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

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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.[1–5] 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.[6,7]

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.[6,8–12] Southern California extends from immediately north of Los Angeles County southward to Mexico. It is bordered on the east by Arizona and Nevada

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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%.[8] A study in 1994 reported between 0 and 15% of LA county shelter dogs were seropositive to *Babesia* species.[9] 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*.[6] Outbreaks of *R. rickettsii* in Northern Mexico and Arizona, and *R. massiliae* in Los Angeles were reported in dogs and people.[13–16] In addition, a focal re-emergence of *Babesia conradae* in dogs in Los Angeles County recently occurred.[17] *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.[18] 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 pathogen. For an antibody-based test to be positive, seroconversion must have occurred before sampling.[18] Furthermore, some CVBD agents including *Bartonella* and *Babesia* species might not consistently induce detectable antibody.[19,20] Therefore, acutely or chronically infected dogs might test seronegative.

Most CVBD PCR assays are highly sensitive. However, many CVBD agents such as *Rickettsia*, *Ehrlichia*, *Bartonella*, and *Babesia* spp. circulate in blood in very low concentration, or intermittently, resulting in a negative PCR test in an infected dog.[20–26] Combining serology and PCR facilitates diagnosis.[7] Analysis of both acute and convalescent serology, sequential PCR testing, and retesting samples with PCR using the same or alternate primers also enhances diagnostic sensitivity.[17,23,24,27] Such additional testing is seldom performed in practice or in prevalence studies, possibly due to financial constraints or lack of clinician awareness regarding its potential value.[6,7]

The objectives of this study were to (1) determine the prevalence of vector-borne pathogens in a cohort of southern California dogs with clinical signs consistent with vector-borne disease using PCR and serologic panels, and (2) determine whether testing convalescent samples and repeating PCR testing on acute samples using the same and different gene targets enhance detection.

Materials and Methods

This study was approved by The Western University College of Veterinary Medicine Institutional Animal Care and Use Committee (# R09iacuc014). Informed consent was required for inclusion. Between December 2009 and May 2011, dogs presenting to 2 specialty practices in southern California[a,b] 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 work 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.[c], and spotted fever group *Rickettsia*[d] were performed as previously described.[23,28–30] 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.[d,17]

Serology[d]. 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.[7,12,28] Antibodies to *E. canis*, *B. burgdorferi*, *Anaplasma* spp., and *D. immitis* were detected using the SNAP®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[d]

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 QIAsymphony[SP] (Qiagen, USA) and QIAsymphony® DNA 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.[23,31,32] Piroplasm species were detected by amplifying a 785-bp region of the 18S rRNA gene using primers Piro18S-144s 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 12.5 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-sodb-s 5′- TGA GGC AAC AGC TGG TGA TTT AGG A -3′ and Ec-sodb-as 5′- GCT CCT CCA CCA TTT TTC TTC ATG G -3′; or Ec-p30-s 5′- GAA TCA TGG ACT GGT GGT ATC ATC CTT -3′ and Ec-p30-as 5′- GCC AAT TAC CCC TGC AAA TCC TAA A -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′- GCT GGA ATA GGT CAT TTT GGT AGT GGA-3′ and Eew-sodb-as 5′- GTT CCC ATA CAT CCA TAG CAA GCA ACG C -3′; or Eew-p28-s 5′- GGT TTT GCT GGA GCC ATT GGA-3′ and Eew-p28-as 5′- GAA CTA TCA ACT TCT CGT GCC AAA AGG -3′, respectively. All reactions were performed in a 25-μL final volume 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 ssp. 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[d]

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 Fischer'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 cephalexin, 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

Table 1. Frequency of hematologic and clinical abnormalities in dogs with and without evidence of exposure to or infection with CVBD.

	Thrombocytopenia	Anemia	Fever	Arthralgia/ polyarthritis	Proteinuria	Ocular inflammation	Neurologic abnormalities	Epistaxis	Myalgia	GI Signs
CVBD	10/14 (71)	5/13 (38)	4/13 (30)	7/14 (50)	2/8 (25)	2/14 (14)	2/14 (14)	0/14 (0)	0/14 (0)	10/14 (71)
No CVBD	12/27 (44)	12/28 (42)	12/27 (44)	9/28 (32)	6/10 (60)	3/28 (10)	1/28 (4)	1/28 (3)	1/28 (3)	17/26 (65)
Total	22/41[c] (54)	17/41[c] (41)	16/40[a] (40)	16/42 (38)	8/18[b] (44)	5/42 (11)	3/42 (7)	1/42 (2)	1/42 (2)	27/40[d] (68)

