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Serum 25-hydroxyvitamin D concentrations in dogs with coccidioidomycosis and variables associated with extent of clinically evident disease

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Abstract

Background: Clinicopathologic variables predictive of disseminated coccidioidomycosis are known in humans but have not been explored in dogs. Serum 25-hydroxyvitamin (OH)D correlates with severity of disease of various etiologies in dogs but its role in coccidioidomycosis is unknown.

Objective: Determine whether serum 25(OH)D concentrations are different in dogs with coccidioidomycosis compared with healthy controls and if clinicopathologic variables are associated with extent of disease.

Animals: Thirty-five dogs with coccidioidomycosis (pulmonary, n = 13; disseminated, n = 15; uncharacterized, n = 7), and 25 healthy control dogs.

Methods: Prospective cohort study. Serum 25(OH)D and C-reactive protein (CRP) concentrations were measured with modified-HPLC and a commercial ELISA kit, respectively.

Results: There was no difference in 25(OH)D concentrations between dogs with coccidioidomycosis (median, interquartile range [IQR]; 31.9 ng/mL, 23.3-49.2) and controls (29.5 ng/mL, 25.6-40.8, $P = .73$). Serum 25(OH)D concentration was lower in dogs with coccidioidomycosis and IgG titers ≥1:32 than dogs with titers below this cut-off ($P = .02$). Dogs with IgG titers ≥1:32 were more likely to have disseminated disease (OR, 7.5; 95% CI: 1.1-68; $P = .03$). Serum CRP concentrations were higher in dogs with IgG titers ≥1:16 (median, IQR; 4474.8 ng/mL, 2885.8-8236.1) than in those below this cut-off (151.2 ng/mL, 30.4-2907.3; $P = .02$). There was a significant inverse association between serum 25(OH)D and CRP at 25(OH)D concentrations ≤33 ng/mL.

Conclusion and Clinical Importance: Serum 25(OH)D concentration was lower for dogs with IgG titers ≥1:32, indicating a potential association between

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; AGID, agar gel immunodiffusion; ANOVA, 1-way analysis of variance; CNS, central nervous system; CRP, C-reactive protein; IQR, interquartile range; VDBP, vitamin D binding protein; VDR, vitamin D receptor; WBC, white blood cell.

semi-quantitative titers and 25(OH)D concentrations in dogs with coccidioidomycosis. IgG titers ≥1:32 yielded higher odds of disseminated disease, but was inadequate as a standalone test to determine form of disease.

KEYWORDS
25(OH)D, coccidioides, dissemination, inflammation, vitamin D

1 | INTRODUCTION

Coccidioides spp., which causes the disease commonly known as Valley Fever, are dimorphic soil-dwelling fungi endemic to the southwestern United States (eg, California, Arizona, Texas, Utah, and New Mexico) and have been identified with increasing frequency in Washington state.[1-4] Disease in dogs can be subclinical, based on positive serology without clinical signs, localize to the respiratory tract (primary pulmonary), or disseminated involving bones, lymph nodes, skin/subcutaneous tissues, eyes, and central nervous system.[4-6]

The specific host immunologic factors in conjunction with fungal strain virulence and inoculum size responsible for the diverse clinical outcomes observed in dogs with coccidioidomycosis are unclear. Vitamin D in various species to enhances several aspects of the innate immune response and modulates potentially deleterious proinflammatory syndromes that, collectively, highlight its importance in mucosal immunity.[7-15] Low serum concentrations or cellular availability of 25-hydroxyvitamin (OH)D, the primary circulating vitamin D metabolite, are associated with *Mycobacterium tuberculosis*, blastomycosis, and community-acquired pneumonia in humans.[16-18] While similar associations were not identified in the single study that investigated vitamin D in humans with coccidioidomycosis, the small study sample could have resulted in limited genotype variability as well as type II error.[19] Vitamin D is a relevant biomarker in dogs with a wide array of infections but has yet to be investigated in dogs with coccidioidomycosis.[20-26] C-reactive protein (CRP) is a positive acute phase protein produced as a reaction to systemic inflammation. Systemic inflammation decreases serum 25(OH)D concentrations.[27,28] Therefore, accounting for the magnitude of inflammation when investigating vitamin D in dogs with coccidioidomycosis is important. In addition, CRP has been used as a biomarker to determine prognosis, treatment response, and relapse in dogs with infectious, neoplastic, and immune-mediated disorders and thus could provide standalone value in dogs with coccidioidomycosis.[29-34]

Differentiation of primary pulmonary and disseminated coccidioidomycosis in dogs is often difficult because of the vague overlap in clinical signs such as fever, altered appetite, lethargy, vomiting, or diarrhea and client aversion to some recommended diagnostic tests because of invasiveness, expense, or both. Understanding the extent of infection is important in this disease, as it can affect prognosis and influence the recommended length of antifungal therapy. Dogs with disseminated disease generally require long-term antifungal therapy, whereas pulmonary infections usually require 6-12 months.[4] Predictive clinicopathologic variables of disseminated coccidioidomycosis have been identified in humans, but have not been reported in dogs.[35] Specifically, high complement fixation titers >1:16 and erythrocyte sedimentation rate.[35]

To begin to understand the role of vitamin D in dogs with coccidioidomycosis and to investigate whether variables associated with extent of disease exist, our study had two objectives (a) to compare serum 25(OH)D concentration in dogs with coccidioidomycosis with healthy control dogs, and (b) to identify clinical and clinicopathologic variables associated with dissemination. We hypothesized that serum 25(OH)D concentration would be lower in dogs with coccidioidomycosis and 1 or more variables would be associated with the presence of dissemination.

