Prevalence of Vector-Borne Pathogens in Southern California Dogs With Clinical and Laboratory Abnormalities Consistent With Immune-Mediated Disease

L. Kidd, B. Qurollo, M. Lappin, K. Richter, J.R. Hart, S. Hill, C. Osmond, and E.B. Breitschwerdt

Background: Studies investigating the prevalence of vector-borne pathogens in southern California dogs are limited. Occult infections might be misdiagnosed as idiopathic immune-mediated disease.

Hypothesis/Objectives: (1) To determine the prevalence of vector-borne pathogens in southern California dogs with compatible clinical findings using PCR and serologic panels and (2) to determine whether testing convalescent samples and repeating PCR on acute samples using the same and different gene targets enhance detection.

Animals: Forty-two client-owned dogs with clinical signs of vector-borne disease presenting to specialty practices in San Diego County.

Methods: Combined prospective and retrospective observational study. Forty-two acute and 27 convalescent samples were collected. Acute samples were prospectively tested for antibodies to Rickettsia, Ehrlichia, Bartonella, Babesia, Borrelia, and Anaplasma species. PCR targeting Ehrlichia, Babesia, Anaplasma, hemotropic Mycoplasma, and Bartonella species was also performed. Retrospectively, convalescent samples were tested for the same organisms using serology, and for Ehrlichia, Babesia, Anaplasma, and Bartonella species using PCR. Acute samples were retested using PCR targeting Ehrlichia and Babesia species.

Results: Evidence of exposure to or infection with a vector-borne pathogen was detected in 33% (14/42) of dogs. Ehrlichia and Babesia species were most common; each was identified in 5 dogs. Convalescent serologic testing, repeating PCR, and using novel PCR gene targets increased detection by 30%.

Conclusions and Clinical Importance: Repeated testing using serology and PCR enhances detection of infection by vector-borne pathogens in dogs with clinical signs of immune-mediated disease. Larger prevalence studies of emerging vector-borne pathogens in southern California dogs are warranted.

Key words: Anaplasmosis; Babesiosis; Ehrlichiosis; Flea; Immune-mediated; Rickettsioses; Tick.

Clinical and laboratory abnormalities that characterize idiopathic immune-mediated diseases are also associated with canine vector-borne disease (CVBD). In addition, CVBD agents might cause immune-mediated disease. Thus, it is important to rule out CVBD before declaring immune-mediated disease idiopathic. Despite recent improvements in serological and molecular-based testing, ruling out CVBD can be challenging.

Knowledge of regional CVBD prevalence helps clinicians determine which organisms to include in testing.

Abbreviations:
CVBD canine vector-borne disease
IFA indirect immunofluorescent assay
PCR polymerase chain reaction

Studies of CVBD prevalence in southern California are limited. Southern California extends from immediately north of Los Angeles County southward to Mexico. It is bordered on the east by Arizona and Nevada.
and on the west by the Pacific Ocean. A recent survey of dogs that included southern California found the seroprevalence of *Borrelia burgdorferi* and *Anaplasma* spp. was 0.1–5% and of *Ehrlichia* spp. was 0–2%. A study in 1994 reported between 0 and 15% of LA county shelter dogs were seropositive to *Babesia* species. The prevalence of other CVBD agents has not been examined. However, a study from central and northern California found 20% of ill dogs had evidence of exposure to, or active infection with *B. burgdorferi*, *A. phagocytophilum*, *Bartonella* sp., *Rickettsia rickettsii*, or *E. canis*. Outbreaks of *R. rickettsii* in Northern Mexico and Arizona, and *R. massiliae* in Los Angeles were reported in dogs and people. In addition, a focal re-emergence of *Babesia conradae* in dogs in Los Angeles County recently occurred. *Rhipicephalus sanguineus* was the suspected vector in these outbreaks. This tick is also an established or suspected vector for *E. canis, B. vogeli, A. platys*, hemotropic *Mycoplasma* and *Bartonella* spp. and its expanding geographic distribution includes southern California. Therefore, investigation into whether these organisms contribute to illness in southern California dogs is warranted.

In addition to deciding which organisms to include in testing, clinicians must consider the sensitivity and specificity of the testing modality. Whether serologic or PCR testing of a single sample is sufficient to diagnose CVBD depends on characteristics of the host and illness in southern California dogs is warranted. Whether serologic or PCR testing was performed at the time of enrollment as part of a separate investigation.

**Materials and Methods**

This study was approved by The Western University College of Veterinary Medicine Institutional Animal Care and Use Committee (# R09iauc014). Informed consent was required for inclusion. Between December 2009 and May 2011, dogs presenting to 2 specialty practices in southern California with 1 or more of the following clinical or laboratory findings were prospectively enrolled in the study: otherwise unexplained fever (defined as a body temperature above 102.5°F); anemia or thrombocytopenia (defined as PCV, hematocrit, or platelet count below the reported reference range from the laboratory where the blood was analyzed); epistaxis; arthralgia or confirmed polyarthritis; evidence of ocular inflammation (scleral or conjunctival inflammation or injection, retinal hemorrhage, uveitis, or retinitis); myalgia; proteinuria; or neurologic abnormalities including hyperesthesia, ataxia, or vestibular disease. Dogs receiving doxycycline at the time of presentation were excluded. Medical records were examined retrospectively to verify clinical and laboratory findings reported at enrollment, to document the occurrence of other underlying disease, and to verify whether owners were asked about tick or flea exposure. Ethylenediaminetetraacetic acid (EDTA)-anticoagulated whole blood and serum were collected at the time of presentation (acute samples) and, for a subset of dogs, 9–42 days later (convalescent samples). Samples were shipped on cold packs overnight to the laboratory. Samples were either immediately tested or stored at −80°C until testing.

**Vector-Borne Disease Testing**

**Initial Acute Sample Testing**

PCR and serologic testing was performed at the time of collection on acute EDTA and serum samples for all dogs (n = 42) enrolled in the study.

