

Mycobacterium bovis vaccination and subsequent experimental infection outcomes are associated with changes in vitamin D status in dairy calves.

Rachel L. Lee,1 Kieran G. Meade,2 Shelley G. Rhodes,3 Tom Ford,4 and Ilias Kyriazakis1

Abstract: Investigations into the role of vitamin D (vitD) in the immune response of cattle are limited. The objectives of this study were therefore to investigate the association between circulating vitD concentration, TB vaccination and Mycobacterium bovis (M. bovis) infection outcomes in 24 dairy calves (<8 weeks old) which were housed throughout and fed a bodyweight-based allowance. The study design incorporated 2 phases: vaccination (experimental wk 0–52) and experimental infection phase (wk 52–65). Vaccinated calves (n = 12) received a subcutaneous injection of a live-attenuated TB strain at wk 0, whereas unvaccinated ones (n = 12) were injected with saline. All animals were infected with 7,600 cfu of M. bovis 52 weeks post-vaccination, and lung and lymph nodes tissues were assessed for pathology following euthanasia after wk 65. Blood samples were taken throughout wk 0–65. Serum 25-hydroxyvitamin D (25(OH)D) concentrations were measured using a commercial ELISA. A mixed-effects linear regression model revealed significantly higher 25(OH)D concentrations in the vaccinates post-infection (wk 65) compared with the non-vaccinates. Linear regression analysis between 25(OH)D concentration and the level of M. bovis-driven pathology revealed a negative linear relationship i.e., higher concentrations were associated with lower pathology scores, irrespective of vaccination status. No correlation was detected between interferon-γ cytokine production and vitD concentration. Overall, the results support a significant role for vitD in the development of effective immunity of M. bovis. Gaining insight into the interaction between TB vaccination, M. bovis infection and vitD could potentially guide the optimization of vaccination protocols and future TB control strategies.

Vitamin D (VitD) has an established role in the regulation of calcium-phosphate homeostasis and skeletal health. However, vitD receptor and activating enzyme 1α-hydroxylase expression have been identified in several cells involved in immune system functions and therefore the role of vitD on immune related functions is of increasing interest (D’Amelio et al., 2012). The optimal levels of circulating 25-hydroxyvitamin D (25(OH)D) for immune function are not well defined, however, concentrations below 30ng/ml have been shown to have a negative impact on immune cell profiles in cattle (Flores-Villalva et al., 2021).

Specifically in relation to mycobacteria, there is evidence for vitD regulation of antimycobacterial responses, e.g., by inhibiting intracellular replication, promoting the maturation and activation of monocytes and macrophages, and the induction of nitric oxide synthase (Rookett et al., 1998; Rode et al., 2017). Lower concentrations of 25(OH)D have been found in human patients with active tuberculosis (TB) in comparison to healthy patients; however, it is unclear if this difference is caused by the infection or if disease progression is favored by lower 25(OH)D levels (Huang et al., 2017). Several in vitro human medicine studies support an anti-inflammatory role of vitD, without a reduction in the antimycobacterial activity, which likely reduces the tissue damage characteristic of TB immunopathology (Papagni et al., 2022; Torres-Juarez et al., 2015).

Vaccination with Bacille Calmette-Guerin (BCG), a live attenuated vaccine form of Mycobacterium bovis (M. bovis), represents a potentially viable control measure for reducing bovine tuberculosis (bTB) prevalence in cattle (Milián-Suazo et al., 2022). Studies of BCG vaccination have shown significant protection against both experimental and natural infection with M. bovis and a recent meta-analysis of the efficacy of BCG supports its use (Srinivasan et al., 2021). Studies in human medicine literature report interactions between 25(OH)D concentrations and vaccination responses, influencing the hypotheses for our study that an interaction between BCG vaccination and M. bovis infection could influence 25(OH)D concentrations (Shahini et al., 2023).

