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Serum 25-hydroxyvitamin D concentration and infectious respiratory disease complex in shelter dogs

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Abstract

Background: Hypovitaminosis D is a risk factor for the development of respiratory infections in humans and repletion can be protective.

Objectives: Determine if serum 25-hydroxyvitamin (OH)D concentrations are lower in shelter dogs and if 25(OH)D concentrations are associated with clinical signs of canine infectious respiratory disease complex (CIRDC) or with time in the shelter.

Animals: One hundred forty-six shelter dogs (clinically ill n = 36, apparently healthy n = 110) and 23 nonshelter control dogs.

Methods: Prospective cohort study. Shelter dogs were grouped as clinically ill or apparently healthy based on the presence or absence, respectively, of clinical signs associated with CIRDC. Serum 25(OH)D concentrations were measured with a competitive chemiluminesence immunoassay. Nucleic acids of agents associated with the CIRDC were amplified by polymerase chain reaction assays.

Results: The concentration of 25(OH)D was 7.3 ng/mL (4.5-9.9, 95% confidence interval [CI]) lower in dogs with signs of CIRDC than apparently healthy shelter dogs ($t(142) = 2.0$, $P = .04$). Dogs positive for DNA of canine herpesvirus (CHV)-1 had serum 25(OH)D concentrations 14.9 ng/mL (−3.7 to 29.6, 95% CI) lower than dogs that were negative ($t(137) = 2.0$, $P = .04$). Serum 25(OH)D concentrations in shelter dogs were not different from control dogs ($t(45) = -1.4$, $P = .17$). Serum 25(OH)D concentration was not associated with duration of time in the shelter ($F(1, 140) = 1.7$, $P = .2$, $R^2 = 0.01$).

Conclusion and Clinical Importance: Vitamin D could have a role in acute respiratory tract infections in shelter dogs.

KEYWORDS

25(OH)D, calcidiol, calcifediol, CIRDC, vitamin D

Abbreviations: [25(OH)D], 25-hydroxyvitamin D; CHV-1, canine herpesvirus-1; CIRDC, canine infectious respiratory disease complex; IQR, interquartile range; PCR, polymerase chain reaction.

1 | INTRODUCTION

Canine infectious respiratory disease complex (CIRDC) is a highly contagious syndrome caused by a variety of infectious agents.[1] This syndrome challenges clinicians globally as it consumes shelter resources, decreases adoption, and could result in euthanasia. Nearly 50% of apparently healthy shelter dogs were polymerase chain reaction (PCR) positive for nucleic acids of ≥1 of the agents that have been associated with CIRDC.[2] These results suggest that exposure to these pathogens is common in crowded environments like shelters. The reason some dogs housed in animal shelters develop clinical signs of CIRDC when exposed to these potential pathogens while others remain healthy is usually not known for individual dogs. However, there are many factors both before (eg, variable immunization, parasiticide administration, and nutritional adequacy) and after (eg, high density housing, common dog-to-dog interaction, dose of the pathogen, or exposure to more pathogenic strains or novel pathogens) shelter admission that could contribute to this complex disease process.

Respiratory infections are 1 of the leading causes of morbidity and mortality in children.[3] Hypovitaminosis D is a risk factor in humans, that is associated with increased susceptibility and severity of acute respiratory infections.[4-7] Meta-analysis revealed that vitamin D supplementation per os in humans was safe and protected against acute respiratory tract infections.[8] The primary circulating metabolite of vitamin D, 25-hydroxyvitamin (OH)D, has been established as a useful biomarker in dogs to predict outcome in various disease processes.[9-13] However, the role of vitamin D in shelter dogs with CIRDC remains unknown.

In order to begin to understand the role that vitamin D has in shelter dogs with CIRDC, our study had 4 objectives (a) to compare serum 25(OH)D concentrations in shelter dogs with signs of CIRDC (ie, clinically ill) with those that are apparently healthy; (b) to compare serum 25(OH)D concentration in all shelter dogs with nonshelter control dogs; (c) to compare serum 25(OH)D concentrations in dogs grouped by PCR-identified CIRDC pathogen nucleic acids; and (d) to determine if an association exists between serum 25(OH)D concentration and time spent in the shelter. We hypothesized that serum 25(OH)D concentrations in clinically ill shelter dogs would be decreased and would also be decreased in all shelter dogs compared to nonshelter control dogs. Furthermore, we hypothesized that there would be differences in serum 25(OH)D concentrations based on PCR-identified pathogen and serum 25(OH)D concentration would have an inverse association with time spent in the shelter.