**PCR.** Conventional PCR assays that amplify DNA of *Mycoplasma* spp., *Neorickettsia/Ehrlichia/Anaplasma* sp., *Babesia* spp., and spotted fever group *Rickettsia* sp. were performed as previously described. For 1 dog, additional PCR testing for *Babesia* species using alternate primers was performed at the time of enrollment as part of a separate investigation.

**Serology.** Acute serum samples from all 42 dogs were tested for antibody to *R. rickettsii, E. canis, B. henselae*, *B. vinsonii* subsp. *berkhoffii, B. vogeli*, and *B. gibsoni* using indirect IFA. Antibodies to *E. canis, B. burgdorferi*, *Anaplasma* spp., and *D. immitis* were detected using the SNAP2 4DX test kit.

**Convalescent Sample Testing**

Convalescent EDTA and serum samples were obtained from 27 of the 42 dogs enrolled in the study. For these 27 convalescent samples, serologic testing was performed to detect exposure to *R. rickettsii* prospectively, whereas more comprehensive PCR and serologic testing was performed retrospectively (see below).

**Retrospective Convalescent Sample Testing**

*PCR.** DNA freshly extracted or previously extracted from convalescent EDTA whole-blood samples and stored at −80°C was used for testing. Freshly extracted DNA was obtained from 200 μL of EDTA whole blood, using QIAasymp (Qiagen, USA) and QIAsymp (Qiagen, USA) Mini Kits (192) (Qiagen, USA cat. no. 931236). DNA was eluted in RNase-free, molecular-grade water and stored at −20°C until ready for PCR analysis. DNA extraction controls and negative reagent controls were included for each PCR. Conventional PCR targeting the spotted fever group *Rickettsia ompA*, *Ehrlichia/Anaplasma* 16S rRNA, *Ehrlichia sodB*, *Babesia* 18S rRNA, and the *Bartonella* 16S-23S ITS region was performed as previously described. *Propliom* species were detected by amplifying a 785-bp region of the 18S rRNA gene using primers Pri18S-1445 5′- ACC GTG CTA ATT GTA GGG CTA ATA CA -3′ and Bab18S-772 5′- ATG CCC CCA ACC GTT CCT ATT A -3′. All reactions were performed in a 25-μL final volume reaction containing 12.3 μL of MyTaq HS Mix (2X) (Bioline cat: BIO-25046), 0.4 μM primers (Sigma-Aldrich), 7 μL of
filter-sterilized, molecular-grade water, and 5 μL of DNA template. Thermocycler conditions consisted of a single hot-start cycle at 95°C for 2 minutes, followed by 55 cycles of denaturation at 94°C for 15 seconds, annealing at 64°C for 15 seconds, and extension at 72°C for 18 seconds.

Additional species-specific quantitative real-time PCRs (qPCRs) targeting either the E. canis sodB and p30 genes or the E. ewingii sodB and p28 genes were performed on Ehrlichia PCR-positive samples. PCR targeting the E. canis sodB and p30 genes was performed by amplifying a 100- to 200-bp region using primers Ec-E. canis sodB samples. PCR targeting the sodB and E. canis sodB targeting either the CTA TCA ACT TCT CGT GCC AAA AGG -3' and Ec-sodb-as 5'-GCT CCT CCA CCA TTT TTC TCT TTC ATG G -3' or Ec-p30-as 5'-GAA TCA TGG ACT GGT GGT ATC ATC -3', respectively. qPCR targeting the E. ewingii sodB and p28 genes was performed by amplifying a 100- to 200-bp region using primers Eew-sodb-s 5'-TTT GGT AGT GGA-3' and Eew-sodb-as 5'-TTT GCT GGA GCC ATT GGA-3', respectively. All reactions were performed in a 25-μL final reaction containing 12.5 μL of SYBR® Green Supermix (Bio-Rad, USA, cat: 172-5271), 0.3 μM primers (Sigma-Aldrich), 7 μL of filter-sterilized, molecular-grade water, and 5 μL of DNA template. Thermocycler conditions consisted of a single hot-start cycle at 98°C for 3 minutes, followed by 40 cycles of denaturation at 98°C for 15 seconds, annealing at 67°C for 15 seconds, and extension at 72°C for 15 seconds. Melting temperature measurements were made between 65 and 88°C at 0.5-second intervals. Amplification was performed in a CFX96™ Real-Time Detection System combined with C1000™ Thermal Cycler (Bio-Rad, USA).

Serology: Banked convalescent serum samples were tested retrospectively for antibody to E. canis, B. henselae, B. vinsonii spp. berkhoffii, B. vogeli, B. gibsoni, and R. rickettsii using indirect IFA as described above, and for antibodies to E. canis, E. ewingii, B. burgdorferi, A. phagocytophilum, A. platys, and D. immitis antigen using the SNAP® 4Dx® Plus test kit.

Retrospective Retesting of Acute Banked Blood Samples Using PCR

Available EDTA-anticoagulated whole blood or DNA previously extracted from acute EDTA-anticoagulated whole blood from the 27 dogs for which both acute and convalescent samples were taken was retested for the presence of Babesia (n = 25 dogs) and Ehrlichia (n = 27 dogs) species DNA using primers targeting the Ehrlichia sodB gene, the Babesia 18S rRNA gene, and the Piroplasm 18S rRNA gene as described above. Banked acute EDTA-anticoagulated whole blood or extracted DNA from the dogs for which convalescent samples were not submitted was also retrospectively retested for Babesia species using the Babesia 18S rRNA gene and the Piroplasm 18S rRNA PCR assays.

Species Verification

DNA amplicons from positive PCR samples were sequenced directly by Genewiz (Research Triangle Park, NC).

