Short communication: Vitamin D status and responses in dairy cows naturally infected with *Mycobacterium avium* ssp. *paratuberculosis*

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ABSTRACT

Serum samples were obtained from Holstein dairy control cows and cows naturally infected with *Mycobacterium avium* ssp. *paratuberculosis* (MAP) to evaluate the effects of disease status on serum 25-hydroxyvitamin D₃ (25OHD₃) levels. Disease status was stratified for infected cows into asymptomatic, subclinical infection (n = 25), and cows demonstrating clinical signs (n = 20), along with noninfected control (n = 12) cows for comparison. In addition, portions of the ileocecal valve were taken from a subsample of cows (n = 5 per treatment group) at necropsy and processed for RNA sequencing gene transcription studies. Genes associated with vitamin D metabolism were queried to determine any association between infection and gene expression. Serum 25OHD₃ levels were significantly lower in cows in the clinical stage of disease compared with either cows in the subclinical stage and noninfected control cows. Differential expression for genes associated with the vitamin D pathway such as *CYP27A1*, *CYP27B1*, vitamin D-binding protein (*DBP*), and *IFNG* was dependent upon infection status. An upregulation of *CYP27A1* was noted for cows in subclinical status, whereas *CYP27B1* expression was enhanced for clinical cows. Increased expression of vitamin D-binding protein was observed for infected cattle, regardless of infection status. In summary, decreases in circulating 25OHD₃ for animals with clinical disease may suggest that these cows have reduced innate immune responses, thereby influencing the ability of animals to fight infection.

Key words: Johne's disease, paratuberculosis, dairy cattle, vitamin D

Short Communication

Vitamin D is an immunomodulator of both innate and adaptive immunity. The active form of vitamin D,

1,25-dihydroxyvitamin D₃ [**1,25(OH)₂D₃**], plays a role in activation of macrophages, resulting in increased killing of intracellular pathogens through toll-like receptor-2 and increased expression of cathelicidin (Liu et al., 2006, 2007). In dairy cattle, intramammary infusion with 100 µg of 25-hydroxyvitaminD₃ (**25OHD₃**) has been associated with enhanced clearance of *Streptococcus uberis* from the mammary gland, along with further correlations with reduced clinical signs of mastitis (Lipopolis et al., 2011). No differences in serum 25OHD₃ were observed in treated cows, suggesting that effects were localized to the mammary gland and likely resulted in increased 1,25(OH)₂D₃ by macrophages present in the infected quarters. In vitro studies demonstrated that the addition of either 1 or 10 nM 1,25(OH)₂D₃ to peripheral blood mononuclear cells isolated from cattle infected with *Mycobacterium bovis* reduced production of antigen-specific IFN-γ, yet increased nitric oxide production (Waters et al., 2001). Stimulation of peripheral blood mononuclear cells with *M. bovis* purified protein derivative (10 µg/mL) for 24 h upon isolation from calves vaccinated for *M. bovis* resulted in upregulation of *CYP27B1* and *IFNG* genes. However, concomitant addition of both 1,25(OH)₂D₃ and *M. bovis* purified protein derivative to cell cultures resulted in downregulation of *IFNG* and *IL17F* gene expression (Nelson et al., 2011).

Vitamin D₃ must undergo dual hydroxylation in the liver and kidney to become the active form, 1,25(OH)₂D₃. Hosts that are unable to perform efficient conversion of dietary vitamin D₃ to the active form may have reduced immune function. Little work has been done to demonstrate a link between circulating levels of vitamin D and cattle with paratuberculosis. However, it is possible that vitamin D plays a role in regulation and mediation of chronic inflammatory responses associated with *Mycobacterium avium* ssp. *paratuberculosis* (MAP) infection. The present study was designed to evaluate effects of disease status on serum levels of 25OHD₃ and expression of vitamin D genes in the target tissue of MAP colonization.