2 | MATERIALS AND METHODS

2.1 | Criteria for selection of cases

A prospective, cohort study was performed. Dogs housed in the Maricopa County Animal Care & Control on June 21, 2019 (ie, date of enrollment for all dogs), that had serum and swabs obtained for other diagnostic purposes were eligible for study inclusion. All dogs were administered intranasal (Intra-Trac3 ADT, Nobivac, MSD Animal Health, Madison, New Jersey) and subcutaneous (Canine 1-DAPPv, Nobivac, MSD, Animal Health) vaccinations at the time of shelter admission. Serum and swabs were collected for the identification, isolation, and treatment of dogs with clinical signs associated with CIRDC. For a shelter dog to be included in the study, a minimum of 0.25 mL of serum obtained from a serum separator tube had to be left over from other diagnostic testing and available for analysis. Physical examinations on shelter dogs were performed by veterinarians. The shelter dogs were grouped as clinically ill or apparently healthy based on the presence or absence, respectively, of the following clinical signs at the time of sample acquisition (ie, June 21, 2019): cough, sneeze, difficulty breathing, tachypnea (ie, ≥40 breaths/minute), conjunctivitis, as well as nasal or ocular discharge.

A second sample of dogs was also enrolled as controls. Control dogs were owned by faculty, students, and staff at the Midwestern University College of Veterinary Medicine (MWU-CVM) and were considered healthy based on history and physical examination. Control dogs could not have any illnesses or been administered any medications or supplements, with the exception of parasitic prevention, within 30 days of enrollment. Client consent was obtained. This study was approved by the MWU-CVM Animal Care and Use Committee (protocol #2981).

Medical records were reviewed for each dog enrolled. The age, sex, and breed were recorded. The following clinical information was retrieved from the medical records of shelter dogs from animal shelter admission to July 1, 2019: admission dates, presence and description of clinical signs, onset of clinical signs, and canine respiratory panel diagnostic results.

2.2 | Canine respiratory panel

Two swabs were obtained from each nonsedated dog: a dry conjunctival swab and a deep pharyngeal swab. Swabs were then combined in sterile blood collection tubes and shipped overnight to the Colorado State University College of Veterinary Medicine. Swabs were stored at −80°C until processed. At the time of processing, the samples were thawed at room temperature for approximately 20 minutes, 1 mL sterile phosphate buffered saline and incubated 2 to 3 hours. The DNA and RNA were coextracted on each swab following a previously published protocol.[14] The DNA/RNA was stored at −20°C until overnight shipment to be tested by PCR assay with a canine respiratory panel (FastPanel Canine Respiratory PCR Panel; agents amplified: *Bordetella bronchiseptica*, Canine respiratory coronavirus, Canine adenovirus type 2, Canine distemper virus, Canine parainfluenza virus, Canine herpesvirus (CHV)-1, Canine influenza virus (H3N8), H1N1 influenza virus, H5N1 influenza virus, *Mycoplasma cynos*, and *Streptococcus equi* subsp *zooepidemicus* (Antech Diagnostics, Fountain Valley, California).

2.3 | Serum 25(OH)D measurement

Serum was stored in freezer resistant plastic tubes, packed with dry ice and shipped overnight for 25(OH)D quantification within 48 hours

of acquisition. Serum was stored at −80°C until quantification. Specimens were measured for 25(OH)D at a commercial laboratory using a direct, competitive chemiluminesence immunoassay. This assay has an intraassay and interassay precision (5 replicates) of 4.0% and 3.4%, respectively. Freeze-thaw testing for 25(OH)D stability revealed there was no significant difference for up to 4 freeze-thaw cycles. This method has been validated in canines and previously reported.[15]

2.4 | Statistical analysis

Statistical analyses were performed using proprietary software (Stata Statistical Software, StatCorp LLC, College Station, Texas). Normality was assessed with tests for skewness and kurtosis. Normally distributed data were reported as means and standard deviation, whereas non-normally distributed data were reported as medians and interquartile range (IQR). Two-tailed *t* tests were used to compare square root transformed 25(OH)D concentrations between clinically ill and apparently healthy dogs. Two-tailed *t* tests with compensation for unequal variances was used to compare all shelter dogs (ie, clinically ill and apparently healthy dogs combined) and nonshelter control dogs. Serum 25(OH)D concentrations were compared between shelter dogs that were PCR-positive for specific pathogens with PCR-negative shelter dogs using 2-tailed *t* tests. The time to clinical signs associated with CIRDC was defined as the number of days between shelter admission and when signs were first recorded. Kaplan-Meier time to event curves were used to analyze the time to clinical signs associated with CIRDC as well as time from shelter admission to sample acquisition. Linear regression was used to examine the relationship between square root transformed 25(OH)D concentration and duration of stay in the shelter. A Lowess line was generated for the concentration of 25(OH)D in shelter dogs over time using a bandwidth of 0.8. The level of significance was set at $P < .05$ for all tests and all results using transformed inputs were reported back-transformed for ease of interpretation.