Statistical Methods

Overall prevalence was calculated as the proportion of dogs enrolled in the study with a positive test result on at least 1 sample. To determine whether combining serology and PCR and repeat testing using the same or alternate primers facilitated detection, the subgroup of dogs from which acute and convalescent samples were taken was examined. The cumulative proportion of dogs with a positive result for acute, convalescent, and retesting of acute samples was calculated. In post hoc analysis, dogs were grouped as to whether they tested positive (CVBD) or negative (no CVBD) for a vector-borne pathogen. Associations between categorical variables were tested using Fisher's exact (2-tailed), and chi-square tests. Statistical significance was set at P < .05. Statistical analysis was performed using GraphPad Prism Statistical Software version 6.0.

Results

Signalment

Forty-two dogs met inclusion criteria. The median age was 7.5 years with a range of 10 weeks to 13 years. There were 14 spayed females, 4 intact females, 18 neutered males, and 6 intact males. There were 10 mixed-breed dogs, 5 golden retrievers, 4 Labrador retrievers, 2 each of Australian shepherd, pitbull, and weimaraner, and 1 each of Anatolian shepherd, basenji, beagle dog, cavalier king charles spaniel, dachshund, Dalmatian, German shepherd, greyhound, keeshond, miniature schnauzer, old English sheepdog, Rhodesian ridgeback, rottweiler, shih tzu, standard poodle, vizsla, and Welsh corgi.

Clinical and Hematologic Findings

Forty-one of 42 dogs had a CBC and 18 dogs had a urinalysis performed within 1 week before study entry. Results of clinical and hematologic findings are presented in Table 1. More than 1 abnormality was reported for 28 dogs; 27 dogs also had accompanying gastrointestinal signs including anorexia vomiting, diarrhea, or both. Ten dogs were receiving antibiotics at the time of enrollment. Three were receiving metronidazole, 5 were receiving a penicillin derivative, 1 was receiving cephalaxin, 1 had received enrofloxacin, and 1 had received Convenia®. Two dogs receiving antibiotics (1 receiving enrofloxacin and 1 receiving metronidazole at the time of testing) were positive for a vector-borne pathogen.

Vector-Borne Disease Testing

Overall Prevalence

When the results of all testing were combined, evidence of exposure to or active infection with at least 1 CVBD agent was 33% (14/42). Five of 42 dogs (12%) had PCR evidence of active infection with Ehrlichia species. Two dogs were E. canis seroreactive and PCR positive and 3 dogs tested PCR positive for an unknown Ehrlichia species closely related to E. ewingii. Five of 42 (12%) of dogs had evidence of infection with Babesia species. Three dogs were B. vogeli PCR positive, and 1 dog was B. gibsoni PCR positive. No dog was seroreactive to B. gibsoni or B. vogeli antigens by IFA testing. Based on PCR testing, 1 dog was coinfected with B. conradae, M. haematoparvum, and M. haemocanis, and was also
seroreactive to \textit{B. vinsonii} subsp. \textit{berkhoffii} antigens. Based upon SNAP\textsuperscript{4DX} results, 1 dog each (2\%) was seroreactive to \textit{A. phagocytophilum} and \textit{B. burgdorferi}. Initially, no dog was \textit{R. rickettsii} seroreactive or PCR positive for spotted fever group \textit{Rickettsia} DNA; however, 2 dogs (4.2\%) subsequently seroconverted to a \textit{Rickettsia} species (Tables 2 and 3).

### Comparison of Combined Acute and Convalescent Testing

Both acute and convalescent samples were obtained from 27 of the 42 dogs initially enrolled in the study. The median time from initial sampling to convalescent sampling was 18 days with a range of 9–42 days. CVBD test results for these 27 dogs were analyzed to determine whether combining serology and PCR and testing more than 1 sample facilitated detection of CVBD agent exposure or infection (Table 3). Combined serologic and PCR testing of the initial blood samples documented infection in 3 of these 27 dogs (11\%). Serology and PCR testing of convalescent samples documented CVBD exposure, infection, or both in 6 additional dogs (9/27: 33\%). Retesting of acute samples using PCR for \textit{Ehrlichia} and \textit{Babesia} spp. combined with PCR and serologic testing of acute and convalescent samples documented evidence of exposure or infection in 11 of 27 dogs (41\%) (Table 3).

### Ehrlichia Testing and Speciation

PCR identified \textit{Ehrlichia} spp. in 2 acute and 5 convalescent samples. \textit{Ehrlichia canis} was verified as the infecting agent for the 2 positive acute samples, both at the time of enrollment and when acute samples were retrospectively retested. Retrospective PCR testing on blood collected 11 days after initial presentation for 1 of these dogs was \textit{E. canis} positive using 3 PCRs (\textit{Ehrlichia} genus sodB, \textit{E. canis} sodB, and \textit{E. canis} p30) and negative using the PCR targeting the \textit{Ehrlichia}/\textit{Anaplasma} 16S rRNA gene. Initial IFA testing revealed an \textit{E. canis} titer of 1:8192 which decreased to 1:64 by day 11. ELISA testing for \textit{E. canis} antibody was positive on both acute and convalescent samples for this dog. For the second dog infected with \textit{E. canis}, a convalescent sample taken 14 days after initial presentation remained positive for \textit{E. canis} DNA by 2 PCRs (\textit{E. canis} sodB and \textit{E. canis} p30) but was negative using the PCR targeting the \textit{Ehrlichia}/\textit{Anaplasma} 16S rRNA gene. Initial IFA testing for an \textit{E. canis} titer of 1:8192 which decreased to 1:64 by day 11. ELISA testing for \textit{E. canis} antibody was positive on both acute and convalescent samples for this dog. For the second dog infected with \textit{E. canis}, a convalescent sample taken 14 days after initial presentation remained positive for \textit{E. canis} DNA by 2 PCRs (\textit{E. canis} sodB and \textit{E. canis} p30) but was negative using the PCR targeting the \textit{Ehrlichia}/\textit{Anaplasma} 16S rRNA gene. Initial IFA testing for this dog revealed an \textit{E. canis} titer of 1:1024 which increased to 1:2048 by day 14. ELISA testing for \textit{E. canis} antibody was negative at presentation but positive by day 14. Both dogs had been treated with doxycycline (Dog A 6.6 mg/kg PO Q12 h and Dog B 10.3 mg/kg PO Q12 h) 2 weeks or less at the time convalescent samples were drawn. One dog (Dog B) was also receiving prednisone 0.5 mg/kg PO Q12 h.

