, IDEXX Laboratories, Westbrook, Maine). Dogs that tested positive using PCR for *B. conradae* were treated with atovaquone and azithromycin for 10 days as part of a separate study.

Any dog that tested positive using PCR for *B. conradae* during the 6-year period of observation was included as cases and was included only once. Control dogs were temporally and location-matched dogs, which tested negative for *B. conradae*. We also attempted select age-similar controls, where possible. For a given time point, dogs were sorted in order of descending age and the next oldest dog that tested negative for *B. conradae* was selected as a control for each case. When this was not possible, the next youngest dog that tested negative for *B. conradae* was chosen as a control, to most closely approximate the age of the case dog. Dogs examined serially were only enrolled once. Our study was approved by the UC Davis Institutional Animal Care and Use Committee (protocol #18956).

## 2.3 | Statistical analysis

Descriptive data is presented as median and interquartile range or median and range for molecular and microbiologic results. For normally distributed data, the Student's *t*-test was used to compare *Babesia* PCR-positive and *Babesia* PCR-negative dogs. Nonparametric data was log transformed before statistical analysis. A Chi-squared test was used to evaluate categorical data, and the Fisher's exact test was used to evaluate categorical data with groups <5. Logistic regression was used to evaluate odds ratios of continuous data on *Babesia* PCR positivity. A four-way ANOVA was performed to evaluate the relationship between hematologic variables and PCR positivity with *B. conradae*, *M. haemocanis*, and "*Candidatus M. haematoparvum*," and positive culture with *B. v. subsp. berkhoffii*. Significance was set as a *P*-value < .05. All statistics were performed on

**TABLE 1** Physical examination findings in 55 coyote-hunting dogs from South-Central California that tested positive and negative for *B. conradae* using real-time PCR

Variable (Unit)		<i>Babesia</i> PCR-negative (range; n = 26)	<i>Babesia</i> PCR-positive (range; n = 29)	P value	Test statistic	Degrees of freedom
Sex	Male	12/26 (46%)	16/29 (55%)	.50	0.446	1
	Female	14/26 (54%)	13/29 (45%)			
Body condition score (1–9)		4 (4–4.5)	4 (4–4)	.46	0.73	41
Rectal temperature (degrees Fahrenheit)		100.7 (100.4–101.0)	100.7 (100.4–101.0)	.98	0.02	41
Heart rate (beats/minute)		120 (88–120)	100 (82–120)	.44	0.78	41
Respiratory rate (breaths/minute)		22 (19–38)	20 (16–36)	.94	0.08	37
Mucous membrane color	White	0/22	2/23 (9%)	<.001	16.052	1
	Pale pink	1/22 (5%)	12/23 (52%)			
	Pink	21/22 (95%)	9/23 (39%)			
Presence of wounds/scars		13/22 (59%)	17/23 (74%)	.29	1.111	1
Splenomegaly		3/22 (14%)	10/22 (45%)	.02	5.350	1
Lymphadenomegaly		7/22 (32%)	6/21(29%)	.59	0.297	1

Values shown indicate median (interquartile range) for continuous data and number of animals (%) for categorical data. For continuous data, t-values are presented and for categorical data,  $X^2$  values are provided in the test statistic column.

commercially available software (SAS version 9.4 2013 SAS Institute Inc, Cary, North Carolina). The tables were removed during the peer review process.