Holstein dairy cows ranged in age from 4 to 9 yr in our study and were placed in 3 groups consisting of

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noninfected control cows ($n = 12$; age 3.9 ± 0.7 yr), cows naturally infected with MAP but asymptomatic (subclinical; $n = 25$; age 5.3 ± 0.4 yr), and cows naturally infected in the clinical stage of disease ($n = 20$; age 4.8 ± 0.3 yr). All cows were maintained on site with separate housing for infected and noninfected control cows and ranged from early to late lactation within treatment group. Cows were fed a TMR consisting of corn silage, chopped hay, cracked corn, and soybean meal with estimated intake of 40,000 IU of vitamin D/d. The stage of infection was determined by fecal shedding of MAP, antigen-specific IFN- γ secretion (Bovigam, ThermoFisher, Waltham, MA), and specific antibody response to MAP (Herdchek, IDEXX Laboratories, Westbrook, ME; Khalifeh and Stabel, 2013). Infection was monitored by bacteriologic culture for the fecal shedding of MAP by standard methods (Stabel, 1997). In the clinical group, MAP shedding exceeded 100 cfu/g of feces and animals demonstrated weight loss and intermittent diarrhea. In the subclinical group, MAP shedding was less than 10 cfu/g of feces and animals were asymptomatic. The noninfected control cows were characterized by repeated negative fecal culture performed semiannually over a period of 3 to 5 yr. In addition, these animals were negative for production of antibodies specific for MAP and antigen-specific IFN- γ responses. Infection status of the cows was also characterized by quantitative culture of MAP, histopathologic scoring of acid-fast stain, and lesion severity in the ileocecal valve (Table 1). All animal related procedures, such as euthanasia and necropsy, were approved by the

Institutional Animal Care and Use Committee (National Animal Disease Center, Ames, IA).

Serum samples were collected from cows while they were housed on-site at the National Animal Disease Center as part of routine screening of infection status. Total 25OHD₃ equivalents were quantified by RIA after extraction of serum with acetonitrile. Serum (50 μ L) was added to 500 μ L of acetonitrile and mixed carefully. Then, 25 μ L of the extracted sample was combined with an equal volume of radioiodinated 25OHD₃ (Diasorin Inc., Stillwater, MN; 10,000 counts/min in 25 μ L of ethanol with 0.01 M sodium phosphate). The samples were then centrifuged at $2,000 \times g$ for 10 min at room temperature, and the pellet was discarded. The RIA then was conducted as described by Hollis et al. (1993). Serum vitamin concentration was analyzed by ANOVA using PROC MIXED procedures of SAS (version 9.3, SAS Institute Inc., Cary, NC), with Tukey-Kramer post-hoc test for comparison of means.

The RNA was extracted from ileocecal valve tissues obtained at necropsy from noninfected control cows ($n = 5$) and naturally infected cows in the subclinical ($n = 5$) and clinical ($n = 5$) stages of disease as previously described (Hempel et al., 2016). Approximately 200 mg of snap-frozen tissue was placed into 2 mL of RNeasy lysis buffer (Life Technologies, Carlsbad, CA) and stored at -80°C according to manufacturer's protocol (<https://www.thermofisher.com/order/catalog/product/AM7030>). Approximately 50 mg of tissue was removed from RNeasy lysis buffer and homogenized in gentleMACS M tubes (Miltenyi Biotec, Bergisch Gladbach, Germa-

Table 1. Tissue results for cows in different *Mycobacterium avium* ssp. *paratuberculosis* (MAP) infection status¹

Infection status	Cow identification	MAP, cfu/g of tissue	MAP, cfu/g of feces	Lesion scores
Control	0017	0	0	0
	0018	0	0	0
	0441	0	0	0
	0490	0	0	0
	8102	0	0	0
Subclinical	0030	0	0	0
	0072	0	0	0
	0787	750	0	0
	1061	425	0	0
	1145	0	0	0
Clinical	0010	TNTC ²	TNTC	4
	0035	TNTC	TNTC	3
	0041	TNTC	TNTC	3
	0789	TNTC	TNTC	4
	5545	TNTC	TNTC	3

¹In addition to tissue results above, stage of infection was determined by fecal shedding of MAP, antigen-specific IFN- γ secretion, and specific antibody response to MAP.