In 3 other dogs, evidence of an \textit{Ehrlichia} species was demonstrated when convalescent samples were tested using PCR targeting the 16S rRNA gene. DNA sequencing of the segment of the 16S gene targeted by the PCR revealed 98\% (1 dog) and 99\% (2 dogs) identical with 100\% coverage to \textit{E. ewingii} GenBank DQ368880 Panola Mtn, \textit{E. ewingii} strain 95E9-TS
Attempts to further amplify DNA and characterize the organism from available acute and convalescent samples using *E. ewingii* species-specific primers were unsuccessful. Acute samples from these 3 *Ehrlichia* convalescent positive dogs were consistently PCR negative. None of these dogs demonstrated antibody to *Ehrlichia* species at any sampling point. One dog had received prednisone, azathioprine, and doxycycline, 1 dog had received prednisone, vincristine, and doxycycline, and 1 dog had received prednisone, mycophenolate mofetil, and doxycycline before convalescent sampling.

**Babesia Species Testing**

On initial PCR testing of the acute samples, *B. vogeli* was amplified from 1 sample using the *Babesia* 18S rRNA primers. Acute samples from 40 of 42 dogs were retrospectively retested using the same primers and *B. vogeli* was detected in 2 additional dogs. These 3 dogs did not seroconvert to *B. vogeli* or *B. gibsoni* antigens. Two were being treated with immunosuppressive medications at the time the convalescent samples were drawn. Convalescent testing using the 18S rRNA PCR revealed *B. gibsoni* infection in another dog; this dog received doxycycline before convalescent sampling.

Forty acute and 27 convalescent samples were tested using the PCR targeting the *Piroplasma* 18S rRNA gene. *B. conradae* infection was demonstrated in a dog that initially tested negative for *Babesia* using the *Babesia* genus-specific 18S rRNA primers. The rest of the samples were PCR negative.

**Associations with Clinical Findings**

Whether or not a dog had been exposed to ticks was specifically noted in the medical record for 14 of 42 dogs. Flea exposure was not noted. Serological or PCR results confirmed exposure/infection in 5 of 5 dogs with a history of tick exposure compared to 1 of 9 with no reported tick exposure. Tick exposure was significantly associated with a positive CVBD test result (Fischer’s exact test: $P = 0.003$).

Thrombocytopenia was documented in 71% (10/14) of the dogs that tested positive for a CVBD and in 44% (12/27) of the dogs that tested CVBD negative (1 dog did not have a CBC performed within a week of presentation). The presence of thrombocytopenia was not significantly associated with a positive CVBD test result (Fisher’s exact test: $P = 0.19$). The presence of anemia, fever, arthralgia, proteinuria, ocular inflammation, or neurologic abnormalities was not significantly higher in dogs with a positive CVBD test.

**Other Diagnoses**

Other than vector-borne disease, the presence of neoplasia (n = 7), suspected neoplasia (n = 3), other infectious diseases (tick-associated dermatitis, and eosinophilic meningitis), and 3 dogs with a history of recent vaccination were noted. Thrombocytopenia was not significantly higher in dogs with a positive CVBD test.

*Table 2. Summary of results of vector-borne disease testing.*

| Testing Modality      | *Ehrlichia spp* | *Anaplasma phagocytophilum* | *Borrelia burgdorferi* | *Rickettsia rickettsii* | *Babesia spp* | *Bartonella spp* | *Mycoplasma spp* |
|-----------------------|-----------------|-----------------------------|------------------------|-------------------------|---------------|-----------------|-----------------|
| Acute Serology n = 42 | 2 *E. canis*    | 1                           | 1                      | 0                       | 0             | 1 *B. vinsonii sp.* | N/A             |
|                        | (1 dog IFA+ and ELISA -) |                     |                        |                         |               |                 |                 |
| Acute PCR n = 42      | 2 *E. canis*    | 0                           | N/A                    | 0                       | 1 *B. vogeli* | N/A             | N/A             |
| Retest Acute PCR n = 27 for *Ehrlichia spp* | 2 *E. canis* | 0                           | N/A                    | 0                       | 3 *B. vogeli* | N/A             | N/A             |
| n = 40 for *Babesia spp* |                |                             |                        |                         | 1 *B. conradae* |                 |                 |
| Convalescent Serology n = 27 | 2 *E. canis* | 0                           | 0                      | 2                       | 0             | 0               | N/A             |
| (Both dogs IFA + and ELISA +) |               |                             |                        |                         |               |                 |                 |
| Convalescent PCR n = 27 | 2 *E. canis* | 0                           | N/A                    | 0                       | 1 *B. gibsoni* | 0               | N/A             |
| 3 *E. spp.* |                                                                                         |                 |                         |                 |

ELISA, Enzyme-linked immunosorbent assay; IFA, indirect immunofluorescent assay; PCR, polymerase chain reaction.

1* dog coinfected with multiple agents.

The 2 *E. canis* positive results were detected in samples taken from the same 2 dogs.