3 | RESULTS

3.1 | Animals

One hundred forty-six shelter dogs fulfilled the inclusion criteria and were enrolled. There were no dogs that were excluded. There were 90 mixed breed dogs and 56 purebred dogs included. Breeds were Pit Bull terrier (n = 41), Labrador retriever (n = 3), Hound dog (n = 2), Chihuahua (n = 2), and 1 each of German Shepherd dog, Dachshund, Hovawart, Chow Chow, Belgian Malinois, Australian Kelpie, Rhodesian Ridgeback, and Welsh Corgi. The median age was 3 years (IQR, 2-5). There were 64 castrated males, 60 spayed females, 13 intact males, and 9 intact females. The median time from shelter admission to acquisition of samples (ie, serum and swabs) was 46 days (IQR, 28-73).

A total of 25% (36/146) of dogs had ≥1 clinical sign of CIRDC at the time of enrollment (ie, time of sample acquisition, June 21, 2019) and were classified as clinically ill. Fifty-eight percent (21/36), 33% (12/36), and 8% (3/36) of dogs exhibited 1, 2, and 3 signs, respectively. The clinical signs associated with CIRDC observed in clinical dogs included nasal discharge 89% (32/36), cough 42% (15/36), sneeze 14% (5/36), ocular discharge 6% (2/36), and conjunctivitis 3% (1/36).

The remaining 75% (110/146) of dogs were apparently healthy at the time of enrollment. However, 45% (49/110) of apparently healthy dogs were noted to have exhibited clinical signs associated with CIRDC before enrollment but had complete resolution before sample acquisition. The median number of days clinical signs had resolved before enrollment was 22 days (IQR, 15-47). Furthermore, 4% (4/110)

TABLE 1 Demographic data for age, sex, breeds for both shelter dogs (ie, clinically ill and apparently healthy) and nonshelter healthy control dogs in addition to duration of stay (ie, number of days between shelter admission and serum sample acquisition [June 21, 2019 for all dogs]) for shelter dogs

	Shelter dogs (n = 146)		
Variable	Clinically ill (n = 36)	Apparently healthy (n = 110)	Nonshelter healthy control dogs (n = 23)
Age (years)	3 (2–5)	3 (2-5)	4 (2-6)
Sex (FS, FI, MC, MI)	11, 7, 8, 10	49, 2, 56, 3	8, 1, 14, 0
Breeds	MBD (n = 20), Pit Bull terrier (n = 11), Hound dog (n = 2), and 1 each of Chihuahua, Dachshund, and Belgian Malinois	MBD (n = 70), Pit Bull terrier (n = 30), Labrador retriever (n = 3), and 1 each of Rhodesian Ridgeback, Welsh Corgi, Chihuahua, Australian Kelpi, Chow Chow, GSD, and Hovawart	MBD (n = 16), Pit Bull terrier (n = 2), and 1 each of GSD, Welsh Corgi, Boston terrier, Golden retriever, American Cocker Spaniel
Duration of stay (days)	24.0 (19.0-30.5)	62.0 (37.8-79.0)	—

Note: Data presented as median (interquartile range).
Abbreviations: FI, female intact; FS, female spayed; GSD, German Shepherd dog; MBD, mixed breed dog; MC, male castrated; MI, male intact; n, number.

FIGURE 1 Violin plot of 25(OH)D concentration in shelter dogs by presence of clinical signs associated with canine infectious respiratory disease complex (CIRDC) at the time of sample acquisition (ie, June 21, 2019) (no signs at sampling = 110 dogs, clinical signs at sampling = 36 dogs, $P = .04$). Distribution of underlying data shown with a kernel density estimate and overlaid with a boxplot. Line at median, box indicates the interquartile range, and lines extending to the upper- and lower-range

of apparently healthy dogs went on to develop clinical signs associated with CIRDC after enrollment (median, IQR; 4 days, 3-4).

Twenty-three nonshelter healthy control dogs were included. The distribution of breed, sex, age, and duration of stay for shelter dogs (ie, clinically ill and apparently healthy) and nonshelter healthy control dogs can be found in Table 1.