The aims of this study were to investigate the effects of vaccination and subsequent infection with M. bovis on the circulating levels of 25(OH)D in dairy cattle, and to then compare these levels of 25(OH)D concentration with pathology in vaccinated and unvaccinated cattle. As interferon-γ (IFN-γ) is both a major protective cytokine for TB and a potent inflammatory mediator, we also investigated whether there was an association of IFN-γ with 25(OH)D (Kumar, 2017). The 3 main hypotheses were: 1) BCG vaccination alone would not affect circulating 25(OH)D levels; 2) there would be differences in circulating 25(OH)D levels between vaccinated and unvaccinated cattle post-infection (pi); 3) vaccina-

1Institute for Global Food Security, School of Biological Sciences, Queen’s University, Belfast, U.K.; 2School of Agriculture and Food Science, University College Dublin, Belfield, Dublin 4, Ireland; 3Department of Bacteriology, Animal and Plant Health Agency, New Haw, Addlestone, Surrey, U.K.; 4Veterinary Sciences Division, Agri-Food and Biosciences Institute, Stormont, Belfast, N.I. © 2024, The Authors. Published by Elsevier Inc. on behalf of the American Dairy Science Association®. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/). Received January 17, 2024. Accepted April 15, 2024.

The list of standard abbreviations for JDSC is available at adsa.org/jdsc-abbreviations-24. Nonstandard abbreviations are available in the Notes.
tion and 25(OH)D concentrations would both play a role in the reduction of TB pathology.

All procedures conducted were approved by the APHA Animal Welfare and Ethical Review Board in accordance with the Animal Scientific Procedures Act (ASPA, 1986) at APHA (PDFS40A5–2-006V2). As a result of existing approval, ethical exemption was provided by the Animal Research Ethics Committee, UCD (AREC-E–22–34-Meade).

Twenty-eight castrated male Danish Holstein or Holstein-crossbred calves were sourced from Denmark (an officially bovine TB-free country) and housed at APHA Weybridge (51° N) throughout the experiment. Calves were 31–46 d old on arrival at APHA in July 2022.

The initial sample size was based on a power analysis with a 5% significance level and 95% power, considering the reduction of pathology as the primary endpoint. Calculated from a median pulmonary lymph node score of 16 (control) and 5 (BCG) (Jones et al., 2022) with an overall SD of 6.6, the target size was 10 animals per group. This was adjusted to 12 animal per group to account for potential losses due to infection.

Calves were raised for 4 weeks on Vita Milk Omega Gold milk replacer (containing 5000 IU Vitamin D₃/kg) and Heygates Course Calf Mixture feed (2200 IU vit D₃/kg) and switched to ForFarmers Grower Pellet (2500 IU vit D₃/kg) for the remainder of the study. They were fed a body weight (BW) based allowance and had access to ad-libitum grass hay and straw.

The study consisted of 2 phases: vaccination (wk 0–52) and experimental infection (wk 52–65). Animals were randomly allocated to treatment groups and balanced by breed. For the vaccination phase, the vaccination group (n = 14) received a 0.5mL subcutaneous injection of a live attenuated TB strain (BCG Danish strain 1331) (AJVaccines, Copenhagen, Denmark) 2 weeks after arrival (wk 0 of the study). The unvaccinated group (n = 14) received a 0.5mL subcutaneous injection of phosphate buffered saline. The groups were kept separately within the barn to link observed effects more confidently to the vaccination, such as potential effects on feed intake. Natural light within the building was not controlled and artificial lights were manually switched on and off by technicians. Pens inside the barn were equidistant from window light sources.

Before M. bovis challenge on wk 52, animals were moved to a CL3 containment unit; animals were randomly assigned to the 6 animal rooms by staff so that 2 vaccines and 2 non-vaccinates were housed together. Two animals were randomly desected for infection due to the capacity of the CL3 unit. Another 2 animals were lost to the study due to study-unrelated causes pre-infection, leaving a total of 24 animals within the study (12 per group). Both groups were experimentally infected via the endotracheal/endo-bronchial route with virulent strain AF2122/97 of M. bovis in wk 52 (Jones et al., 2022). The CL3 facility was under negative pressure, with each pen having a separate high efficiency particulate air filtered duct from each room. The light within the containment facility was scheduled.