1 of the 3 positive *B. vogeli* results was from the same dog PCR positive for *B. vogeli* on initial testing of an acute sample.
Table 3. Detection of vector-borne disease pathogens using repeated testing in 27 dogs.

| Testing Modality       | Ehrlichia spp | Anaplasma phagocytophilum | Borrelia burgdorferi | Rickettsia rickettsii | Babesia spp | Bartonella spp | Cumulative Overall Prevalence |
|------------------------|---------------|---------------------------|----------------------|----------------------|-------------|---------------|-----------------------------|
| Acute Serology n = 27  | 2 E. canis$^a$ | 0                         | 0                    | 0                    | 0           | 0             | 2/27 (7%)                  |
|                        |               |                           |                       |                      |             |               | 1 dog IFA$^+$ and ELISA $-$ |
| Acute PCR n = 27       | 2 E. canis$^a$ | 0                         | N/A                  | 1 B. vogeli          | 0           | 0             | 3/27 (11%)                 |
| Convalescent Serology  | 2 E. canis$^a$ | 0                         | 2                    | 0                    | 0           | 0             | 5/27 (18%)                 |
|                        |               |                           |                       |                      |             |               | (Both dogs IFA$^+$ and ELISA$^+$) |
| Convalescent PCR n = 27| 2 E. canis$^a$ | 0                         | N/A                  | 1 B. gibsoni         | 0           | 0             | 9/27 (33%)                 |
|                        |               |                           |                       |                      |             |               | 3 E. spp.                  |
| Retest Acute PCR n = 25| 2 E. canis$^a$ | 0                         | N/A                  | 3 B. vogeli$^b$      | 0           | 0             | 11/27 (41%)                |
|                        |               |                           |                       |                      |             |               | 27 Babesia spp             |
|                        |               |                           |                       |                      |             |               | 27 Ehrlichia spp           |

ELISA, Enzyme-linked immunosorbent assay; IFA, indirect immunofluorescent assay; PCR, polymerase chain reaction.

$^a$The 2 E. canis positive results were detected in samples taken from the same 2 dogs.

$^b$1 of the 3 positive B. vogeli results was from the same dog PCR positive for B. vogeli on initial testing of an acute sample.

$^c$Total number of dogs testing positive for at least 1 agent.

Discussion

We found that overall 33% (14/42) of southern California dogs with clinical and laboratory findings compatible with an immune-mediated disease had evidence of CVBD exposure or infection. Consistent with other studies, we found that convalescent serologic testing, sequential PCR testing, and targeting additional bacterial or protozoal genes by PCR facilitate CVBD documentation.\(^{17,21,25,33}\) Evidence of CVBD exposure or infection was higher in this study of ill dogs than has been reported in previous serosurvey testing for fewer infections in dogs.\(^{12,34,35}\) Our results also support microbiological testing trends that recommend the diagnostic use of combined serological and PCR panels.\(^{7,36}\)

The clinical and laboratory abnormalities such as anemia, thrombocytopenia, fever, arthralgia, CNS signs, and ocular inflammation that served as inclusion criteria for this study are commonly reported in association with CVBD. However, these disease manifestations are also associated with idiopathic immune-mediated diseases, neoplasia, and other infections. As expected, many non-CVBD underlying disease processes were documented in the study population. This likely explains the lack of a CVBD statistical association among clinical signs and laboratory abnormalities that occur in association with numerous diseases. However, it is worthy to note a history of a tick bite was associated with documentation of 1 or more CVBD, and documentation of infection or exposure was found in some dogs diagnosed with an idiopathic immune-mediated disease or in dogs with an open diagnosis. Although tick bites often go unnoticed in dogs and people infected with vector-borne agents, these results suggest using both serological and PCR panels and sequential or repeated testing should be strong diagnostic considerations in ill dogs with a history of tick bite.

Rhipicephalus sanguineus is a tick commonly found in southern California.\(^{16,37}\) This tick transmits E. canis, B. vogeli, and R. rickettsii, and it is the suspected vector for B. conradae, A. platys, hemotropic Mycoplasma, and Bartonella vinsonii ssp. berkholfi.\(^{17,18,38,39}\) Infection with an Ehrlichia or Babesia species was most frequently documented in this study. Investigation into the prevalence of Babesia species in either healthy or ill dogs in this area has not been reported since 1994 when 0–15% of shelter dogs in LA county tested seropositive to Babesia species.\(^9\) A more recent study documented exposure to Ehrlichia spp. in up to 2% southern California dogs using serology.\(^8\) Whether these dogs were ill or healthy was not specified. In the study reported here, inclusion criteria specified that all dogs had clinical signs consistent with CVBD, samples were tested using PCR in addition to serology, and sequential and repeated testing was performed. This likely explains the discrepancy in prevalence of Ehrlichia exposure between these 2 studies. In this study, 1 of 2 E. canis-infected dogs with positive PCR and significant IFA titers did not initially have detectable antibodies using the SNAP$^\text{®}$4Dx$^\text{®}$ ELISA test. This rare phenomenon has been
demonstrated previously. It has been suggested that differences in the nature of the target antigen or low titers may explain the discordant results between different types of serologic tests. This explanation is consistent with the observation that the SNAP®/4DX® ELISA became positive after the titer increased to 1:2048 in this dog.

Importantly, these same 2 E. canis-infected dogs tested negative during doxycycline treatment using conventional PCR targeting the 16S rRNA gene, but the presence of circulating Ehrlichia DNA was documented using a PCR targeting the sodB gene and using qPCR, targeting the p30 gene. These results underscore the importance of PCR assay conditions and gene targets used in molecular diagnostics. Differences in sensitivity of PCR or very low numbers of circulating (live or dead) organisms may complicate detection of this organism.

PCR testing of convalescent samples from 3 dogs was positive for an uncharacterized Ehrlichia species most closely matching E. ewingii based on sequencing of the 16S RNA gene. This was an unexpected finding based on sequencing of the 16S RNA gene, but the presence of circulating Ehrlichia DNA was documented using a PCR targeting the sodB gene and using qPCR, targeting the p30 gene. These results underscore the importance of PCR assay conditions and gene targets used in molecular diagnostics. Differences in sensitivity of PCR or very low numbers of circulating (live or dead) organisms may complicate detection of this organism.

PCR testing of convalescent samples from 3 dogs was positive for an uncharacterized Ehrlichia species most closely matching E. ewingii based on sequencing of the 16S RNA gene. This was an unexpected finding based on sequencing of the 16S RNA gene, but the presence of circulating Ehrlichia DNA was documented using a PCR targeting the sodB gene and using qPCR, targeting the p30 gene. These results underscore the importance of PCR assay conditions and gene targets used in molecular diagnostics. Differences in sensitivity of PCR or very low numbers of circulating (live or dead) organisms may complicate detection of this organism.