²TNTC = too numerous to count.

ny) containing cold RLT Plus buffer (Qiagen, Hilden, Germany) with β -mercaptoethanol (diluted 1:100). After centrifugation at $2,000 \times g$ for 5 min at 4°C to remove tissue debris, the supernatant was removed and used for RNA extraction using the RNeasy Mini Plus Kit (Qiagen). Approximately $4 \mu\text{g}$ of total RNA from each sample was used to create an RNA sequencing library using the mRNA Seq Single Read Library preparation kit per manufacturer's instructions (<https://www.illumina.com/products/by-type/sequencing-kits/library-prep-kits/truseq-stranded-mrna.html>; Illumina Inc., San Diego, CA). Complementary DNA libraries were sequenced using Illumina sequencing technology (Illumina HiSeq 2500) in 5 lanes of the sequencing flow cell. Libraries were prepared and sequenced at the Iowa State University DNA Facility (Ames, IA). Sequences were analyzed for quality by FastQC (version 0.10.1; <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and aligned to the *Bos taurus* genome as previously described (Hempel et al., 2016). Trimmed reads were aligned to the *Bos taurus* genome (Ensembl UMD 3.1, version 70; Elsik et al., 2016) with TopHat splice junction mapper (version 2.0.11; Kim et al., 2013) using the Bowtie method (version 2.2.2; Langmead and Salzberg, 2012) and sorted using SAMtools (version 0.1.19; Li et al., 2009; Anders et al., 2014) to obtain raw counts per transcript. Differential expression analysis was performed on the raw counts in the R statistical programming environment (R Core Team, 2016) using the EdgeR (version 3.8.6) Bioconductor package (Robinson and Smyth, 2007; Robinson et al., 2010). All the libraries were sequenced within 5 lanes of 1 Illumina flow cell, generating an average of 122.2 million reads per lane. The average number of reads per sample was 40.7 million (Hempel et al., 2016). Alignment of the RNA sequencing reads to the *Bos taurus* genome (Ensembl UMD 3.1) yielded a mean of 36.9 million reads per sequenced library. Genes ($n = 36$) associated with vitamin D pathways were parsed, and those that were differentially expressed between treatment groups (control vs. subclinical; control vs. clinical; and subclinical vs. clinical) are discussed.

Serum 25OHD_3 levels were significantly ($P < 0.01$) lower for clinical cows compared with the other treatment groups (Figure 1). Serum obtained from a subset of these same cattle ($n = 10$ per treatment group) just before necropsy demonstrated further reductions in serum $25(\text{OH})$ -vitamin D_3 , with an additional 20% drop observed for clinical (58.8 ± 6.7 vs. 46.3 ± 7.3) cows but not controls or subclinical cows (97 ± 5.1 vs. 103.7 ± 11.4 and 95.2 ± 4.6 vs. 84.6 ± 8.2 , respectively). Antigen-stimulated $\text{IFN-}\gamma$ responses were higher ($P <$

0.01) for subclinical and clinical cows compared with noninfected controls (Figure 2).

Genes associated with the vitamin D pathway that demonstrated differential expression between one or more treatment groups are shown in Table 2. Interferon- γ expression was upregulated in tissue of clinical cows versus control ($P < 0.04$) and clinical cows versus subclinical cows ($P < 0.03$). We found no difference in *IFNG* gene expression between subclinical cows and control cows. Expression of *CYP27A1* and *CYP27B1*, 2 genes that control hydroxylation of vitamin D_3 , were dissimilar in their responses. Gene expression of *CYP27A1* was not different for clinical cows but was downregulated ($P < 0.05$) for subclinical cows when compared with controls. In contrast, expression of *CYP27B1* was upregulated for clinical cows compared with subclinical cows ($P < 0.04$). Interestingly, vitamin D-binding protein (*VDBP*) was upregulated in both clinical and subclinical cows compared with control cows ($P < 0.01$ and $P < 0.03$, respectively). We noted no differences in gene expression of *CYP2R1* or *VDR* in the tissue for these cows regardless of treatment group (data not shown).

In the present study, reduced levels of serum 25OHD_3 were associated with cattle in the clinical stage of

Figure 1. Serum $25(\text{OH})$ -vitamin D_3 concentrations (ng/mL) for dairy cows in different states of *Mycobacterium avium* ssp. *paratuberculosis* infection. Cows were stratified into noninfected control cows and naturally infected cattle in subclinical or clinical infection status. Boxplots shown represent the upper and lower quartiles of the data with a line representing the median value. Whiskers on the plots represent the maximum and minimum values in the dataset. Means with different symbols (*, **) are different ($P < 0.01$).