2 | MATERIALS AND METHODS

2.1 | Criteria for selection of cases

Client-owned dogs that had an initial diagnosis of coccidioidomycosis between April 2019 and September 2020 were eligible for inclusion in this prospective cohort study. Dogs were included in the study after obtaining informed owner consent or if serum left-over from other diagnostic purposes was available. This study was conducted in accordance with guidelines for clinical studies and approved by the Midwestern University Animal Care and Use Committee (protocol # 2929). Diagnostic testing for coccidioidomycosis and antifungal treatment decisions were made by the attending clinician, not the research investigators. Serological testing for IgM and IgG against *Coccidioides* spp. was performed by agar gel immunodiffusion (AGID) through commercial laboratories (Protatek Reference Laboratory, Mesa, AZ; Antech Diagnostics, Irvine, CA, IDEXX Laboratories, Westbrook, ME). Dogs were considered to have coccidioidomycosis if they had nonspecific clinical signs (eg, lethargy, pyrexia, vomiting, diarrhea, weight loss) with or without organ-specific clinical signs, in addition to ≥1 of positive AGID IgM or IgG titer result, positive culture, or if *Coccidioides* spp. organisms were identified on cytological or histopathological examination. Dogs with coccidioidomycosis were categorized into three subgroups: pulmonary, disseminated, or uncharacterized. Requirements for the pulmonary subgroup included respiratory signs (eg, cough, wheeze, increased respiratory effort, exercise intolerance, tachypnea, or syncope) with thoracic imaging abnormalities characteristic of *Coccidioides* spp. fungal pneumonia and no clinical evidence of

dissemination.[36,37] Dogs in the disseminated subgroup had confirmation or strong clinical suspicion of disease in ≥1 extrapulmonary organ. The uncharacterized subgroup included dogs with insufficient clinical or diagnostic evidence to determine pulmonary vs disseminated disease. Radiographs were reviewed by a single board-certified veterinary radiologist (Eric T. Hostnik). Exclusion criteria included dogs that were pregnant, lactating, had a known history of hypercalcemia of malignancy, hyperparathyroidism, hypoparathyroidism, chronic kidney disease, or were administered vitamin D or calcium supplements. In addition, dogs were excluded if antifungal therapy was administered for >24 hours before enrollment, or if there was previous treatment with antifungal therapy for a historical diagnosis of coccidioidomycosis.

A second sample of dogs was enrolled as a control group. Control dogs were owned by faculty, students, and staff at the Midwestern University College of Veterinary Medicine and were considered healthy based on history, physical examination, and after review of hematology, serum chemistry, and urinalysis results by a single board-certified small animal internist (JAJ). Control dogs could not have had any illnesses or been administered any medications, except monthly parasiticides within 60 days of enrollment. Control dogs were also required to have negative anti-*Coccidioides* spp. antibody (IgM and IgG) titer results at the time of enrollment.

2.2 | Sample collection

Medical records were reviewed for each dog enrolled. The age, sex, weight, and breed were recorded. The following clinical information was extracted when available: clinical signs, physical examination findings, medications, maintenance diet, hematology and chemistry results, cytological and histopathological examination reports, microbiology results, radiographs, and follow-up information.

Blood samples were collected into serum separator blood tubes, allowed to clot, centrifuged, and serum collected within 1 hour of sample collection. Serum was placed in freezer-resistant conical microcentrifuge tubes and stored at −80°C for batch analysis of serum 25(OH)D and CRP concentrations. Serum concentration of 25(OH)D were determined in thawed serum using a previously reported HPLC method with the following modifications: the volume of assayed serum was halved to 0.5 mL and omission of addition of the internal standard, laurophenone, to liquid the extraction solvent, acetonitrile. For internal standard, 3-epi-25-hydroxyvitamin D_2 (25 ng in 5 µL of methanol) was mixed into serum and left to equilibrate for 14–16 hours at 4°C before sample extractions. Validity of the assay for detection of 25(OH)D in dog serum was previously reported.[38,39] Serum CRP was measured with a commercially available canine-specific sandwich ELISA (Abcam, Cambridge, UK) as previously described.[40] ELISA samples were measured in duplicate with concurrent standard curves using kit-provided canine standards; the lower limit of detection for CRP was 1.1 ng/mL. Mean absorbance was used to calculate concentration. Most neat samples from dogs with coccidioidomycosis yielded CRP concentrations outside the range of the kit standards. To correct for this, samples were first diluted 3× in Sample Diluent NS and then diluted 8× in Sample Diluent 25BS according to manufacturer recommendations. The optical density of the samples was determined with a Biotek Cytation 3 microplate reader (Biotek, Vermont, United States) set to a wavelength of 450 nm and background absorbance was measured at 700 nm and subtracted from sample absorbance. Sample CRP concentration was determined by plotting the kit standards using a linear curve, calculating dilute sample concentrations from this curve, and multiplying concentrations by 24 to adjust for the serial dilution.

2.3 | Statistical analysis

Statistical analyses were performed using proprietary software (SigmaPlot, Systat Software Inc and STATA 17, StataCorp LLC). Normality was assessed using the Shapiro-Wilk test. Normally distributed data were presented as mean and SD, while data that were not normally distributed were presented as median and interquartile range (IQR), which was expressed as the 25th and 75th percentiles. Categorical data were presented as proportions. The primary aim was to compare serum 25(OH)D concentrations in dogs with coccidioidomycosis to healthy controls. The secondary aim was to determine whether anti-*Coccidioides* spp. antibody titers or other biochemical data were associated with disseminated disease as compared with pulmonary disease in dogs with coccidioidomycosis. Statistical testing was performed from a priori hypotheses. Student's *t*-test was used for two group comparisons of normally distributed continuous variables, and Mann-Whitney rank sum test for non-normally distributed continuous or ordinal variables. A One-Way Analysis of Variance (ANOVA) test was used for multigroup comparisons of serum 25(OH)D concentration. A Kruskal-Wallis 1-way ANOVA on ranks test, followed by a Dunn's test for multiple comparisons was used for multi-group comparisons of serum CRP concentration. Fisher's exact test was used for categorical associations. Linear regression was used to quantify the relationship between serum 25(OH)D and CRP below a cut point determined from the inflection of a Lowess curve superimposed on a scatterplot of 25(OH)D and CRP. Anti-*Coccidioides* spp. IgG titer cut points were determined similarly for 25(OH)D and CRP from the inflection of non-linear best-fit lines superimposed over scatterplots of IgG titer and the respective measure. Nonparametric Receiver Operating Characteristic (ROC) analysis was used to determine whether IgG titers were predictive of disseminated vs pulmonary disease in dogs with coccidioidomycosis. The optimal cut point was determined using the Youden method. Exact logistic regression was used to determine the odds ratio for disseminated as compared with pulmonary disease ≥ the cut point identified via the ROC analysis. Only hematologic and serum