Results are represented as number with abnormality/total number in group (percent with abnormality).
[a]Temperature was not measured in 2 dogs.
[b]18 dogs had a urinalysis.
[c]1 dog did not have a CBC performed within 1 week before presentation.
[d]The presence or absence of signs of gastrointestinal disease was not noted in 2 dogs.

seroreactive to *B. vinsonii* subsp. *berkhoffii* antigens. Based upon SNAP®4Dx® results, 1 dog each (2%) was seroreactive to *A. phagocytophilum* and *B. burgdorferi*. Initially, no dog was *R. rickettsii* seroreactive or PCR positive for spotted fever group *Rickettsia* DNA; however, 2 dogs (4.2%) subsequently seroconverted to a *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 *Ehrlichia* and *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 *Ehrlichia* spp. in 2 acute and 5 convalescent samples. *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 *E. canis* positive using 3 PCRs (*Ehrlichia* genus sodB, *E. canis sodB,* and *E. canis p30*) and negative using the PCR targeting the *Ehrlichia*/*Anaplasma* 16S rRNA gene. Initial IFA testing revealed an *E. canis* titer of 1:8192 which decreased to 1:64 by day 11. ELISA testing for *E. canis* antibody was positive on both acute and convalescent samples for this dog. For the second dog infected with *E. canis*, a convalescent sample taken 14 days after initial presentation remained positive for *E. canis* DNA by 2 PCRs (*E. canis sodB* and *E. canis p30*) but was negative using the genus-specific sodB PCR and the PCR targeting the *Ehrlichia*/*Anaplasma* 16S gene. Initial IFA testing for this dog revealed an *E. canis* titer of 1:1024 which increased to 1:2048 by day 14. ELISA testing for *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 *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 *E. ewingii* GenBank DQ365880 Panola Mtn, *E. ewingii* strain 95E9-TS

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*[b] (1 dog IFA+ and ELISA −)	1	1	0	0	1 *B. vinsonii ssp. berkhoffi*[a]	N/A
Acute PCR n = 42	2 *E. canis*[b]	0	N/A	0	1 *B. vogeli*	0	1 *M. haematoparvum*[a] 1 *M. hemocanis*[a]
Retest Acute PCR n = 27 for *Ehrlichia spp* n = 40 for *Babesia spp*	2 *E. canis*[b]	0	N/A	0	3 *B. vogeli*[c] 1 *B. conradae*[a]	0	N/A
Convalescent Serology n = 27	2 *E. canis*[b] (Both dogs IFA + and ELISA +)	0	0	2	0	0	N/A
Convalescent PCR n = 27	2 *E. canis*[b] 3 *E. spp.*	0	N/A	0	1 *B. gibsoni*	0	N/A

ELISA, Enzyme-linked immunosorbent assay; IFA, indirect immunofluorescent assay; PCR, polymerase chain reaction.
[a]1 dog coinfected with multiple agents.
[b]The 2 *E. canis* positive results were detected in samples taken from the same 2 dogs.
[c]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.

(U96436.1), and *E. ewingii* Stillwater strain (NR-044747.1). 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 $P = .003$).

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). Thrombocytopenia was not associated with a positive CVBD test result (Fischer's exact test $P = .19$). 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 = 1), other infectious disease (endocarditis, bite wound abscess, discospondylitis, and coccidiomycosis) (n = 4), protein losing nephropathy (n = 3), history of recent vaccination (n = 2), pancreatitis (n = 1, this dog also had a bite

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[c]
Acute Serology n = 27	2 *E. canis*[a] (1 dog IFA+ and ELISA −)	0	0	0	0	0	2/27 (7%)
Acute PCR n = 27	2 *E. canis*[a]	0	N/A	0	1 *B. vogeli*	0	3/27 (11%)
Convalescent Serology n = 27	2 *E. canis*[a] (Both dogs IFA + and ELISA +)	0	0	2	0	0	5/27 (18%)
Convalescent PCR n = 27	2 *E. canis*[a] 3 *E. spp.*	0	N/A	0	1 *B. gibsoni*	0	9/27 (33%)
Retest Acute PCR n = 25 *Babesia spp* n = 27 *Ehrlichia spp*	2 *E. canis*[a]	0	N/A	0	3 *B. vogeli*[b]	0	11/27 (41%)

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.

wound abscess), and protein losing nephropathy with acute renal failure (n = 1) were documented at the time of presentation. A final diagnosis was not reported for 7 dogs. Idiopathic immune-mediated disease was suspected or diagnosed in 14 dogs. Serological or PCR evidence of a CVBD was ultimately documented in 4 dogs with suspected idiopathic immune-mediated disease (2 with presumed immune-mediated thrombocytopenia, 1 with presumed immune-mediated thrombocytopenia and immune-mediated neutropenia, and 1 with presumed immune-mediated pancytopenia), 4 of the dogs with an open diagnosis, 1 dog with PLN, and 2 dogs with evidence of other infections (endocarditis and suspected discospondylitis).