3.2 | Canine respiratory panel

A total of 96.5% (141/146) of dogs had results from the canine respiratory PCR panel available. Of the 141 dogs with available panel results, 47 (32%) were positive for ≥1 pathogen including CHV-1 6 (4%), canine distemper virus 1 (1%), *B bronchiseptica* 11 (8%), and *M cynos* 32 (23%). Nucleic acids of adenovirus, parainfluenza, H1N1, H5N1, H3N8, or *S equi* var. *zooepidemicus* were not amplified from any dog. Most of the PCR positive dogs were apparently healthy at the time of sampling (85%), whereas 19% of clinically ill dogs were PCR positive for CHV-1 (n = 3), *B bronchiseptica* (n = 3), or *M cynos* (n = 3). Although most dogs were PCR positive for only a single pathogen, DNA of both CHV-1 and *B bronchiseptica* were amplified from 1 apparently healthy dog and DNA of CHV-1, *B bronchiseptica*, and *M cynos* were amplified from 1 clinically ill dog. A total of 29 clinically ill dogs (81%) were negative for nucleic acids of all agents.

3.3 | Serum 25(OH)D concentrations

Serum 25(OH)D levels were determined for 146 shelter dogs, 141 of which had concurrent PCR panel results for interpretation. The

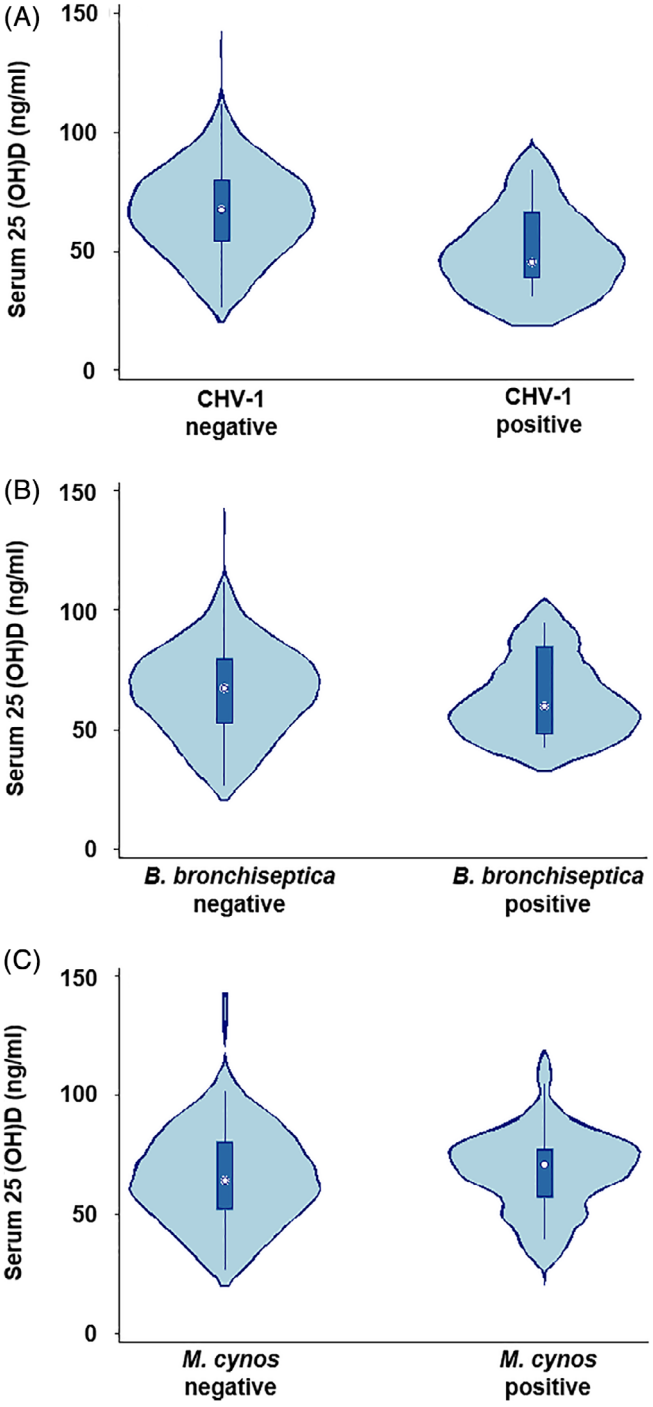

FIGURE 2 Violin plots of 25(OH)D concentration in shelter dogs by presence of the nucleic acids of (A) CHV-1 (negative = 135, positive = 6, $P = .04$), (B) *Bordetella bronchiseptica* (negative = 130, positive = 11, $P = .83$), (C) *Mycoplasma cynos* (negative = 109, positive = 32, $P = .47$). Distribution of underlying data shown with a kernel density estimate and overlaid with a boxplot. Line at median, box indicates the interquartile range, and lines extending to the upper- and lower-range

median concentration of serum 25(OH)D for all shelter dogs was 64.5 ng/mL (IQR, 53-78; n = 146). The distribution of 25(OH)D had a slight right skew, and normality was achieved with a square root