### 3 | RESULTS

Twenty dogs were initially identified and have been separately described as the first cohort because their history and diagnostic investigation were more comprehensive than those in subsequent annual visits. In this first cohort, there were 11 males (all intact) and 9 females (1 spayed). The dogs were held in open, individual runs on a cattle property and were used for breeding as well as hunting coyotes on the property. Sheep and horses were also raised on the property. Twelve dogs were born on the property; the remaining 8 dogs were acquired from breeders in Georgia (4), Texas, Louisiana, California, and New Mexico (1 each). Only 1 dog had spent time out of California since acquisition (6 months in Georgia for breeding). Nine dogs had a history of tick exposure and eleven had no known history of tick exposure. There were no observed tick infestations on the property since 2009 when the owner started applying amitraz collars (Preventic, Virbac Corporation, Fort Worth, Texas). No other medications were administered on a regular basis. Eight dogs had a history of illness including 6 dogs who had episodes of anemia between 2008 and 2011. One of these dogs received a plasma transfusion during cesarean for dystocia 16 months before the study reported here began; no other dogs had received blood product transfusions. One dog had a history of rattlesnake envenomation 1 month before initial examination. Fifteen dogs had a history of previous treatment with cephalexin or trimethoprim-sulfamethoxazole (unknown doses, typically short duration) for treatment of fever or anemia. All dogs were fed a mixture of commercial adult kibble and occasional raw food including chicken legs and tripe.

From the 2 kennels, a total of 55 dogs were included for statistical analysis, consisting of 28 intact males and 27 females (26 intact and

1 spayed). All were either greyhound or greyhound mixes. Dogs ranged in ages from 6 months to 132 months (median 36 months). No dogs had known history of trauma or aggressive interactions with domestic animals, but 36 dogs had a history of aggressive interactions with coyotes (14 in the original cohort).

On physical examination, all 55 dogs were docile, alert, and interactive and assessed to have normal hydration and vital signs including body temperature. All dogs were in lean body condition (median body condition score 4/9). Over the entire 6-year period, ticks were only identified on 1 dog on one occasion, which was found to have several ticks in the inguinal region. Ticks were not identified on any other greyhound. Physical examination findings included palpable splenomegaly (n = 13), peripheral lymphadenomegaly (n = 13), mucosal pallor (n = 15), gingival bleeding (n = 1), and scars over the muzzle, trunk and limbs (n = 30; Table 1). No evidence of infective endocarditis was observed on echocardiography of the first cohort.

Twenty-nine of the 55 dogs tested positive for *B. conradae* using PCR (median CT value 25.99, range 18.95–37.01) using real-time PCR. Dogs testing positive for *B. conradae* were significantly more likely to have splenomegaly (P = .02), and mucosal pallor (P < .001), but not lymphadenomegaly (P = .59). Dogs that tested positive using PCR for *B. conradae* were older than dogs that tested negative (OR 2.1, CI 1.3–3.3, P < .001) and were more likely to have a history of known aggressive interaction with coyotes (OR 31.2, CI 3.6–272.3, P < .001).

PCR-positive dogs were more likely to be anemic (P < .001) with macrocytosis (P < .001) and hypochromasia (P < .001, Table 2). They were also more likely to be leukopenic (P < .001) and neutropenic (P < .001) than dogs that were PCR-negative for *B. conradae*. Finally, dogs that were PCR-positive for *B. conradae* were more likely to be thrombocytopenic (P < .001) and have macroplatelets (P < .001) than dogs testing PCR-negative for *B. conradae* (Table 2).

Dogs that tested PCR-positive for *B. conradae* had higher serum total protein (P < .001) and globulin (P < .001) concentrations, but lower albumin concentration (P < .001), sodium concentration (P < .001), and alanine aminotransferase activity (P = .04) than control

**TABLE 2** CBC Findings in 55 coyote-hunting dogs from South-Central California that tested positive and negative for *B. conradae* using real-time PCR