In this study, acute samples from 3 dogs tested positive for B. vogeli using PCR. Acute blood samples taken from 1 dog tested positive both initially and upon retrospective testing. For the other 2 dogs, B. vogeli DNA was not detected when acute samples were initially tested. However, B. vogeli DNA was detected when banked acute samples were retrospectively tested. (Table 3) This is likely because Babesia species can circulate in low copy number, and therefore, the organism may not be present in each aliquot of blood used for PCR testing. This illustrates that clinicians should consider retesting samples using PCR to detect infection in some patients.

3 B. vogeli PCR-positive dogs did not have detectable antibodies in either acute or convalescent sera. Two of these 3 dogs were being treated with immunosuppressive drugs, 1 for presumed immune-mediated thrombocytopenia and the other for pancytopenia. Immunosuppression has been hypothesized to contribute to a lack of seroconversion in some Babesia-infected dogs, but to our knowledge, this possibility has not been tested in a controlled laboratory study. One dog testing B. gibsoni PCR positive on the convalescent sample was also B. gibsoni seronegative. For this dog, infection occurring after initial presentation cannot be ruled out. The Babesia conradae PCR-positive sample was taken from a household involved in a R. sanguineus bite, and the same house also had concurrent R. rickettsii infection with initially low numbers of organisms detected in this dog are thought to be R. massiliae sp. and how inclusive testing for all relevant species in a geographic locale has important diagnostic and treatment implications.

R. sanguineus is also an important vector for R. rickettsii in Arizona and Mexico, geographic regions adjacent to southern California, and, more recently, in Oklahoma. Like B. vogeli, Rh. sanguineus is the suspected vector for this organism.
convalescent titers to *R. rickettsii* were due to cross-reacting antibodies to another bacteria or infection with another *Rickettsia*, such as *Rickettsia felis*, *R. philipii*, *R. massiliae*, or a nonpathogenic rickettsial endosymbiont of ticks.\(^{16,49,50}\) Considering the presence of competent tick vectors and proximity to outbreaks of RMSF in Arizona and Mexico, veterinarians in southern California should remain vigilant.\(^{13,35,48}\)

Limitations of this study include the small sample size and that cases were from San Diego County rather than across southern California. Furthermore, due to initial funding limitations, testing of convalescent samples and additional testing using PCR of acute samples was performed on stored samples 3 years after the initial study period. In addition, all PCR assays performed initially on the acute sample were not repeated during retrospective testing, limiting direct comparisons of assay results. Samples from all dogs initially enrolled in the study were not available for additional testing, and although no dogs were receiving doxycycline at the time of enrollment, other antibiotics such as metronidazole and enrofloxacin might have decreased circulating numbers of protozoal or rickettsial organisms, respectively.\(^{51,52}\) Therefore, the overall CVBD prevalence reported here might be less than the true CVBD prevalence in this group of dogs. In addition, screening for secondary causes of immune-mediated diseases was not standardized, so it is possible undetected illness could have contributed to clinical signs in CVBD seroreactive or PCR-positive dogs. Indeed, 2 dogs in this study, 1 with bacterial endocarditis and another with suspected discospondylitis, tested positive for vector-borne disease. Finally, due to retrospective testing after initial presentation, it was not possible to evaluate response to treatment. However, lack of response to immunosuppressive treatment is a common cause of euthanasia in dogs with idiopathic immune-mediated disease. The results of this study suggest that occult vector-borne disease could potentially contribute to treatment failure in some of these patients.

**Conclusion**

Overall, we found that 33% of a small group of southern California dogs presenting to private specialty hospitals with clinical or laboratory abnormalities consistent with immune-mediated disease had serological or molecular evidence of CVBD exposure or infection. Infection with *Ehrlichia* and *Babesia* spp. was documented most frequently. Convalescent serologic testing, sequential testing, and the use of novel bacterial and protozoal PCR gene targets enhanced CVBD detection. Clinicians should critically determine whether a sick dog with clinical or hematomatological findings indicative of an immune-mediated disease has ever experienced a flea or tick infestation. In addition to combined serologic and PCR testing at the time of initial presentation, clinicians should consider testing convalescent samples using PCR and serology, or additional testing using PCR on previously acquired samples to detect infection. In addition, laboratories should use multiple gene targets if needed to enhance PCR sensitivity for organisms in a given geographic locale. Larger studies are warranted to determine the overall prevalence of CVBD in southern California dogs and to further define the prognostic and therapeutic implications of unrecognized CVBD in dogs with suspected idiopathic immune-mediated disease.

**Footnotes**

\(^a\) Veterinary Specialty Hospital San Diego CA
\(^b\) California Veterinary Specialists Carlsbad CA
\(^c\) Performed at Colorado State University Center for Companion Animal Studies
\(^d\) Performed North Carolina State University College of Veterinary Medicine Vector Borne Disease Diagnostic Laboratory
\(^e\) Thomas JE, Chandrashekar R, Leutnegger C. et al. Infection of *Babesia conraui* in hunting greyhounds from Oklahoma. *abs. J. Vet. Int. Med* DOI 10.1111/jvim.12699 full

**Acknowledgments**

Conflict of Interest Declaration: E. Breitschwerdt directs the Vector Borne Disease Diagnostic Laboratory and the Intracellular Pathogens Research Laboratory at the Institute for Comparative Medicine at North Carolina State University. He also is chief scientific officer at Galaxy Diagnostics and has been a paid consultant and researcher for IDEXX Laboratories. B. Qurullo is a research assistant professor at the Vector Borne Disease Diagnostic Laboratory at North Carolina State University, and part of her salary is funded by IDEXX Laboratories. M. Lappin oversees diagnostic services at the Center for Companion Animal Studies at Colorado State University and Dr. Lappin has been a paid consultant and researcher for IDEXX and Antech Diagnostics. L. Kidd has consulted for ANTECH and IDEXX Laboratories, and been a paid consultant for Zoetis and Merck Animal Health, and gives lectures sponsored by Zoetis. Antech has provided discounted services for research projects for L. Kidd.