Blood samples (per animal; one clotted sample tube for serum collection and one heparin-coated sample tube for IFN-γ) were collected on experimental wk 0 (pre-BCG), 4, 6, 8, 16, 24, 32, 40, 45, 47, 50, 53 (1 week pi), 54, 58, 62 and 65. BW measurements were obtained at all sampling points before the infection (wk 0–52) using calibrated scales. All animals were euthanized 3–5 d after wk 65 samples were taken, using an appropriate method under the ASPA (1986) Schedule 1, consisting of captive bolt stunning and exsanguination.

Serum samples were analyzed for concentrations of total 25(OH)D using a commercially available ELISA (VID3-K01, Eagle BioScience, Nashua, NH) with customized bovine standards (Nelson et al., 2016). The concentrations of 25(OH)D were determined by measuring the absorbance at a wavelength of 450nm with a reference wavelength of 620nm using a CLARIOstar Plus BMG LabTech microplate reader. The resulting absorbance values were fitted against the bovine standard concentrations to a standard curve using cubic spine plotting in GraphPad Prism v9 software to convert to 25(OH)D concentrations. Standards were run in duplicate, and samples were run singly. The intra-assay CV was 1.41% based on measurements of replicates of the bovine standards within a single assay. The inter-assay CV was 3.60%, determined from 9 independent assays performed on separate days. IFN-γ was assessed using BOVIGAM™ ELISA provided by APHA (Holder et al., 2023).

Post-mortem examinations were conducted 3–5 d following the last blood sampling week (wk 65) to determine the level of pathology observed in the lungs and lymph nodes (LNs) using a previously published method and scoring system (Jones et al., 2022; Lyashchenko et al., 2004). Briefly, the LNs (submandibular, medial retropharyngeal, mediastinal, bronchial, tracheobronchial) and lung lobes were meticulously examined post-mortem. Individual LN/lung scores were added up to calculate the total scores.

All statistical analyses were conducted in R (version 4.2.3), including but not limited to packages ‘nlme’, ‘lme4’, ‘anova’ (Bates, 2000). Two unvaccinated calves were excluded from the analysis due to missing datapoints throughout wk 0–65, therefore the final analysis included 12 vaccinated and 10 unvaccinated calves.

The impact of vaccination status on BW and 25(OH)D concentrations was analyzed using linear mixed effects (LME) models. The main fixed effects in both models were date of breed, vaccination status and time; BW at allocation (BW0) was a covariate. Calf ID was the random effect, as it formed the repeated measure variable. The normality of residuals was assessed using QQ plots and the Shapiro-Wilk test, concluding normal distribution of the residuals. Heteroscedasticity was evaluated through visual inspection of residual plots associated with the LME model. The absence of discernible patterns or trends suggested that the assumption of homogeneity of variance was met across the observed data points. Boxplots of BW and 25(OH)D concentrations against time were plotted to determine statistical outliers. The screening of interaction terms assessed their impact by systematically evaluating significance with the Akaike Information Criterion (AIC). Non-contributing terms were then identified and removed. The choice of covariance structures in the mixed-effects model was determined through systematic evaluation based on AIC and BIC (Supplementary Table). Two structures (spatial power and ante-dependence) were considered due to the uneven spacing of the sampling points. The ante-dependence structure was selected for its lowest AIC value, indicating superior fit. Significance was determined at P < 0.05 and tendency was declared at 0.05 ≤ P < 0.10. Post-hoc analysis was conducted to identify where significant interactions occurred, and estimated marginal means were obtained. Pairwise
comparisons were performed using Tukey’s HSD multiple comparison test on the interaction between vaccination status and time. An investigation into the relationship between the independent variables, vaccination status, and 25(OH)D concentration and the dependent variable pathology score utilized separate linear regression models for each time point pi, using the ‘lm’ function in R. Before model fitting, assumptions of normality, linearity, and homoscedasticity were assessed as above. To confirm the linearity assumption, scatter plots of the predictor variables against the fitted values and residuals were examined. Focused on time points where 25(OH)D concentrations tended to or significantly influenced pathology scores (wk 62 and 65), Pearson correlation coefficients (ρ) were computed to quantify the linear relationship. Finally, the relationship between 25(OH)D concentrations and IFN-γ, was analyzed through a partial correlation analysis. The analysis controlled for the effects of vaccination status and time. The partial correlation coefficient (r) was computed using the ‘pcor’ function.