Variable (units)	Reference interval	<i>Babesia</i> PCR-negative (n = 26)	<i>Babesia</i> PCR-positive (n = 29)	P value	Test statistic	Degrees of freedom
Hematocrit (%)	40–55	54.3 (52.0–57.2)	42.1 (33.1–44.6)	<.001	7.58	37.991
Anemic (%)		4% (1)	45% (13)	<.001	15.263	1
Mean corpuscular volume (fL)	65–75	70.1 (69.1–71.9)	75.2 (72.2–78.6)	<.001	–5.01	36.947
Macrocytosis		0% (0)	52% (15)	<.001	18.491	1
Mean corpuscular hemoglobin concentration (g/dL)	33–36	33.4 (32.9–34.0)	31.5 (30.3–32.6)	<.001	6.52	48.146
Hypochromia		30% (8)	86% (25)	<.001	19.803	1
Red cell distribution width (%)	11–14	12.8 (12.2–13.0)	14.4 (13.4–15.2)	<.001	–6.03	37.904
White blood cells (10 <sup>3</sup> cells/μL)	6.0–13.0	9.6 (8.5–11.4)	6.1 (5.3–8.0)	<.001	6.10	45.769
Leukopenic (%)		4% (1)	41% (12)	<.001	10.700	1
Neutrophils (10 <sup>3</sup> cells/μL)	3.0–10.5	5.5 (5.0–7.6)	3.3 (2.6–4.8)	<.001	5.20	42.454
Neutropenic (%)		4% (1)	45% (13)	<.001	12.134	1
Lymphocytes (10 <sup>3</sup> cells/μL)	1.0–4.0	2.7 (2.1–3.2)	2.1 (1.7–2.7)	.005	2.94	45.105
Monocytes (cells/μL)	150–1200	530 (400–800)	450 (310–596)	.009	2.76	42.67
Eosinophils (cells/μL)	0–1500	488 (56–826)	170 (125–262)	.017	2.52	34.507
Basophils (cells/μL)	0–50	30 (23–42)	20 (10–29)	.016	2.48	53
Platelets (10 <sup>3</sup> cells/μL)	150–400	201 (174–226)	96 (73–163)	<.001	4.28	41.643
Thrombocytopenic (%)		7% (2)	69% (20)	<.001	21.446	1
Mean platelet volume (fL)	7–13	10.2 (9.3–10.9)	17.6 (14.2–19.3)	<.001	–9.62	35.459
Macroplatelets (%)		11% (3)	83% (24)	<.001	30.992	1

Values shown indicate median (interquartile range) or percent affected (number). For continuous data, *t*-values are presented and for categorical data,  $\chi^2$  values are provided in the test statistic column.

dogs (Table 3). Significant differences were observed between groups in anion gap, serum calcium, and phosphorus concentrations but mean values for both groups fell within reference intervals. Despite there being more proteinuric cases than controls on urinalysis, proteinuria was mild, and differences were not statistically significant ( $n = 20$ , median 25 mg/dL, range 0–150 mg/dL,  $P = .07$ , Table 4). Only 1 dog had a urine protein : creatinine ratio performed (urine proteinuria 150 mg/dL, UPC 4.5).

### 3.1 | Other blood-borne infections and coinfections

In the first cohort, 9 of the 20 dogs tested positive for *B. conradae* using real-time PCR. Of the 9 dogs that were positive for *B. conradae*,

3 had no coinfections, 3 were coinfecting with *M. haemocanis* alone, 1 was coinfecting with “*Candidatus M. haematoparvum*” alone and 2 were coinfecting with both *M. haemocanis* and “*Candidatus M. haematoparvum*.” Of the 11 dogs that tested negative for *B. conradae*, 4 tested negative for all blood-borne pathogens while 6 were infected with *M. haemocanis*, and 1 was coinfecting with both *M. haemocanis* and “*Candidatus M. haematoparvum*,” 1 dog tested positive for *E. canis* by PCR (but tested negative on ELISA serology) and 1 dog tested positive for a *Hepatozoon felis*-like organism by PCR (96.94% identity to the *H. felis* 18S rRNA gene sequence, HQ829439.1). All 20 dogs were seronegative for *F. tularensis* antibodies. Nine dogs were seropositive for *Bartonella* antibodies (3 *B. henselae*, range 1 : 64–1 : 1024; 4 *B. clarridgeiae*, range

**TABLE 3** Serum biochemistry results in 52 coyote-hunting dogs from South-Central California that tested positive and negative for *B. conradae* using real-time PCR