Off-label Antimicrobial Declaration: Doxycycline was prescribed for some dogs in this study.

**References**

1. Grindem CB, Breitschwerdt EB, Perkins PC, et al. Platelet-associated immunoglobulin (antiplaet antibody) in canine Rocky Mountain spotted fever and ehrlichiosis. *J Am Anim Hosp Assoc* 1999;35:56–61.
2. Bexfield NH, Villiers EJ, Herrtage ME. Immune-mediated haemolytic anaemia and thrombocytopenia associated with *Anaplasma phagocytophilum* in a dog. *J Small Anim Pract* 2005;46:543–548.
3. Goodman RA, Breitschwerdt EB. Clinicopathologic findings in dogs seroreactive to *Bartonella henselae* antigens. *Am J Vet Res* 2005;66:2060–2064.
4. Farwell GE, LeGrand EK, Cobb CC. Clinical observations on Babesia gibsoni and Babesia canis infections in dogs. J Am Vet Med Assoc 1982;180:507–511.
5. Day MJ. The immunopathology of canine vector-borne diseases. Parasit Vectors 2011;4:48.
6. Foley D, Drazenovich N, Leutenegger CM, et al. Association between polyarthritis and thrombocytopenia and increased prevalence of vector-borne pathogens in California dogs. Vet Rec 2007;160:159–162.
7. Maggi RG, Birkenheuer AJ, Hegarty BC, et al. Comparison of serological and molecular panels for diagnosis of vector-borne diseases in dogs. Parasit Vectors 2014;7:127.
8. Little SE, Beall MJ, Bowman DD, et al. Canine infection with Dirofilaria immitis, Borrelia burgdorferi, Anaplasma spp., and Ehrlichia spp. in the United States, 2010–2012. Parasit Vectors 2014;7:257.
9. Yamane I, Gardner IA, Ryan CP, et al. Serosurvey of Babesia canis, Babesia gibsoni and Ehrlichia canis in pound dogs in California, USA. Preventive Vet Med 1994;18:293–304.
10. Bowman D, Little SE, Lorentzen L, et al. Prevalence and geographic distribution of Dirofilaria immitis, Borrelia burgdorferi, Ehrlichia canis, and Anaplasma phagocytophilum in dogs in the United States: Results of a national clinic-based serologic survey. Vet Parasitol 2009;160:138–148.
11. Carrade D, Foley J, Sullivan M, et al. Spatial distribution of seroprevalence for Anaplasma phagocytophilum, Borrelia burgdorferi, Ehrlichia canis, and Dirofilaria immitis in dogs in Washington, Oregon, and California. Vet Clin Pathol 2011;40:293–302.
12. Yancey CB, Hegarty BC, Qurollo BA, et al. Regional seroreactivity and vector-borne disease co-exposures in dogs in the United States from 2004–2010: Utility of canine surveillance. Vector Borne Zoonotic Dis 2014;14:724–732.
13. Eremeeva ME, Zambrano ML, Anaya L, et al. Rickettsia rickettsii in Rhipicephalus ticks, Mexico City, Mexico. J Med Entomol 2011;48:418–421.
14. Demma LJ, Eremeeva M, Nicholson WL, et al. An outbreak of Rocky Mountain spotted fever associated with a novel tick vector, Rhipicephalus sanguineus, in Arizona, 2004: Preliminary report. Ann N Y Acad Sci 2006;1078:342–343.
15. McQuiston JH, Guerra MA, Watts MR, et al. Evidence of exposure to spotted fever group rickettsiae among Arizona dogs outside a previously documented outbreak area. Zoonoses Public Health 2011;58:85–92.
16. Beeler E, Abramowicz KF, Zambrano ML, et al. A focus of dogs and Rickettsia massiliae-infected Rhipicephalus sanguineus in California. Am J Trop Med Hyg 2011;84:244–249.
17. Di Cicco MF, Downey ME, Beeler E, et al. Re-emergence of Babesia conradae and effective treatment of infected dogs with atovaquone and azithromycin. Vet Parasitol 2012;187:23–27.
18. Nicholson WL, Allen KE, McQuiston JH, et al. The increasing recognition of rickettsial pathogens in dogs and people. Trends Parasitol 2010;26:205–212.
19. Birkenheuer AJ, Levy MG, Savary KC, et al. Babesia gibboni infections in dogs from North Carolina. J Am Anim Hosp Assoc 1999;35:125–128.
20. Breitschwerdt EB, Maggi RG, Chomel BB, et al. Bartonellosis: An emerging infectious disease of zoonotic importance to animals and human beings. J Vet Emerg Crit Care (San Antonio) 2010;20:8–30.
21. Starkey LA, Barrett AW, Beall MJ, et al. Persistent Ehrlichia ewingii infection in dogs after natural tick infestation. J Vet Intern Med 2015;29:552–555.
22. Breitschwerdt EB, Hegarty BC, Qurollo BA, et al. Intravascular persistence of Anaplasma platys, Ehrlichia chafeensis, and Ehrlichia ewingii DNA in the blood of a dog and two family members. Parasit Vectors 2014;7:298.
23. Kidd L, Maggi R, Diniz PP, et al. Evaluation of conventional and real-time PCR assays for detection and differentiation of Spotted Fever Group Rickettsia in dog blood. Vet Microbiol 2008;129:294–303.
24. Jeffries R, Ryan UM, Jardine J, et al. Babesia gibsoni: Detection during experimental infections and after combined atovaquone and azithromycin therapy. Exp Parasitol 2007;117:115–123.
25. Harrus S, Waner T, Aizenberg I, et al. Therapeutic effect of doxycycline in experimental subclinical canine monocytic ehrlichiosis: Evaluation of a 6-week course. J Clin Microbiol 1998;36:2140–2142.
26. Baneth G, Harrus S, Ohnoma FS, et al. Longitudinal quantification of Ehrlichia canis in experimental infection with comparison to natural infection. Vet Microbiol 2009;136:321–325.
27. Otranto D, Testini G, Dantas-Torres F, et al. Diagnosis of canine vector-borne diseases in young dogs: A longitudinal study. J Clin Microbiol 2010;48:3316–3324.
28. Birkenheuer AJ, Levy MG, Breitschwerdt EB. Development and evaluation of a seminested PCR for detection and differentiation of Babesia gibsoni (Asian genotype) and B. canis DNA in canine blood samples. J Clin Microbiol 2003;41:4172–4177.
29. Lappin MR, Breitschwerdt EB, Jensen WA, et al. Molecular and serologic evidence of Anaplasma phagocytophilum infection in cats in North America. J Am Vet Med Assoc 2004;225:879–882.
30. Jensen WA, Lappin MR, Kamkar S, et al. Use of a polymerase chain reaction assay to detect and differentiate two strains of Haemobartonella felis in naturally infected cats. Am J Vet Res 2001;62:604–608.
31. Eddleston SM, Diniz PP, Neer TM, et al. Doxycycline clearance of experimentally induced chronic Ehrlichia canis infection in dogs. J Vet Intern Med 2007;21:1237–1242.
32. Qurollo BA, Riggins D, Conyn A, et al. Development and validation of a sensitive and specific sodB-based quantitative PCR assay for molecular detection of Ehrlichia species. J Clin Microbiol 2014;52:4030–4032.
33. Starkey LA, Barrett AW, Chandrashekar R, et al. Development of antibodies to and PCR detection of Ehrlichia spp. in dogs following natural tick exposure. Vet Microbiol 2014;173:379–384.
34. Balakrishnan N, Musulin S, Varanat M, et al. Serological and molecular prevalence of selected canine vector borne pathogens in blood donor candidates, clinically healthy volunteers, and stray dogs in North Carolina. Parasit Vectors 2014;7:116.
35. Diniz PP, Beall MJ, Omark K, et al. High prevalence of tick-borne pathogens in dogs from an Indian reservation in northeastern Arizona. Vector Borne Zoonotic Dis 2010;10:117–123.
36. Allison RW, Little SE. Diagnosis of rickettsial diseases in dogs and cats. Vet Clin Pathol 2013;42:127–144.
37. Fritz CL, Kriner P, Garcia D, et al. Tick infestation and spotted-fever group Rickettsia in shelter dogs, California, 2009. Zoonoses Public Health 2012;59:4–7.
38. Latrofa MS, Dantas-Torres F, Giannelli A, et al. Molecular detection of tick-borne pathogens in Rhipicephalus sanguineus group ticks. Ticks Tick Borne Dis 2014;5:943–946.
39. Pappalardo BL, Correa MT, York CC, et al. Epidemiologic evaluation of the risk factors associated with exposure and seroreactivity to Bartonella vinsonii in dogs. Am J Vet Res 1997;58:467–471.
40. Harrus S, Allemme AR, Bark H, et al. Comparison of three enzyme-linked immunosorbent assays with the indirect immunofluorescent antibody test for the diagnosis of canine infection with Ehrlichia canis. Vet Microbiol 2002;86:361–368.
41. O’Connor TP, Hanesco JL, Hegarty BC, et al. Comparison of an indirect immunofluorescence assay, western blot
42. Chandrashekar R, Mainville CA, Beall MJ, et al. Performance of a commercially available in-clinic ELISA for the detection of antibodies against *Anaplasma phagocytophilum*, *Ehrlichia canis*, and *Borrelia burgdorferi* and *Dirofilaria immitis* antigen in dogs. Am J Vet Res 2010;71:1443–1450.