A main effect of time on BW was found pre-infection, with animals increasing in BW as time progressed (F = 670, P < 0.001). Main effects of vaccination status and breed on BW were not significant, nor were the effects of their interactions.

An interaction between vaccination status and time on 25(OH)D concentrations was observed (F(15, 298) = 2.67, P = 0.0008). There was no effect of vaccination on 25(OH)D concentration before infection, but there were higher 25(OH)D concentrations in the vaccinated calves compared with the unvaccinated ones pi (wk 65: 72.4 ± 2.73 ng/ml versus 53.9 ± 2.99 ng/ml (P = 0.038)). No interaction effects with breed on 25(OH)D concentrations were found. A main effect of vaccination status on 25(OH)D concentrations was also observed (F(1, 19) = 5.02, P = 0.037): the mean concentrations of the vaccinated group were higher than those of the unvaccinated group (42.8 ± 1.47 ng/ml versus 37.8 ± 1.60 ng/ml). Time was found to affect 25(OH)D concentrations (F(15, 298) = 66.97, P < 0.001), with concentrations increasing with time up to wk 54 and flattening out subsequently (Figure 1). A main effect of breed on 25(OH)D concentrations was observed (F(1, 19) = 6.87, P = 0.017), with Holstein-crossbred cattle having higher 25(OH)D levels than purebred cattle (43.2 ± 1.47 ng/ml versus 37.4 ± 1.62 ng/ml).

A main effect of vaccination status on LN pathology scores was observed (F(1,19) = 7.36, P = 0.014), with previously vaccinated calves having lower LN pathology scores than unvaccinated ones. An effect of 25(OH)D concentrations on LN pathology scores was found at wk 62 (10 weeks pi) (F(1, 18) = 8.72, P = 0.009) followed by a tendency at wk 65 (13 weeks pi) (F(1, 19) = 4.17, P = 0.055) (Figures 2A and 2B). The vaccination status affected the intercept of the linear relationship (P = 0.022) but did not affect the slope of the relationship. Vaccination status did not affect the slope or the intercept of the relationship at wk 65 (13 weeks pi).

A main effect of vaccination status on lung pathology scores was found (F(1,19) = 6.87, P = 0.017), with previously vaccinated calves having lower pathology scores than unvaccinated ones. 25(OH)D concentrations tended to affect lung pathology scores at wk 65 (13 weeks pi) (F(1, 19) = 3.47, P = 0.078). The vaccination

Figure 1. Boxplot of the relationship between 25(OH)D concentrations and time stratified by vaccination status (red bars unvaccinated, blue bars vaccinated calves). Vaccination with BCG took place on wk 0, while infection with M. bovis happened on wk 52. Time points are indicated for M. bovis infection. The x-axis is discontinuous, with breaks marked by separators ‘\///’; with a transition to continuous from wk 50 onward. The central line represented the median, and the box spanned the IQR. The mean was represented with a yellow point. SD error bars are included for each mean. The horizontal dotted line represents the suggested vitamin D insufficiency reference point at 30 ng/ml. Significant difference (P < 0.05) is denoted by *.
status affected only the linear relationship at wk 62 (10 weeks pi) \( (P = 0.009) \). Vaccination status did not affect the slope or the intercept of the relationship at wk 65 (13 weeks pi).