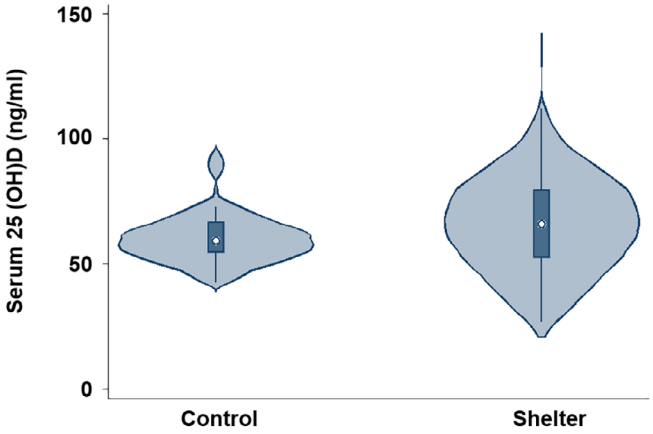

FIGURE 3 Violin plot of serum 25(OH)D concentration between all shelter dogs (n = 146) and nonshelter control dogs (n = 23). Serum 25(OH)D concentration was not significantly different between shelter and nonshelter control dogs (P = .33). Distribution of underlying data shown with a kernel density estimate and overlaid with a boxplot. Line at median, box indicates the interquartile range, and lines extending to the upper- and lower-range

transformation. The serum 25(OH)D concentration of clinically ill shelter dogs was 7.3 ng/mL lower than apparently healthy shelter dogs (t(142) = 2.0, P = .04; Figure 1). Dogs that were PCR-positive for DNA of CHV-1 had significantly lower serum 25(OH)D concentration (14.9 ng/mL) compared to dogs that were PCR-negative for CHV-1 (t(137) = 2.0, P = .04; Figure 2). There was no difference in serum 25(OH)D concentration for dogs that were PCR-positive for DNA of *B bronchiseptica* or *M cynos* and dogs that were negative for these agents, (t(137) = 0.2, P = .83 and t(137) = −0.7, P = .47, respectively; Figure 2). There was no difference in serum 25(OH)D concentration between shelter dogs PCR-positive for ≥1 CIRDC agent as compared to dogs that were PCR-negative for all agents (t(137) = −0.1, P = .95). Similarly, there was no difference between shelter dogs that were PCR-positive in conjunction with clinical signs associated with CIRDC and those that were either PCR-negative or did not have clinical signs (t(142) = 1.7, P = .09). Comparison of serum 25(OH)D concentration between all shelter dogs (ie, clinically ill and apparently healthy dogs combined) and nonshelter control dogs, controlling for unequal variances, showed no difference (t(45) = −1.4, P = .17; Figure 3). Lastly, there was no difference in serum 25(OH)D concentrations in shelter dogs that were PCR-positive for ≥1 CIRDC agent that were clinically ill compared to those that were apparently healthy (t(48) = −1.4, P = .17).

3.4 | Time to event analysis

Time to event analysis of the time from admission to the shelter until onset of clinical signs associated with CIRDC revealed that the median time for dogs that would show clinical signs was before day 19 (Figure 4). A time-to-event curve demonstrating when dogs were sampled (median 46 days) showed that most dogs were sampled after the