biochemical data obtained from the Midwestern University Companion Animal Clinic at the corresponding visit associated with dog's coccidioidomycosis diagnosis were used in comparisons. Hematologic and serum biochemical continuous data were also described categorically after a qualitative transformation with respect to variables below or above their respective reference interval. Because of no prior data available for serum 25(OH)D concentrations in dogs with coccidioidomycosis, the sample size analysis performed during study design was based on a study of serum 25(OH)D concentrations in dogs with blastomycosis as compared with healthy controls that found a difference of 53 ng/mL between the median values for 25(OH)D.[26] Using an alpha of 0.05, beta of 0.2, and an estimated population variation of 25 ng/mL to calculate a sample size for a Mann-Whitney rank sum test resulted in a sample size of at least 25 dogs per group. A *P*-value of <.05 was considered significant.

3 | RESULTS

3.1 | Dogs

Thirty-five dogs with coccidioidomycosis were enrolled and no screened dogs were excluded. Thirty-one dogs were initially eligible to be included as healthy controls. Six dogs were subsequently excluded because of anti-*Coccidioides* spp. IgG positivity (n = 2), administration of a nutraceutical containing vitamin D (n = 2), moderate liver enzymopathy (n = 1), and diarrhea the day of sample collection (n = 1). Twenty-five healthy dogs were included as controls. There was no difference in age ($P = .25$), weight ($P = .34$), or sex distribution ($P = .69$) between dogs with coccidioidomycosis and controls. A complete summary of demographic data (ie, age, weight, sex, breed) can be found in Table 1. Maintenance diet information was available for 80% (28/35) of dogs with coccidioidomycosis. Most dogs (96%, 27/28) were fed a commercially available diet. One dog was fed a home-prepared diet. Control dogs were all fed a commercially available diet.

Coccidioidomycosis was most commonly diagnosed via serology for anti-*Coccidioides* spp. antibodies alone (n = 27), or in combination with cytology (n = 4), histopathology (n = 1), cytology and histopathology (n = 1), or cytology, histopathology, and culture (n = 1). One dog was euthanized without serologic testing and was diagnosed with histopathology alone on post-mortem examination. Thirty-four dogs with coccidioidomycosis had serology results evaluated for both IgM and IgG. Most dogs had anti-*Coccidioides* spp. antibody titers performed at Antech Diagnostics (71%, 24/34) while the remaining 10 dogs had antibody titers measured at Protatek Reference Laboratory (21%, 7/34) and IDEXX Laboratories (8%, 3/34). A total of 27% (9/34) of dogs had positive IgM results. All 34 dogs had positive IgG results. Semi-quantitative IgG titers ranged from 1:2 to 1:128, with a median titer of 1:16. The mean rectal temperature at the examination visit corresponding with disease diagnosis was 102.4 °F (SD, 1.0). Thirty-four percent (12/35) of dogs had a rectal temperature ≥ 103 °F. The most common clinical signs included cough (24/35, 69%), lethargy (14, 40%), decreased appetite (13, 37%), lameness (8, 23%), and weight loss (6, 17%). The complete distribution of clinical signs can be found in Table S1.

Thirteen dogs were diagnosed with pulmonary coccidioidomycosis with anticoccidioidal antibody titer positivity in conjunction with respiratory signs and thoracic imaging abnormalities. One of the 13 dogs (8%) had a positive IgM result and all 13 dogs (100%) had positive IgG results. Semi-quantitative IgG titers ranged from 1:2 to 1:128, with a median titer of 1:16. The most common signs of dogs with pulmonary coccidioidomycosis included cough (13/13, 100%), lethargy (9/13, 69%), and decreased appetite (5/13, 38%). Increased respiratory effort and exercise intolerance were reported in 1 dog (1/13, 8%); tachypnea was reported in 1 dog (1/13, 8%). Sixty-nine percent (9/13) of dogs had a solitary pulmonary pattern of which, a bronchial pattern was most common (56%, 5/9), followed by nodular pattern (33%, 3/9), and unstructured interstitial pattern (11%, 1/9). Thirty-one percent (4/13) of dogs had multiple concurrent pulmonary patterns. The combination of pulmonary patterns varied and included: (i) nodular and alveolar, (ii) nodular, unstructured interstitial, alveolar, and bronchial, (iii) nodular and bronchial, and (iv) alveolar and bronchial. Intrathoracic lymphadenomegaly was identified in 31% (4/13) of dogs. Two dogs had tracheobronchial lymphadenomegaly alone while another dog only had cranial mediastinal lymphadenomegaly. The last dog had a combination of tracheobronchial lymphadenomegaly and cranial mediastinal lymphadenomegaly. There was a single dog that had pleural effusion.

Disseminated coccidioidomycosis was diagnosed in 15 dogs. Twelve dogs had a single extra-thoracic site of dissemination that included bone (n = 6), subcutaneous nodules/masses ± draining tracts (n = 4), and 1 each within the central nervous system (CNS) and eye. Three dogs had >1 extra-thoracic site of dissemination. One dog was euthanized and post-mortem examination identified infection in the majority of long bones/lumbar vertebrae, multifocally within bone marrow, multiple lymph nodes, spleen, liver, lungs, and brain. The remaining 2 dogs had surgical exploratory procedures; 1 dog for a subcutaneous mass and draining tract in the ventral cervical region and the other for severe ascites. The dog with disease in the ventral cervical region had infection confirmed by histopathology and culture in subcutaneous tissue, regional lymph nodes, skeletal muscle, and thyroid. The dog with ascites had disease confirmed by cytology as well as histopathology in intra-abdominal body wall nodules, testicles, and lymph node. Forty-three percent (6/14) of dogs had IgM positivity. All 14 dogs that had IgG titers available were positive. Semi-quantitative IgG titers ranged from 1:2 to 1:128, with a median titer of 1:32. Cough was reported in 40% (6/15) of dogs with disseminated disease, of which 83% (5/6) had either thoracic radiography or computed tomography performed. Three of these dogs had unremarkable thoracic radiographs. One dog had a bronchial pattern with multicentric intrathoracic lymphadenomegaly (tracheobronchial, cranial mediastinal, and sternal). The 1 dog that had