Variable (units)	Reference interval	<i>Babesia</i> PCR-negative (n = 25)	<i>Babesia</i> PCR-positive (n = 27)	P value	Test statistic	Degrees of freedom
Blood urea nitrogen (mg/dL)	11–33	15 (13–16)	13 (12–17)	.93	–0.28	39.19
Creatinine (mg/dL)	0.8–1.5	1.0 (0.9–1.1)	0.9 (0.8–1.1)	.57	0.52	47
Total protein (g/dL)	5.4–6.9	6.1 (5.8–6.4)	7.6 (6.6–8.4)	<.001	–4.69	34.072
Albumin (g/dL)	3.4–4.3	3.6 (3.5–3.8)	2.9 (2.3–3.2)	<.001	6.56	40.545
Globulin (g/dL)	1.7–3.1	2.5 (2.1–2.7)	4.7 (3.8–5.6)	<.001	–7.10	32.667
Total bilirubin (mg/dL)	0.0–0.2	0.2 (0.1–0.2)	0.3 (0.2–0.4)	.20	–1.33	26.054
Sodium (mEq/mL)		145 (145–146)	142 (140–144)	<.001	4.48	25.034
Alanine transferase (IU/mL)		49 (39–70)	39 (26–46)	.04	2.17	47

Values shown indicate median (interquartile range). *T*-values are presented in the test statistic column.

**TABLE 4** Urinalysis results in 14 coyote-hunting dogs from South-Central California that tested positive and negative for *B. conradae* using real-time PCR

Variable (units)	<i>Babesia</i> negative (range; n = 7)	<i>Babesia</i> positive (range; n = 7)	P value	Test statistic	Degrees of freedom
Urine specific gravity	1.021 (1.017-1.027)	1.023 (1.013-1.029)	1.0	0.00	12
pH	5 (5-6)	5 (5-5)	.42	0.84	10.566
Protein (mg/dL)	0 (0-0)	25 (0-150)	.07	-2.20	6
Bilirubin (mg/dL)	0 (0-1)	0 (0-3)	.14	-1.67	

Values shown indicate median (interquartile range). T-values are presented in the test statistic column.

1 : 64-1 : 1024; 9 *B. vinsonii* subsp. *berkhoffii*, range 1 : 64-1 : 2048). Finally, *B. vinsonii* subsp. *berkhoffii* was isolated from the blood of 6 dogs (1 coinfecting with *B. conradae*). Dogs that tested positive for *B. conradae* were more likely to seroreact to *B. v.* subsp. *berkhoffii* ( $P = .03$ ,  $\chi^2$  value 4.74) but were less likely to be culture-positive for *B. v.* subsp. *berkhoffii* ( $P = .03$ ,  $\chi^2$  value 4.90) than dogs that tested PCR-negative for *B. conradae* (Table 5). There was no difference in hematocrit ( $P = .35$ ,  $t$ -value  $-0.96$ ) or platelet count ( $P = .38$ ,  $t$ -value  $-0.91$ ), but there was a significantly higher white blood cell count in dogs with a positive blood culture for *B. vinsonii* subsp. *berkhoffii* ( $P = .003$ ,  $t$ -value  $-3.67$ ).

When both cohorts of *B. conradae* PCR-positive dogs were evaluated together, 18/29 (62.1%) had coinfections with other blood-borne pathogens (Table 5). Out of these 18 dogs, 8 were PCR-positive for *M. haemocanis* alone, 4 dogs were PCR-positive for "*Candidatus M. haematoparvum*" alone and 4 dogs were PCR-positive for both *M. haemocanis* and "*Candidatus M. haematoparvum*." One dog was seropositive but PCR-negative for *E. canis*. Overall, 9 dogs tested negative for all blood-borne pathogens, 24 had only a single infection, 18 had 2 infections and 4 dogs had 3 coinfections. Thirteen dogs were positive for both *M. haemocanis* and *B. conradae* and the 4 dogs with 3 infections tested positive for *B. conradae* and both hemotropic mycoplasmas.

Out of the *B. conradae* PCR-negative dogs, 17/26 (65.4%) had infections with other blood-borne pathogens (Table 5). Seven dogs were PCR-positive for *M. haemocanis* alone, 1 dog was PCR-positive for "*Candidatus M. haematoparvum*" alone and 5 dogs were coinfecting with *M. haemocanis* and "*Candidatus M. haematoparvum*." All 55 dogs tested negative for antibodies to *A. phagocytophilum* and *B. burgdorferi*, and *D. immitis* antigen.