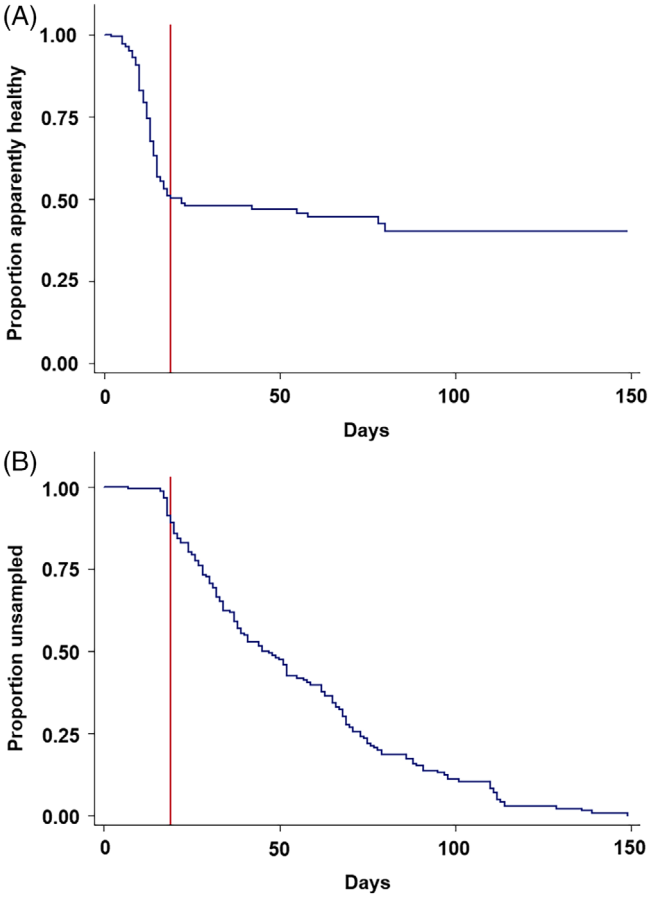

FIGURE 4 Kaplan-Meier curves plotted for 146 shelter dogs for (A) days until clinical signs associated with canine infectious respiratory disease complex (CIRDC) and (B) days until sample acquisition (ie, June 21, 2019). Vertical lines at median time to display respiratory tract infection signs (18 days)

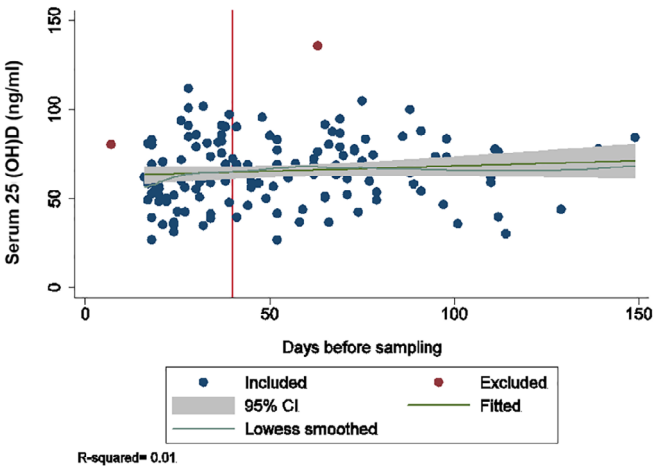

FIGURE 5 Scatterplot of the serum concentration of 25(OH)D by days before sampling for 146 shelter dogs overlaid with a linear best fit line with 95% confidence intervals and a Lowess line calculated using a bandwidth of 0.8. Vertical line at 40 days. Two points excluded from best fit and Lowess lines because of high leverage or outlier status

period of time when they were most likely to begin clinical signs (Figure 4).

3.5 | Serum 25(OH)D concentration and duration of stay

Visual inspection of a scatterplot of serum 25(OH)D concentration and days in the shelter before sampling overlaid with best fit and Lowess lines (Figure 5) did not reveal a linear relationship between 25(OH)D concentration and duration of stay in the shelter. A leverage versus residual squared plot indicated that a dog sampled unusually early (at 7 days) exerted high leverage and a dog with an unusually high 25(OH)D level (136 ng/mL, resulting in a studentized residual of 3.2) was a significant outlier, so both were excluded from the best fit and Lowess lines. Even after these exclusions, there was no significant linear association ($F(1, 140) = 1.7$, $P = .19$) and R^2 was only 1%.

4 | DISCUSSION

This study investigated the role of vitamin D in shelter dogs with and without clinical signs associated with CIRDC. The results from this study indicate that serum 25(OH)D concentrations were decreased in clinically ill dogs compared to those that were apparently healthy when sampled. However, there was no difference between all shelter dogs (ie, clinically ill and apparently healthy dogs combined) and nonshelter control dogs. Dogs that were PCR-positive for CHV-1 had decreased serum 25(OH)D concentrations compared to those that were PCR-negative for CHV-1, which also suggests that vitamin D could have varying roles dependent on the type of infection. Lastly, there was no association between serum 25(OH)D concentrations and duration of stay.