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.[17,21,23,33] Evidence of CVBD exposure or infection was higher in this study of ill dogs than has been reported in previous serosurvey testing for fewer CVBD pathogens that presumably included a population of both healthy and sick dogs.[8] Although the CVBD prevalence in southern California is lower than some other regions of the United States, our results document serological or PCR evidence of several CVBD 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.

Rhiphicephalus 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. berkhoffi*.[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 s*pecies.[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®4Dx® ELISA test. This rare phenomenon has been

demonstrated previously.[40–42] 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.[41,42] 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 rRNA gene. This was an unexpected finding as *Amblyomma americanum*, the vector for *E. ewingii,* has not been reported in southern California. *E. ewingii* infection is most commonly documented in the southeast and mid-atlantic regions of the United States. However, the geographic distribution of *E. ewingii* is expanding, and the organism was recently amplified from a cat in California.[43,44] These 3 *Ehrlichia* PCR-positive dogs were SNAP®4DX Plus® ELISA seronegative for *Ehrlichia* species, and *E. canis* IFA negative. *Ehrlichia* DNA was not PCR-amplified from their acute blood samples, and amplicons were not obtained from the convalescent samples using alternate *E. ewingii*-specific primers. When retested by 16S rRNA PCR, 1 of the 3 dogs had repeatable PCR and DNA sequencing results. Infection with a novel *Ehrlichia* species, chronic *E. ewingii* infection with initially low numbers of organisms and lack of seroconversion, or infection after initial presentation and before seroconversion might explain these results. All 3 dogs were treated with immunosuppressive medications in addition to doxycycline. This may have affected the hosts' ability to mount a humoral immune response, while facilitating an increase in circulating organisms (target DNA for PCR amplification). All 3 dogs were thrombocytopenic, 1 dog had concurrent neutropenia, and another had arthralgia. These clinical signs have been described with *E. ewingii* infection in dogs and people.[44,45] Rigorous protocols that include physical separation of DNA extraction, and PCR amplification and analysis are standard operating procedures for the laboratory and DNA extraction controls and reagent controls were negative for these samples, making false-positive result due to contamination unlikely. Additional studies are needed to define the identity and medical importance of this potentially novel *Ehrlichia*.

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.[24] This illustrates that clinicians should consider retesting samples using PCR to detect infection in some patients.

The 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.[19] 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.

Babesia conradae causes thrombocytopenia and severe hemolytic anemia in dogs in southern California, and, more recently, in Oklahoma.[c,17,46] Like *B. vogeli*, *Rh. sanguineus* is the suspected vector for this organism.[17,47] The *Babesia* 18S PCR that was designed to selectively amplify *B. vogeli* and *B. gibsoni* is not as sensitive as the broader *Piroplasm* 18S primer set that was used in this study for amplification of *Babesia conradae*.[17] Repeat testing with the less species-specific primers confirmed *B. conradae* infection in 1 dog who initially tested negative using the *Babesia* 18S PCR. The dog had severe hemolytic anemia and thrombocytopenia and was adopted from a household involved in a *B. conradae* outbreak (reported previously).[17] This dog was also PCR positive for 2 hemotropic *Mycoplasma* species and seroreactive to *Bartonella vinsonii* ssp. *berkhoffii* antigens. All of the organisms detected in this dog are thought to be transmitted by *Rh. sanguineus*. The CVBD testing results from this dog illustrate the limitations of a PCR assay designed to specifically amplify 2 more common *Babesia* sp. and how inclusive testing for all relevant species in a given geographic locale has important diagnostic and treatment implications.

Rh. sanguineus is also an important vector for *R. rickettsii* in Arizona and Mexico, geographic regions adjacent to southern California, and was a suspected vector for *R. massiliae* infection in dogs and people in Los Angeles.[16,35,48] In the present study, 2 dogs seroconverted to *R. rickettsii*. One of these dogs had transient febrile illness that resolved without antibiotic treatment and the other dog was diagnosed presumptively with discospondylitis that responded clinically after treatment with a cephalosporin. It is not unexpected that PCR would be negative in a dog infected with a *Rickettsia* due the endotheliotropic nature of these organisms and the low numbers of rickettsiae found in systemic circulation throughout infection.[23] However, because the serologic response was not robust and the clinical signs were not typical of Rocky Mountain spotted fever, it is possible that the weakly positive

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 samples 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 hematological 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 conradae* in hunting greyhounds from Oklahoma. *abs*. J. Vet. Int. Med DOI 10.1111/jvim.12609 full

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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. Qurollo 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.

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