There was not a significant association between *B. conradae* infection and coinfection with *M. haemocanis* or "*Candidatus M. haematoparvum*" ( $P = .70$ ,  $\chi^2$  value 0.1471). Additionally, there was no difference in hematocrit ( $P = 0.14$ ,  $t$ -value  $-1.50$ ), white blood cell count ( $P = 0.20$ ,  $t$ -value  $-1.31$ ), or platelet count ( $P = .28$ ,  $t$ -value  $-1.09$ ) in dogs testing PCR-positive for *M. haemocanis* or "*Candidatus M. haematoparvum*" when compared with those testing PCR-negative.

When comparing mean hematologic variables between coinfections identified by PCR and culture, only *B. conradae* PCR positivity was associated with lower hematocrit ( $P = <.001$ ,  $F$  value 31.16) and lower WBC count ( $P = .005$ ,  $F$  value 11.35).

Finally, when comparing hematologic variables between dogs with any infection identified by PCR or culture (46) and those negative for all tested pathogens (9), there was a significantly lower hematocrit ( $P < .001$ ,  $t$ -value 4.57) and platelet count ( $P = .016$ ,  $t$ -value 2.70) but

**TABLE 5** Coinfections in 55 coyote-hunting dogs from South-Central California that tested positive and negative for *B. conradae* using real-time PCR in the case-control group

	<i>Babesia</i> negative (n = 26)	<i>Babesia</i> positive (n = 29)	P value	Test statistic	Degrees of freedom
<i>Mycoplasma haemocanis</i> <sup>a</sup>	12/26 (44%)	12/29 (41%)	.72	0.127	1
" <i>Candidatus Mycoplasma haematoparvum</i> " <sup>a</sup>	6/26 (23%)	8/25 (32%)	.53	0.397	1
<i>E. canis</i> <sup>a</sup>	0/21	0/24	N/A		
<i>Ehrlichia</i> spp. <sup>b</sup>	0/9	1/6 (17%)	.20	1.607	1
<i>Bartonella</i> spp. <sup>a</sup>	0/21	0/24	N/A		
<i>B. henselae</i> <sup>c</sup>	1/8 (13%)	2/9 (22%)	.31	1.021	1
<i>B. clarridgeae</i> <sup>c</sup>	1/8 (13%)	3/9 (33%)	.31	1.022	1
<i>B. vinsonii</i> subsp. <i>berkhoffii</i> <sup>c</sup>	2/8 (25%)	7/9 (78%)	.03	4.735	1
<i>B. vinsonii</i> subsp. <i>berkhoffii</i> <sup>d</sup>	5/8 (63%)	1/9 (11%)	.03	4.898	1
Any non- <i>Babesia</i> infection	17/26 (65%)	17/29 (59%)	.61	0.2657	1

<sup>a</sup>Real-time PCR on whole blood samples.

<sup>b</sup>ELISA on whole blood (IDEXX Snap 4Dx Plus).

<sup>c</sup>Immunofluorescence Assay (IFA) serology.

<sup>d</sup>*Bartonella* culture on whole blood.

Values shown indicate number of animals (%) infected.  $\chi^2$  values are provided in the test statistic column.

no significant difference in absolute white blood cell count ( $P = .186$ ,  $t$ -value 1.38) in infected dogs.