43. Hegarty BC, Qurollo BA, Thomas B, et al. Serological and molecular analysis of feline vector-borne anaplasmosis and ehrlichiosis using species-specific peptides and PCR. Parasit Vectors 2015;8:320.

44. Harris RM, Couturier BA, Sample SC, et al. Expanded geographic distribution and clinical characteristics of *Ehrlichia ewingii* infections, United States. Emerg Infect Dis 2016;22:862–865.

45. Goodman RA, Hawkins EC, Olby NJ, et al. Molecular identification of *Ehrlichia ewingii* infection in dogs: 15 cases (1997–2001). J Am Vet Med Assoc 2003;222:1102–1107.

46. Kjemtrup AM, Wainwright K, Miller M, et al. *Babesia conradae*, sp. Nov., a small canine Babesia identified in California. Vet Parasitol 2006;138:103–111.

47. Kjemtrup AM, Conrad PA. A review of the small canine piroplasms from California: *Babesia conradae* in the literature. Vet Parasitol 2006;138:112–117.

48. Nicholson WL, Gordon R, Demma LJ. Spotted fever group rickettsial infection in dogs from eastern Arizona: How long has it been there? Ann N Y Acad Sci 2006;1078:519–522.

49. Johnston SH, Glaser CA, Padgett K, et al. *Rickettsia* spp. 364D causing a cluster of eschar-associated illness, California. Pediatr Infect Dis J 2013;32:1036–1039.

50. Hii SF, Kopp SR, Abdad MY, et al. Molecular evidence supports the role of dogs as potential reservoirs for *Rickettsia felis*. Vector Borne Zoonotic Dis 2011;11:1007–1012.

51. Breitschwerdt EB, Davidson MG, Aucoin DP, et al. Efficacy of chloramphenicol, enrofloxacin, and tetracycline for treatment of experimental Rocky Mountain spotted fever in dogs. Antimicrob Agents Chemother 1991;35:2375–2381.

52. Lin MY, Huang HP. Use of a doxycycline-enrofloxacin-metronidazole combination with/without diminazene diacetate to treat naturally occurring canine babesiosis caused by *Babesia gibsoni*. Acta Vet Scand 2010;52:27.