Shelter dogs that exhibited clinical signs associated with CIRDC had lower serum 25(OH)D concentrations compared to apparently healthy shelter dogs in our study. This result supports our hypothesis and corroborates relationships between vitamin D and acute respiratory infections in humans. Whether the decreased serum 25(OH)D concentrations in the clinically ill shelter dogs was a cause of respiratory infection or a consequence of it remains unclear. The majority of circulating vitamin D is tightly bound to vitamin D binding protein (80%-90%) and albumin (10%-20%). These negative acute phase proteins decrease with systemic inflammatory stimuli resulting in increased renal loss of unbound filtered vitamin D.[16] Therefore, it is reasonable to expect that systemic inflammation associated with respiratory infection in shelter dogs contributed to decreased serum 25(OH)D concentrations. Alternatively, genetic variants in vitamin D handling proteins that confer decreased plasma concentrations or receptor affinity to vitamin D in humans are associated with increased risk for many respiratory infections including respiratory syncytial virus,[17] *Mycobacterium tuberculosis*,[18,19] and *Blastomyces dermatitidis*.[20] Mendelian randomization analyses in large, longitudinal, cohort studies in humans have illustrated that genetic variants affecting plasma 25(OH)D concentrations can be causal for disease rather than just a consequence of it.[21] Similar molecular studies have not yet been explored in veterinary medicine. Regardless of whether decreased serum 25(OH)D concentrations was a cause, effect, or a combination of both in the clinically ill shelter dogs in our study, there is potential that an increase in circulating 25(OH)D concentration with oral vitamin D supplementation could decrease the incidence, morbidity, or both of CIRDC in shelter dogs. Future randomized, double-blinded, placebo-controlled clinical trials are needed to investigate the safety and therapeutic benefit of oral supplementation of vitamin D in this population.

Serum 25(OH)D concentrations were lower in dogs that were PCR-positive for CHV-1 compared to those that were PCR-negative for CHV-1; however, differences were not identified between dogs that were PCR-positive for *B bronchiseptica* or *M cynos* and their PCR-negative counterparts. To the authors' knowledge, there are no studies in the human literature that have identified differences in systemic 25(OH)D concentrations based on the type of respiratory pathogen. One explanation for the decreased serum 25(OH)D concentrations in PCR-positive CHV-1 dogs in our study is that a higher proportion of those dogs were clinical (50%) compared to dogs that were PCR-positive for *B bronchiseptica* (27%) or *M cynos* (9%) at the time of serum sample acquisition. This means that a greater proportion of dogs that were PCR-positive for CHV-1 could have had serum 25(OH)D concentrations affected by systemic inflammation. Our study was limited by its design to accurately investigate for clinically important differences in serum 25(OH)D concentrations based on pathogen PCR-positivity. Our investigatory efforts were secondary to standard of care diagnostics aimed to control a CIRDC outbreak in an animal shelter. Therefore, the timing of sample acquisition could have influenced our results. Studies that obtain serum and swabs for 25(OH)D measurement and respiratory pathogen PCR identification, respectively, at the onset of clinical signs associated with CIRDC are needed to better understand if respiratory pathogen related differences in 25(OH)D concentration exist in shelter dogs.

A difference in serum 25(OH)D concentration was not identified when all shelter dogs (ie, clinically ill and apparently healthy dogs combined) were compared to nonshelter control dogs. We had anticipated that shelter dogs would have had lower serum 25(OH)D concentrations because of variable nutritional adequacy before shelter admission. The biosynthesis of vitamin D in many species begins with exposure to ultraviolet light from the sun, wherein 7-dehydrocholesterol in the skin is transformed to pre-vitamin D_3.[22,23] Unlike humans, dogs are unable to synthesize vitamin D_3 in the skin and as a result are entirely reliant on dietary supplementation with vitamin D to meet requirements.[22,23] Serum samples were acquired from shelter dogs in our study a median of 46 days after shelter admission. Therefore, nutritional deficiencies including low serum 25(OH)D could have been improved upon by the time serum samples were obtained because all dogs were fed balanced commercial dog foods in the shelter. Serum 25(OH)D measurements should be obtained at the time of shelter admission in future studies to better understand systemic vitamin D status in shelter dogs.