## 4 | DISCUSSION

Our long-term monitoring of *B. conradae* infections in 2 dog kennels and similar reports suggest that babesiosis caused by this piroplasm is an under-recognized disease.<sup>7,8</sup> In our study, we examined dogs from 2 kennels in the south-central valley of California and *B. conradae* infection was identified in 29 dogs over 6 years. In this population, infection with *B. conradae* was significantly associated with increasing age and a history of aggressive interactions with coyotes. Previous investigators hypothesized that *B. conradae* was vector-borne and implicated *Rh. sanguineus* as the likely vector being the most prevalent dog tick in the region.<sup>4</sup> In the population herein, ticks were only found on a single dog infected with *B. conradae* over the 6-year span. Furthermore, tick and flea infestations were not reported by the dogs' owners. In addition, *E. canis* seroreactivity was present in only 1 dog; if *Rh. sanguineus* was the likely vector, a higher prevalence of *E. canis* seroreactivity might have been anticipated. Although *Rh. sanguineus* has been implicated as a vector for *M. haemocanis*,<sup>14</sup> there was no association between *M. haemocanis* PCR positivity and *B. conradae* PCR positivity. Because tularemia can be tick-borne and transmission of *F. tularensis* by coyote bites has been reported,<sup>15</sup> we investigated the presence of potential exposure in these dogs. However, all tested dogs were seronegative for that pathogen, indicating a low risk of exposure in southern California.

Had fleas been the likely vector, a positive association between *Bartonella* infection and *B. conradae* infection might have been expected. Dogs that were bacteremic with *Bartonella* were less likely to be infected with *B. conradae*, but there was an association with seroreactivity to *B. v. subsp. berkhoffii*. Infection with *B. gibsoni* has been associated with dog fighting among Japanese tosa dogs and pit bull terriers.<sup>16,17</sup> It seems that transmission of *B. conradae* to these dogs may have resulted from aggressive interactions with coyotes, and only friendly behavioral interactions were observed by the owner or authors of our study among the hunting dogs themselves.

Prior studies have suggested that dogs infected with *B. conradae* were often anemic, but these studies lacked control groups and often included incomplete data on study animals which may have led to biased conclusions.<sup>7</sup> In our case-control study we attempted to provide a more comprehensive clinical picture of infection with *B. conradae* in dogs including hematologic, biochemical and thorough infectious disease testing. In our study, similar to findings from previous smaller studies, dogs testing positive for *B. conradae* were more likely to be anemic and had lower median hematocrits than control dogs.<sup>1,7,18</sup> Reticulocyte counts were not routinely performed as part of the present study, but dogs that tested positive for *B. conradae* were more likely to have a macrocytic, hypochromic anemia with an increased red cell distribution width, suggestive of a regenerative anemia. The mechanism for this anemia is likely multifactorial, resulting from increased erythrocyte fragility and intravascular hemolysis because of the presence of an intracellular parasite in addition to immune-mediated extravascular haemophagocytosis.<sup>18</sup> Early

experimental studies of *B. gibsoni* found that dogs become Coombs' positive and develop splenomegaly as a result of expanded red and white pulp with increased numbers of macrophages, phagocytized erythrocytes or hemosiderin granules along with increased plasma cells with persistent parasitemia.<sup>18</sup> Similarly, many of the dogs in our study were found to have splenomegaly on physical exam and 1 dog who died acutely after diagnosis was found to have moderate, diffuse plasmacytosis, histiocytosis, and hemosiderosis on histopathology consistent with reactive, erythrophagocytic hemolysis.

Dogs that tested PCR-positive for *B. conradae* were also more likely to be thrombocytopenic and have lower median platelet counts than negative dogs. However, platelet counts in the case dogs were often not sufficiently low to explain the spontaneous bleeding observed in these dogs (median 96 000 platelets/ $\mu$ L, interquartile range 73 000–163 000 platelets/ $\mu$ L). Only 2 dogs had platelet counts lower than 30 000/ $\mu$ L, corroborating previous reports, which have shown only mild to moderate thrombocytopenia in spite of sometimes significant bleeding diatheses.<sup>1,7</sup> A platelet function defect might also explain the hemorrhagic tendencies observed. In contrast, recent studies have shown that although dogs infected with *B. rossi* tend to be thrombocytopenic, they tend to lack clinical evidence of bleeding as they possess large, activated platelets, and maintain adequate platelet counts for appropriate coagulation.<sup>19</sup> We found that *B. conradae* infected dogs were more likely to have macroplatelets, but many of the dogs from these kennels were reported to have previous bleeding diatheses. Further studies into platelet function are required to better understand the suspected thrombocytopathia associated with *B. conradae* infection.