Table 2. Regulation of vitamin D₃-associated genes between naturally infected dairy cattle in subclinical or clinical state of Johne's disease compared with noninfected control cows

Gene ID	Description	Clinical vs. control		Subclinical vs. control		Clinical vs. subclinical	
		Log ₂ fold change	P-value	Log ₂ fold change	P-value	Log ₂ fold change	P-value
ENSBTAG000000012529	<i>Bos taurus</i> interferon, gamma (IFNG)	1.281	0.039	-0.096	0.989	1.388	0.027
ENSBTAG000000013489	<i>Bos taurus</i> cytochrome P450, family 27, subfamily A, polypeptide 1 (CYP27A1)	0.04	0.935	-0.931	0.046	0.934	0.149
ENSBTAG000000013718	Group-specific component, vitamin D binding protein	1.617	0.009	2.817	0.028	-0.826	0.581
ENSBTAG000000016906	<i>Bos taurus</i> cytochrome P450, family 27, subfamily A, polypeptide 1 (CYP27B1)	3.779	0.082	-0.019	0.501	3.858	0.041

Johne's disease. Neonatal calves are most susceptible to MAP infection, generally through exposure to MAP shed into the feces or milk by infected dams. Although neonates are born with very low levels of vitamin D₃, they can rapidly achieve adequate nutritional status through proper ingestion of colostrum and milk replacer. If proper nutritional management is not followed, deficiencies might contribute to the susceptibility to MAP infection; however, that was beyond the scope of the current study. Under normal husbandry, adult dairy cattle receive ample vitamin D₃ in their diets, averaging 800 to 2,000 IU/kg, depending upon stage of lactation (NRC, 2001), suggesting that intake of vitamin D₃ is not a contributing issue to the effect on disease state. Serum 25OHD₃ is the major form of vitamin D₃ in the serum, ranging from 53 to 82 ng/mL, and is measured to assess vitamin D status (Prietl et al., 2013; Nelson et al., 2016). A previous field report demonstrated an inverse correlation between serum 25OHD₃ concentrations and serum MAP antibody in dairy cattle, with serum 25OHD₃ averaging 5.3 ng/mL lower in MAP antibody-positive cows (Sorge et al., 2013). Cattle in the present study were characterized by multiple diagnostic tests including serum antibody, IFN- γ release, and fecal shedding and, therefore, were able to be grouped more definitively into subcategories of subclinical and clinical infection. Cows in the clinical

Figure 2. Whole blood (WB) IFN- γ (ng/ μ L) for dairy cows in different states of *Mycobacterium avium* ssp. *paratuberculosis* infection. Cows were stratified into noninfected control cows and naturally infected cattle in either subclinical or clinical infection status. Boxplots shown represent the upper and lower quartiles of the data with a line representing the median value. Whiskers on the plots represent the maximum and minimum values in the dataset. Means with different symbols (*, **) are different ($P < 0.01$).

cal group averaged a sample-to-positive ratio of 2.07 ± 0.24 for serum antibody, compared with a sample-to-positive ratio of 0.17 ± 0.10 for cows with subclinical status. It is clear that cattle afflicted with clinical disease are unable to absorb or metabolize vitamin D₃ as well as asymptomatic cattle or noninfected controls. However, hosts that are diseased may be to unable to adequately absorb nutrients from the diet efficiently. Johne's disease (paratuberculosis) is characterized by a chronic inflammation of the small intestine, a target tissue for colonization of MAP. During the clinical stage of disease, the small intestine becomes corrugated and thickened due to the influx of macrophages and lymphocytes into the region. Animals become unable to properly absorb nutrients across the thickened wall and suffer from weight loss and cachexia. As vitamin D₃ is absorbed via the small intestine, it is likely that disease state would affect its uptake (Norman and Henry, 2007), which would be corroborated by the further decline in serum 25OHD₃ for clinical cows at posting. Culling of noninfected control cows and subclinical cows was routinely performed due to age of animals and hip, foot, or udder problems, unassociated with the presence of infectious disease. However, culling of clinical cows was strongly associated with signs of clinical disease, including profuse diarrhea and severe weight loss. Studies in humans have demonstrated that patients suffering from disorders associated with severe inflammation, such as knee replacements, acute pancreatitis, congestive heart failure, and infections, have low serum 25OHD₃ concentrations (Autier et al., 2014). Further, it has been shown that proinflammatory markers, such as tumor necrosis factor- α or C-reactive protein, are correlated with reduced serum 25OHD₃ (Peterson and Heffernan, 2008; Murr et al., 2012). One proposed mechanism for shifts in vitamin D metabolism during intracellular infections is the induction of *CYP27B1* expression by proinflammatory cytokines. Interestingly, we observed a significant ($P < 0.01$) correlation between *IFNG* and *CYP27B1* gene expression in this study, suggesting that upregulation of *IFNG* is inducing *CYP27B1* expression. The work of Fabri et al. (2011) would support these finding, as they observed that treatment of human monocytes with exogenous *IFNG* increased *CYP27B1* enzymatic activity and gene expression. This results in increased conversion of 25OHD₃ to 1,25(OH)₂D₃, which induces *CYP24A*. This enzyme degrades both 25OHD₃ and 1,25(OH)₂D₃ to inactive products and decreases their half-life, ultimately resulting in lowered serum 25OHD₃ (Nelson et al., 2012).