TABLE 1 Demographic data for 35 dogs with coccidioidomycosis and 25 healthy control dogs

Variable	Coccidioidomycosis dogs (n = 35)			Control dogs (n = 25)
	Pulmonary (n = 13)	Disseminated (n = 15)	Uncharacterized (n = 7)	
Age (years)[a]	8 (5.6)	6 (3.4)	5.3 (2.7)	5 (5.7)
Weight (kg)[b]	17.7 (11.2)	25.6 (13.7)	24.1 (12.8)	19.3 (11.3)
Sex (MN, MI, FS, FI)	7, 1, 4, 1	7, 3, 5, 0	5, 1, 1, 0	9, 0, 15, 1
Breeds (n)	MBD (5), Labrador retriever (2), Jack Russel terrier (1), Pit bull terrier (1), Catahoula leopard dog (1), Miniature pinscher (1), Portuguese water dog (1), Cairn terrier (1),	MBD (4), Boxer (3), Pit bull terrier (3), Golden retriever (1), Greyhound (1), Heeler (1), Shih tzu (1), Dogue de Bordeaux (1)	MBD (2), German shorthair pointer (1), Pit bull terrier (1), Chihuahua (1), Miniature Australian shepherd (1), Labrador retriever (1)	MBD (13), Miniature schnauzer (2), Miniature Australian shepherd (1), Havanese (1), Goldendoodle (1), Golden retriever (1), Dutch shepherd (1), German shepherd (1), Chihuahua (1), Bull terrier (1), Border collie (1), Australian shepherd (1)

Abbreviations: FI, female-intact; FS, female-spayed; kg, kilogram; MBD, mixed breed dog; MI, male-intact; MN, male-neutered; n, number.
[a]Data presented as median (interquartile range).
[b]Data presented as mean (SD).

a thoracic CT performed had numerous small pulmonary nodules. Thoracic radiographs were unremarkable in the 5 dogs without reported respiratory signs that had thoracic imaging performed. Signs of respiratory disease were not reported in the dog with *Coccidioides* spp. organisms identified in the lungs on post-mortem examination.

Seven dogs were classified as having uncharacterized coccidioidomycosis. The most common reported signs included cough (5/7, 71%), decreased appetite (4/7, 57%), lameness (2/7, 29%), and lethargy (2/7, 29%). Radiographic examination was declined by owners of all dogs with reported cough or lameness. All 7 dogs had either anti-*Coccidioides* spp. IgM (1/7, 14%) or IgG (7/7, 100%) positivity. Semi-quantitative IgG titers ranged from 1:2 to 1:64, with a median titer of 1:8. One dog was lost to follow-up. Clinical signs resolved in the remaining 6 dogs after initiation of antifungal therapy. Four dogs had follow-up titers available for review. Three dogs had ≥1-fold decrease in IgG titer and 1 dog became seronegative.

3.2 | Serum 25(OH)D and C-reactive protein comparisons

Serum 25(OH)D concentration was not different between dogs with coccidioidomycosis (median, IQR; 31.9 ng/mL, 23.3-49.2) and controls (29.5 ng/mL, 25.6-40.8; $P = .73$). Coccidioidomycosis subgroup analysis also revealed no significant differences ($P = .51$, Figure S1). Serum from dogs with coccidioidomycosis (median, IQR; 425 days, 333-542) was stored frozen for longer than healthy controls before measurement of 25(OH)D (150 days, 129-157.5; $P < .001$). Sufficient serum was unavailable to measure CRP concentration in 10 dogs (coccidioidomycosis, n = 5; controls, n = 5).

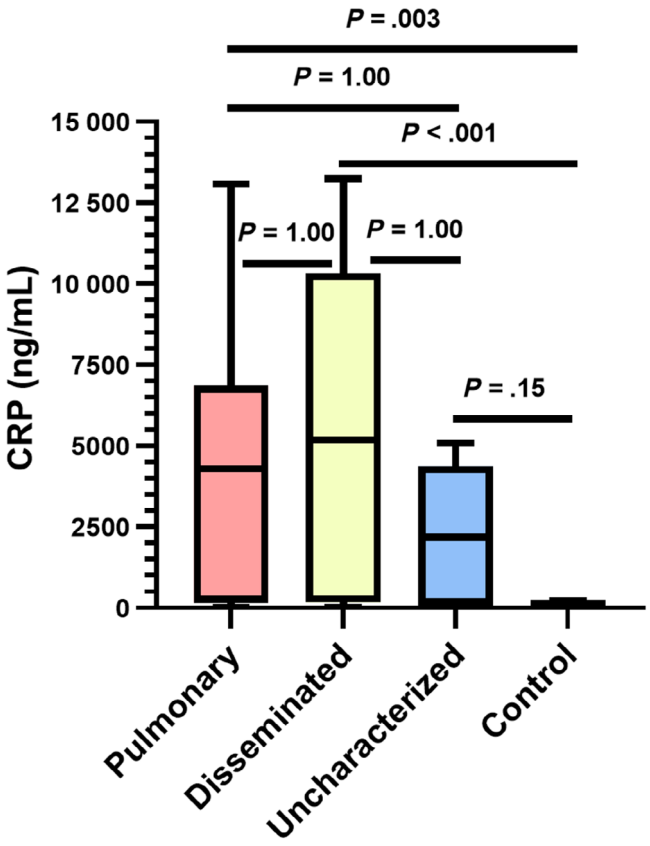

FIGURE 1 Box and whiskers plot comparing serum C-reactive protein (CRP) concentration in dogs with coccidioidomycosis (pulmonary, n = 10; disseminated, n = 13; uncharacterized, n = 7) and healthy control dogs (n = 20). The top and bottom of the boxes represent the 75th and 25th quartiles, respectively with the black horizontal line representing the median. The whiskers represent the range of data