As expected, dogs that tested PCR-positive for *B. conradae* had higher globulin concentrations and lower albumin concentrations, both expected findings in dogs with antigenic stimulation and subsequent T-helper immune response.<sup>20,21</sup> Interestingly, dogs that tested PCR-positive for *B. conradae* were more likely to be hyponatremic than controls. The cause of hyponatremia was not clear, but in humans, severe malaria infections have been associated with a syndrome of inappropriate antidiuretic hormone.<sup>22</sup> Although statistically significant, it might not be clinically relevant or could represent type I error.

No association was found between proteinuria and *B. conradae* infection, but there was a trend approaching significance and may be a result of type II error. Previous studies have shown that dogs infected with *B. gibsoni* and *B. microti*-like organisms sustain damage to their glomeruli and are often profoundly proteinuric, stemming from their circulating immune complexes.<sup>18,23</sup> Notably, before our study commenced, many dogs in these kennels had died of acute kidney injury, potentially related to glomerulopathies.

As a case-control observational study there are some limitations to our study; it was impossible to account for all variables during the study period. Namely, many dogs in this cohort were coinfecting with multiple blood-borne infections, which may have affected their clinical or clinicopathologic findings despite attempts control such limitation by performing multivariate statistical analysis. Furthermore, although this report has included a more comprehensive clinical evaluation of dogs with *B. conradae* than previously reported, not all dogs received the same evaluation. Dogs enrolled later in the study period did not

have a urinalysis, echocardiogram, *F. tularensis* or *Bartonella* serology and *Bartonella* culture because of logistical and economic constraints. Finally, all dogs enrolled in our study were sighthound or sighthound mixed breed dogs and their hematologic variables may differ from other breeds potentially limiting external validity.<sup>24</sup> Breed impact on clinical pathology variables were controlled by including only sight-hounds from the same kennels as control dogs, thus although absolute values may not be able to extrapolate to other breeds, the trends likely do. Despite such limitations, our study represents a clinical evaluation of dogs naturally infected with *B. conradae* to date.

In conclusion, dogs known to have aggressive interactions with coyotes have a greater risk of becoming infected with *B. conradae*. As with other *Babesia* organisms, infected dogs may manifest clinical signs including pallor, splenomegaly, and lethargy. Likewise, laboratory testing may reveal regenerative anemia, leukopenia, thrombocytopenia, and hyperglobulinemia. Infection with *B. conradae* should be considered a differential diagnosis for any dog with these signs, particularly when associated with a history of exposure to coyotes or, potentially, other wild canids. Further investigation is needed to fully establish the geographic distribution and epidemiology of this disease, including the role of wildlife and arthropod vectors in *B. conradae* transmission as well as the response to treatment with anti-babesial compounds.

#### ACKNOWLEDGMENTS

The authors acknowledge Dr Geri Lake-Bakaar, DVM, DACVIM (Cardiology) for performance and interpretation of echocardiograms on some of the dogs in our study. This work was performed at the Veterinary Medical Teaching Hospital, University of California, Davis and field locations in central California. This work was supported by a grant from the Center for Companion Animal Health and gifts from the Judith McBean Foundation. A part of our study was presented as a scientific report at the 2012 ACVIM Forum, New Orleans, LA.

#### CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

#### OFF-LABEL ANTIMICROBIAL DECLARATION

Azithromycin (Zithromax Pfizer Inc, New York) and atovaquone (Mepron, GlaxoSmithKline, Durham, North Carolina) were used in an off-label manner to treat dogs infected with *Babesia conradae*. Doxycycline (Vibramycin, Pfizer Inc, New York) was used off-label for the treatment of hemotropic *Mycoplasma* infections.

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

Our study was approved by the University of California, Davis, IACUC (protocol #18956).

#### ORCID

Jonathan D. Dear  <http://orcid.org/0000-0002-7166-1442>

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**How to cite this article:** Dear JD, Owens SD, Lindsay LL, et al. *Babesia conradae* infection in coyote hunting dogs infected with multiple blood-borne pathogens. *J Vet Intern Med.* 2018;32:1609–1617. <https://doi.org/10.1111/jvim.15233>