Upon absorption from the diet, vitamin D is metabolized from 7-dehydrocholesterol in the skin to form pre-vitamin D₃ that isomerizes to vitamin D₃. Vitamin

D₃ is then hydroxylated in the liver to form 25OHD₃ via the enzymes encoded primarily by microsomal *CYP2R1* gene and, to a lesser extent, a mitochondrial *CYP27A1* gene (Bergadà et al., 2014). The *CYP27A1* gene also codes for a 27-hydroxylase enzyme associated with reduced lipid accumulation within the macrophage, thereby promoting innate defenses of the host (Quinn et al., 2005). In the present study, *CYP27A1* expression was influenced by stage of disease, as expression trended higher in clinical cows and was down-regulated in subclinical cows compared with controls. As the MAP organism has a high lipid content, it is probable that the accumulation of MAP in the tissue of clinically affected cows triggered the upregulation of this gene. The *CYP27B1* gene that encodes the 1- α hydroxylase enzyme was upregulated for clinical cows compared with both subclinical cows and noninfected controls. The 1- α hydroxylase enzyme encoded by this gene is predominantly found in the kidney, but extra-renal sources of the enzyme also exist, including cells of the monocyte or macrophage lineage (Monkawa et al., 2000). Gene expression of *CYP27B1* was induced in peripheral blood mononuclear cells from cattle vaccinated with *M. bovis*-BCG (bacillus Calmette-Guérin) upon stimulation of cells with *M. bovis* antigen (Nelson et al., 2011). Although direct measurements of 1,25(OH)₂D₃ have not been done in macrophages of cattle, 1,25(OH)₂D₃ was localized by immunohistochemistry within the granulomatous lesions of lymph nodes isolated from *M. bovis*-infected cattle (Rhodes et al., 2003). Although the cells were not defined specifically, they were determined to be mononuclear cells by morphology. Studies with humans have demonstrated that patients with active tuberculosis had significantly higher 1- α hydroxylase activity in monocytes than those in the frequent tuberculosis contact group (Tung et al., 2013). The local production of 1,25(OH)₂D₃ may be a compensatory mechanism in hosts that are diseased, increasing autocrine and paracrine activation of macrophages and associated cells.

An interesting association of increased gene expression for *VDBP* with MAP infection, regardless of stage of disease, was observed. Previously, *VDBP* had been shown to be a key serum biomarker in calves experimentally infected with MAP for 12 mo (Seth et al., 2009). Another study screened serum samples from calves that were infected with *M. bovis* for various periods of time and serum *VDBP* was found to highly sensitive and specific for *M. bovis* detection (Lamont et al., 2014). Additionally, serum *VDBP* levels were highly elevated in human patients with active tuberculosis, indicating this is a key marker of mycobacterial infections (Tanaka et al., 2011). Influence of disease status on IFN- γ was

included, as one mechanism of action for 1,25(OH)₂D₃ in cattle is the downregulation of this cytokine in the host (Nelson et al., 2012). The inverse relationship between IFN- γ and serum 25(OH)D₃ was observed only for cows in the clinical stage of disease, fitting the pattern host responses in mycobacterial infections.

In conclusion, it is clear that the stage of disease is critical to interpretation as to a role for vitamin D₃ in cattle with Johne's disease. The reduction in serum 25OHD₃ may be due to impedance of absorption of dietary vitamin D₃ due to a thickened intestinal wall in animals with end-stage disease. In addition, a state of chronic inflammation associated with clinical disease may have also contributed to the decline. The upregulation of *CYP27B1* suggests that animals in clinical status may invoke a localized compensatory mechanism to maintain immunologic function of the macrophages. This field of research is compelling and suggests that further work would be key to understanding critical mechanisms for controlling MAP infection in the host.

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