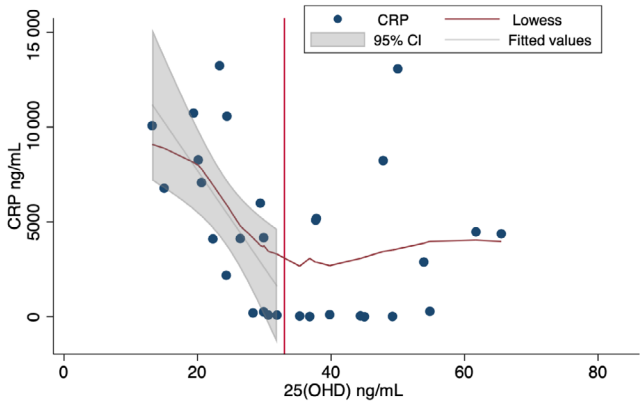

FIGURE 2 Scatterplot of C-reactive protein (CRP) and 25-hydroxyvitamin (OH)D overlaid with Lowess curve. Vertical line at inflection point of 33 ng/mL. Best fit line and 95% confidence interval overlaid for 25(OHD) values <33 ng/mL (n = 16)

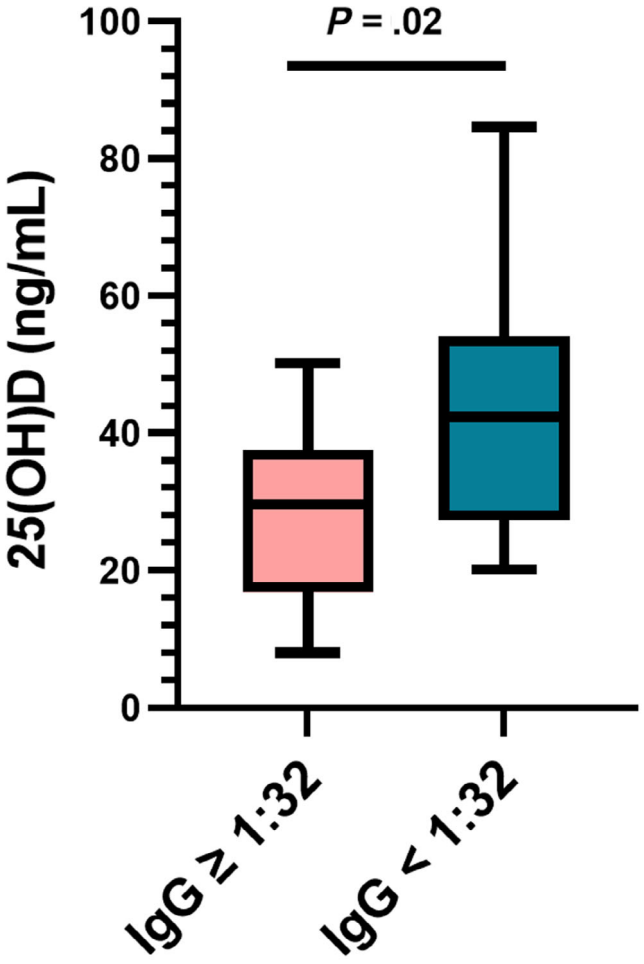

FIGURE 3 Comparison of serum 25-hydroxyvitamin (OH)D concentration in dogs with coccidioidomycosis that had anti-*Coccidioides* spp. IgG titers ≥1:32 (n = 16) and those with titers <1:32 (n = 18). The top and bottom of the boxes represent the 75th and 25th quartiles, respectively with the black horizontal line representing the median. The whiskers represent the range of data

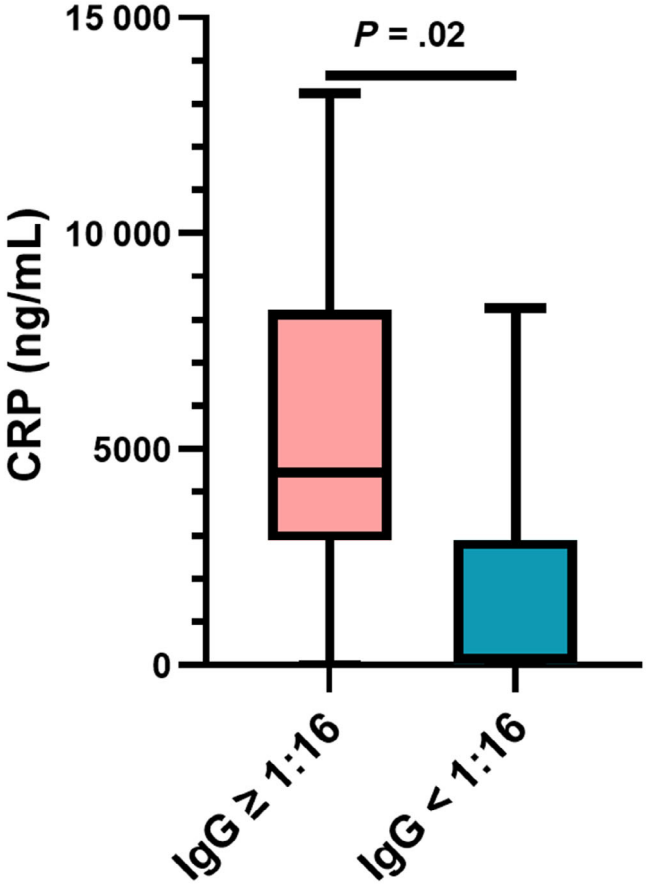

FIGURE 4 Box and whisker plots comparing serum C-reactive protein (CRP) concentration in dogs with coccidioidomycosis that had anti-*Coccidioides* spp. IgG titers ≥1:16 (n = 19) and those with titers <1:16 (n = 10). The top and bottom of the boxes represent the 75th and 25th quartiles, respectively, with the black horizontal line representing the median. The whiskers represent the range of data

Dogs with coccidioidomycosis (median, IQR; 4143 ng/mL, 106-7078, n = 30) had greater serum CRP concentration than controls (24 ng/mL, 17-51, n = 20; $P < .001$). Subgroup comparisons of serum CRP concentration revealed significant differences ($P < .001$); dogs with pulmonary (median, IQR; 4289 ng/mL, 196-6780, $P = .003$) or disseminated (5187 ng/mL, 254-10 082, $P < .001$) disease had greater CRP concentration than controls (24 ng/mL, 17-51, Figure 1). There was no difference in CRP concentration among coccidioidomycosis subgroups or between dogs with uncharacterized coccidioidomycosis and controls (Figure 1). Serum from dogs with coccidioidomycosis (median, IQR; 618.5 days, 342-722.3) was stored frozen for longer than healthy controls before measurement of CRP (63 days, 61-84.5; $P < .001$). There was a linear relationship between CRP and 25(OH)D at 25(OH)D concentrations ≤33 ng/mL, with CRP decreasing by 512 ng/mL (95% CI: 751-273) for each 1 ng/mL increase in 25(OH)D (n = 16; $P = .001$; Figure 2). At 25(OH)D concentrations >33 ng/mL there was no relationship (n = 14; $P = .42$) with CRP.