There was not a significant association between serum 25(OH)D concentration and duration of stay in our study. This result was in

contrast with our hypothesis that an inverse association would be identified. We had anticipated that the risk for opportunistic infections and other inflammatory stimuli would be increased with duration of stay and with it a decrease in serum 25(OH)D concentration. A potential confounding factor that could have offset a gradual decrease in vitamin D was the incorporation of a balanced diet after shelter admission. The lack of an association (positive or negative) between serum 25(OH)D concentration and duration of stay should be interpreted cautiously because the kinetics of vitamin D and influential variables were not investigated in this study and there was a lack of data from the first 2 weeks after admission. Although significant associations were not identified, there was a trend of increasing serum 25(OH)D concentrations suggested by the Lowess curve between days 16 and 40. These results are important because they support that diet could help increase vitamin D concentrations over time but also highlights the improbability that diet alone will increase serum 25(OH)D concentrations fast enough in shelter dogs to yield clinical effects as the onset of CIRDC occurs within 2 to 3 weeks. Commercial dog foods contain vitamin D primarily as vitamin D_3 (cholecalciferol), and some vitamin D_2 (ergocalciferol).[24] It takes 9 to 10 weeks to significantly increase serum 25(OH)D concentration for dogs fed commercial diets along with daily vitamin D_3 (cholecalciferol) supplemented at 5 times the National Research Council recommended allowance.[24] Alternatively, supplementation with oral 25(OH)D results in a much more rapid and efficient increase in serum 25(OH)D concentrations dogs.[25] Therefore, future clinical trials in shelter dogs that aim to investigate the clinical benefits of vitamin D will need to target oral 25(OH)D supplementation in addition to a balanced diet.

The primary problem with all studies amplifying nucleic acids of CIRDC agents is the failure for these assay results to correlate to clinical signs of disease.[26] Apparently healthy dogs are commonly positive for each of these potential pathogens.[2] Positive PCR assay results can be from infection by field strains of the agents, but for many of the potential pathogens, modified live vaccine administration can also result in positive assay results.[27] The only modified live vaccines administered to the dogs of this study that could cause positive PCR assay results were *B bronchiseptica* (11 dogs) and CDV (1 dog). In a previous abstract studying experimental puppies, RNA of CDV was not amplified from conjunctival samples after parenteral administration of a modified live vaccine and so this dog was likely a natural exposure (Burton JH, Veir JK, Pearce L, Hawley JR, Lappin MR. Detection of canine Distemper virus RNA from blood and conjunctival swabs collected from healthy puppies after administration of a modified live vaccine. Paper presented at: Proceedings from the 2008 American College of Veterinary Internal Medicine Forum; June 4-7, 2008; San Antonio, TX). For dogs with previous intranasal administration of a *B bronchiseptica* vaccine, most were negative by day 14.[27] There are no vaccines for CHV-1 or *M cynos*. Thus, the positive dogs described herein were likely from natural exposure to the agents. Negative PCR assay results can indicate lack of infection or the agent in question may have already finished the detectable shedding period as has been described with H3N8.[26] Although the details of the assays are proprietary, the PCR laboratory adheres to standard operating procedures including the use of positive and negative controls, thus erroneous results are unlikely. Results for some of the specific targets in this PCR panel were previously reported.[27] Based on these limitations, our data attempting to correlate PCR assay results to vitamin D concentrations should be interpreted cautiously.

Our study had several other limitations that were mostly related to its design. Although there was a statistically significant difference in serum 25(OH)D concentration between clinically ill and apparently healthy shelter dogs, it is of questionable clinical relevance. Our investigatory efforts were secondary to standard of care procedures aimed to control a CIRDC outbreak. Many dogs with severe respiratory signs (eg, respiratory difficulty, pyrexia, tachypnea) were humanely euthanized before June 21, 2019 (ie, the day diagnostic samples were procured). The removal of these dogs likely skewed our clinical shelter dogs to being comprised of mild to moderate cases of CIRDC. Therefore, it is likely serum 25(OH)D concentration differences detected between clinically ill and apparently healthy shelter dogs as well as all shelter dogs and control dogs were underestimated. The difference in sample sizes between control and shelter dogs also could have led to the unequal variances that were observed. Compensation for the unequal variances decreased the power, leading to an increased chance for type II error.

5 | CONCLUSION

Shelter dogs that exhibit clinical signs associated with CIRDC have lower serum 25(OH)D concentrations compared to apparently healthy shelter dogs and shelter dogs positive for DNA of CHV-1 had lower serum 25(OH)D concentrations than their negative counterparts. Moreover, there was no association between serum 25(OH)D concentration and time spent in the shelter. These results indicate that like humans, vitamin D could have a role in dogs with acute respiratory tract infections.

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CONFLICT OF INTEREST DECLARATION

Dr Randy Ringold is employed by VDI Laboratory which offers testing for vitamin D 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

Approved by the Midwestern University College of Veterinary Medicine dThanAnimal Care and Use Committee (protocol 2981).

HUMAN ETHICS APPROVAL DECLARATION

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

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