Next, we wanted to determine if serum 25(OH)D and CRP concentrations were associated with IgM positivity or semi-quantitative IgG titer in all dogs with coccidioidomycosis regardless of subtype. There was no difference in 25(OH)D ($z = 0.8$, $P = .45$) or CRP ($z = -0.1$, $P = .94$) for dogs positive for IgM. Dogs with IgG titer ≥1:32 had lower serum 25(OH)D concentration than those with results <1:32 ($P = .02$, Figure 3). Serum CRP concentration was greater in dogs with IgG titer ≥1:16 compared with those with results <1:16 ($P = .02$, Figure 4). Comparisons of serum 25(OH)D and CRP concentration were performed using significant IgG titer cut-offs identified from visual inspection of scatterplots overlaid with nonlinear best fit-line (Figure S2).

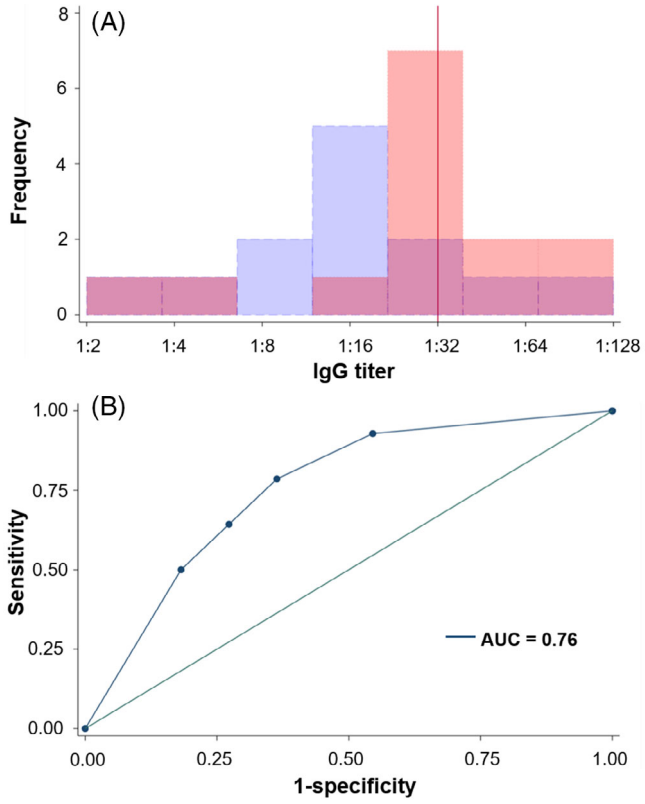

FIGURE 5 Use of IgG titer 1:32 (red vertical line) as a discriminator between pulmonary and disseminated disease as visualized by (A) overlapping histograms of pulmonary (blue dashed outline) and disseminated (red dot outline) disease, with purple areas demonstrating overlap, and (B) Receiver Operating Characteristic curve demonstrating discriminator performance. The diagonal green line represents the reference line

3.3 | Variable association with pulmonary or disseminated coccidioidomycosis

A ROC analysis of the IgG titer's ability to discriminate between pulmonary and disseminated disease yielded an area under the curve (AUC) of 0.76 (95% CI: 0.56-0.95) at IgG titers ≥1:32 (Figure 5). Sensitivity was 78.6% (95% CI: 49.2-95.3) and specificity 69.2% (95% CI: 38.6-90.9), with 74% of cases correctly classified (Table 2). The odds ratio for this cut-off to predict disseminated disease was 7.5 (95% CI: 1.1-68, $P = .03$). There was no significant association between serum anti-*Coccidioides* spp. IgM positivity, breed, sex, or altered status with extent of coccidioidomycosis (Table 3). Sixty-two percent (8/13) and 60% (9/15) of dogs with pulmonary and disseminated disease, respectively, had hematology and serum chemistry results available. A descriptive summary of clinicopathologic variables after qualitative transformation can be found in Table S2. Dogs with pulmonary coccidioidomycosis had higher absolute white blood cell (WBC; $P = .03$) and neutrophil ($P = .02$) counts than dogs with disseminated disease (Table 4). There was no significant difference in age, weight, rectal temperature, serum globulin concentration, absolute lymphocyte count, absolute eosinophil count, absolute monocyte count, platelet

TABLE 2 Test performance of characteristics for anti-*Coccidioides* spp. IgG titer used to discriminate disseminated from pulmonary coccidioidomycosis

IgG cut point	Sensitivity (95% CI)	Specificity (95% CI)	Correctly classified (%)
≥1:2	100% (76.8-100)	0.0% (0.0-24.7)	52%
≥1:4	92.9% (66.1-99.8)	7.69% (0.20-36.0)	52%
≥1:8	85.7% (57.2-98.2)	15.4% (1.92-45.4)	52%
≥1:16	85.7% (57.2-98.2)	30.8% (9.1-61.4)	59%
≥1:32	78.6% (49.2-95.3)	69.2% (38.6-90.9)	74%
≥1:64	28.6% (8.4-58.1)	84.6% (54.6-98.1)	56%
≥1:128	14.3% (0.0-23.2)	92.3% (75.3-100)	52%

TABLE 3 Association of serum anti-*Coccidioides* spp. IgM positivity, breed (purebred/mixed breed), sex (female/male), altered status (neutered/intact) with coccidioidomycosis subtype (ie, pulmonary or disseminated)

Variable	Pulmonary	Disseminated	P-value	OR	95% CI
IgM positivity	1/13 (8%)	6/14 (43%)	0.07	9.00	0.90-89.60
Purebred	8/13 (62%)	11/15 (73%)	0.69	0.58	0.12-2.88
Male	8/13 (62%)	10/15 (67%)	1.00	1.25	0.27-5.89
Neutered	11/13 (85%)	12/15 (80%)	1.00	1.38	0.19-9.83

Abbreviations: CI, confidence interval; IgM, immunoglobulin M; OR, odds ratio.

TABLE 4 Comparative clinical and clinicopathologic variables of dogs with either pulmonary or disseminated coccidioidomycosis

Variable	n	Pulmonary	n	Disseminated	Reference range	*P*-value
Age (years)	13	7.3 (2.7)	15	5.8 (2.9)	—	0.16[a]
Weight (kg)	13	17.7 (11.2)	15	25.6 (13.7)	—	0.11[a]
Temperature (°F)	13	102.7 (1.0)	15	102.3 (1.0)	<103	0.42[a]
HCT (%)	8	42.3 (6.3)	9	47.1 (5.9)	36-60	0.12[a]
WBC ($\times 10^3$ cells/μL)	8	18.7 (5.3)	9	12.6 (7.3)	4.0-15.5	0.03[b]
Neutrophils ($\times 10^3$ cells/μL)	8	15.5 (4.0)	9	10.8 (3.5)	2.1-10.6	0.02[a]
Monocytes ($\times 10^3$ cells/μL)	8	1.1 (0.5)	9	0.9 (0.3)	0-0.8	0.11[b]
Lymphocytes ($\times 10^3$ cells/μL)	8	2.1 (0.8)	9	2.0 (0.6)	0.7-4.5	0.79[a]
Eosinophils ($\times 10^3$ cells/μL)	8	0.4 (0.6)	9	0.5 (0.4)	0-1.2	1.00[b]
PLT ($\times 10^3$ cells/μL)	8	300.8 (122.5)	9	286.9 (72.9)	170-400	0.78[a]
Globulins (g/dL)	8	4.8 (1.0)	9	4.5 (1.0)	1.6-3.6	0.67[a]

Abbreviations: F, fahrenheit; HCT, hematocrit; kg, kilogram; n, number; PLT, platelet; WBC, white blood cell.
[a]Student's *t*-test, data presented as mean (SD).
[b]Mann-Whitney rank sum test, data presented as median (interquartile range).

count, or hematocrit between dogs with pulmonary and disseminated coccidioidomycosis (Table 4).

4 | DISCUSSION

This prospective cohort study investigates in dogs with coccidioidomycosis the association between serum 25(OH)D concentration and the extent of clinically evident coccidioidomycosis (ie, pulmonary vs disseminated). Median serum 25(OH)D concentration was no different in dogs with coccidioidomycosis when compared with healthy controls. However, serum 25(OH)D concentration was lower in dogs with coccidioidomycosis that had IgG titers ≥1:32 compared with dogs with results below this cut-off. Moreover, dogs with IgG titers ≥1:32 were more likely to have disseminated coccidioidomycosis. Serum CRP concentration was higher in dogs that had IgG titers ≥1:16 than those below this titer cut-off and there was an inverse association between serum 25(OH)D and CRP concentration at 25(OH)D concentrations ≤33 ng/mL. Lastly, dogs with pulmonary coccidioidomycosis had higher absolute WBC and neutrophil counts than dogs with disseminated disease. As no adjustments for multiple comparisons were made in this exploratory study, future studies with pre-planned hypotheses based on these findings must be conducted to confirm associations.

Dogs with anti-*Coccidioides* spp. IgG titers ≥1:32 had lower serum 25(OH)D concentration than dogs with results below this cut-off. This suggests that vitamin D could have clinical implications as a biomarker for prognosis and treatment monitoring in dogs with coccidioidomycosis and higher semi-quantitative IgG titers. Further investigation is required to confirm or refute this theory. The specific reason serum 25(OH)D concentration was lower in these dogs is unknown but is likely multifactorial. One such explanation is that vitamin D decreases with systemic inflammation in dogs and humans.[27,28] The majority of vitamin D in circulation is bound to either vitamin D binding protein (VDBP; 70%-80%) or albumin (10%-20%), both of which decrease with systemic inflammation causing an increased fraction of unbound vitamin D and renal loss.[27,41] Proinflammatory cytokines are also theorized to stimulate immunologic cells to uptake 25(OH)D from circulation and convert it to $1,25(OH)_2D$ (ie, calcitriol), the active form of vitamin D.[42-45] The results of our study support the possibility that increased inflammation at higher IgG titers caused a reduction of serum 25(OH)D concentration, as an inverse association between serum 25(OH)D and CRP was identified at low 25(OH)D concentrations. Similar associations between serum 25(OH)D and markers of systemic inflammation (eg, acute phase proteins and inflammatory cytokines) have been reported in dogs and humans.[15,46-50]

The direction of association between 25(OH)D and CRP in our study is unknown and thus it is possible that low serum 25(OH)D concentrations contributed to a more exuberant inflammatory response in dogs with higher IgG titers. This theory is substantiated by the results of a previous study in dogs that identified an inverse relationship between serum 25(OH)D concentration and lipopolysaccharide-stimulated leukocyte production of tumor necrosis factor (TNF)-α, a pro-inflammatory cytokine, in vitro.[15] As serum 25(OH)D concentrations decreased, the magnitude of induced inflammation increased. Moreover, in vitro studies in dogs have highlighted that incubation of blood with calcitriol attenuates leukocyte production of TNF-α in a concentration-dependent manner.[9-11,15] Additional studies with larger sample sizes and more extensive diagnostic databases are needed to better understand the role of vitamin D in dogs with coccidioidomycosis.

The median serum 25(OH)D concentration was no different between dogs with coccidioidomycosis and healthy controls. These results are in contrast with our hypothesis but are similar to findings from the only study to investigate vitamin D in humans with coccidioidomycosis.[19] There were two primary limitations that could have hindered identification of vitamin D differences in our study as well as

the human *Coccidioides* spp. study by Thompson et al[19] in 2013. Both study samples were small, which could have contributed to being underpowered to identify a difference (ie, type II error), as well as limited genetic variability. Several prospective cohort studies investigating genes involved with vitamin D metabolism in humans have revealed associations between variants and risk for infections (eg, *Mycobacterium tuberculosis*, blastomycosis) as well as other outcomes.[17,18,51,52] These genetic variants have yielded several phenotypic derangements such as low circulating 25(OH)D concentrations, decreased cellular availability because of increased VDBP affinity, or lower cellular utilization because of decreased number or affinity for vitamin D receptors (VDR).[17,18,51,52] Results from two recent studies that included molecular analyses of VDR gene polymorphisms suggest that genetics could potentially influence the vitamin D metabolic pathway and have clinical value in dogs, as it does in humans.[24,53]

The odds of disseminated disease were 7.5 time higher in dogs with quantitative IgG titers ≥1:32 than dogs with results below this cut-off, although this result should be interpreted cautiously because of the wide confidence intervals and the classification performance of the cut-off value. While the current study is the first to report an association between increasing baseline serum IgG titers and form of coccidioidomycosis in dogs, this relationship was identified in humans more than 70 years ago.[54] This study evaluated 39 500 serologic tests and concluded that complement fixation titers >1:16 (in other words ≥1:32) were predictive of disseminated coccidioidomycosis.[54] A relatively recent follow-up study found that a serum IgG titer >1:16 had a 70% sensitivity and 85% specificity to predict disseminated coccidioidomycosis in humans.[35] In our study, the diagnostic performance for IgG titers had a slightly higher sensitivity (78.6%) but lower specificity (69.2%). The AUC value of 0.76 indicates that this is a fair test, although AUC values >0.8 would be necessary for a good test. The lower bound of the 95% CI was >0.5, indicating statistical significance.[55] Interpretation of the AUC should be cautious because of this study's small sample population. In addition, it is possible that some dogs with pulmonary coccidioidomycosis and high IgG titers had occult dissemination that was not clinically evident and were misclassified in our study. Future studies with larger sample populations are needed to further investigate the utility of IgG titers to predict form of disease in dogs with coccidioidomycosis. Collectively, our results highlight that generally, dogs with disseminated coccidioidomycosis have higher IgG titers; however, serologic tests alone are inadequate at definitively confirming the form of disease. Dogs and humans with primary pulmonary coccidioidomycosis can have high serum IgG titers.[35,36] In addition, negative or low titers can be found in cases with disseminated infection, either early in the course of disease or when there are sequestered extrapulmonary foci that prevent an adequate antibody response, or in patients with immunodeficiencies.[2,35,36,56,57]

Dogs with pulmonary coccidioidomycosis had higher absolute WBC and neutrophil counts than dogs with disseminated disease in this study. There are no previous data comparing clinicopathologic variables in dogs divided by pulmonary vs disseminated disease. The Crum et al[35] study in 2005 found no differences in leukon parameters based upon extent of coccidioidomycosis in humans. Qualitative assessment of clinicopathologic parameters such as leukocytosis (53%), neutrophilia (30%-63%), monocytosis (8%-50%), and hyperglobulinemia (35%-53%) have been reported in dogs with coccidioidomycosis, irrespective of disease extent, and were similar to results found in our study.[36,56,58] Collectively, these findings demonstrate that an absence of expected abnormal clinicopathologic results cannot be used to exclude a diagnosis of coccidioidomycosis, nor can abnormal results reliably determine extent and localization of disease.

Our study had several limitations that must be considered. Dissemination could have been present in some dogs classified as having primary pulmonary disease. Definitive exclusion of dissemination is impossible in client-owned dogs with naturally acquired coccidioidomycosis, as it would require necropsy data. The subgroup classification criteria used in this study was purposefully strict as to leave little doubt of group classification. This approach led to less dogs being available for statistical tests aimed at identifying variables associated with extent of infection. This exploratory study had a relatively small population size, which could have contributed to being underpowered to identify statistically significant differences or associations. Regarding limitations on analyses involving serum titers, especially because the number of dogs in the study was small, it should be noted that there could be variability in serology results from different laboratories based on differing reagents and methods.[59,60] However, 71% (24/34) of the titers examined herein came from a single laboratory and the impact was likely small. To specifically address laboratory differences would require a separate prospective study testing the same serum at multiple laboratories to identify a systematic difference which could be accounted for statistically. Immune complex glomerulonephritis is a reported complication in dogs with coccidioidomycosis and decreased serum vitamin D is a sequela of protein losing nephropathies in dogs.[61,62] Our study did not account for the potential influence of proteinuria on serum 25(OH)D concentrations. Serum from dogs with coccidioidomycosis was stored frozen at −80°C for longer than healthy controls before measurement of 25(OH)D and CRP. The long-term stability of serum 25(OH)D and CRP in dogs has not been published but both remain stable frozen in serum from humans for many years.[63,64] Finally, it is possible that some dogs with coccidioidomycosis had occult comorbid conditions that could have lowered circulating vitamin D concentrations. The attending clinician made decisions regarding diagnostic investigations and thus a comprehensive evaluation was not available for all dogs in our study. Therefore, some dogs might have had a disorder that could have affected serum 25(OH)D concentrations.

5 | CONCLUSION

Serum 25(OH)D concentration was lower for dogs with IgG titers of ≥1:32, indicating a potential association between semi-quantitative IgG titer and 25(OH)D concentrations in dogs with

coccidioidomycosis. Serum CRP concentrations were higher for dogs with IgG titers of ≥1:16, suggesting an association between IgG titers and inflammation. In addition, serum CRP concentrations were also inversely associated with 25(OH)D concentrations for 25(OH)D concentrations ≤33 ng/mL, although this study could not determine causality. Similar to coccidioidomycosis in humans, an IgG titer of ≥1:32 was a fair discriminator of disseminated disease as compared with pulmonary, with further work required to more precisely determine the sensitivity and specificity.

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CONFLICT OF INTEREST DECLARATION

Cyndi Holland is employed by Protatek Reference Laboratory which offers infectious disease testing in companion animals. No other authors have a conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

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

This study was conducted in accordance with guidelines for clinical studies and approved by the Midwestern University Animal Care and Use Committee (protocol # 2929).

HUMANS ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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