Similar effects of vaccination status and 25 D concentrations on total (LN plus lung) pathology scores were also identified. 25(OH)D concentrations tended to affect total pathology scores at wk 62 (10 weeks pi) \( (F(1, 19) = 3.01, P = 0.099) \), and had an effect on total pathology scores at wk 65 (13 weeks pi) \( (F(1, 18) = 4.64, P = 0.044) \). The vaccination status affected the intercept of the linear relationship only at wk 62 (10 weeks pi) \( (P = 0.011) \).

The partial correlation coefficient for the relationship between 25(OH)D concentrations and IFN-\( \gamma \), while controlling for vaccination status and time, was \(-0.066\) (p-value = 0.500). This is consistent with findings from Ragab et al. (2016), however others have reported a correlation between higher vitD levels and reduced IFN-\( \gamma \) production in vitro (Barker et al., 2013).

We accept there may be some limitations in this study e.g., the absence of food intake data, as investigating vitD was an opportunist additional piece of work in a TB vaccination-protection investigation. Nevertheless, efforts were made to ensure uniform treatment of all animals regarding accommodation, food, and exposure to light sources.

Consistent with the first hypothesis, there was no difference in 25(OH)D concentrations between the vaccinated and unvaccinated calves’ pre-infection. We found no differences in BW between the vaccinated and unvaccinated animals, suggesting that the vaccination did not significantly impact feed intake, as all animals were offered a BW-based allowance (Golder et al., 2021). Similar studies with BCG vaccination in cattle report that the animals did not show adverse effects (Balseiro et al., 2020). However, Williams et al. (2022) reported inappetence in calves as a transient factor only in the first 14 d post-vaccination. Differences in baseline 25(OH)D levels between the purebred Holstein and the crosses were also identified, emphasizing the importance of accounting for breed-related variations in vitD research in cattle. We also note that all animals had 25(OH)D concentrations lower than the suggested vitD insufficiency threshold of 30ng/ml until experimental wk 6 (~3 mo of age), and many were still below this level up to wk 16 (~5 mo of age).

Regarding the second hypothesis of the study – that \( M. \text{bovis} \) infection would result in differences in 25(OH)D between vaccinated and unvaccinated cattle, in keeping with a role for vitD in vaccine-induced protection – BCG-vaccinated calves did show a divergence in 25(OH)D levels compared with unvaccinated cattle pi. A transient drop in mean 25(OH)D was observed 6 weeks pi (wk 58) in both groups – potentially associated with a transient reduction in food intake (pathogen-induced anorexia), that is consequently reflected in their circulating 25(OH)D concentrations (Asçkar et al., 2018). Unfortunately, BW was not recorded pi to confirm.

Previous literature reports that the lag time between the point of infection and the first signs of anorexia for bacterial infections can vary based on the time course of recognition of the pathogen by host immunity (Kyriazakis et al., 1998). The temporal developments in 25(OH)D measurement supports a potential faster rate of recovery from pathogen-induced anorexia in vaccinated animals compared with unvaccinated calves (Sandberg et al., 2007). Vaccinated
calves, having acquired immunity to the *M. bovis* pathogen, might exhibit a quicker and more effective immune response, possibly resulting in a shorter anorexia duration, if any (Plata-Salaman, 2001; Waters et al., 2016). However, the statistically significant difference in 25(OH)D concentrations were observed at wk 65 only, and caution should be exercised in drawing generalized conclusions.

The third hypothesis of the study – that serum 25(OH)D levels would associate with the level of pathology – was upheld by the significant negative correlation of higher pathology score with lower 25(OH)D concentrations. This was the case in lung, LN and total pathology scores, linking higher 25(OH)D levels with effective immunity (less pathology) (Hope et al., 2023).

Although there is a lack of research in cattle, there are proposed mechanisms in other species which could potentially explain the effects of 25(OH)D concentrations on the expression of immunity in infected hosts (Thirumadas et al., 2021). The inflammatory response and granuloma formation is essential in the early stages of TB disease; however, persistent granulomatous inflammation is the main cause of tissue damage and clinical manifestation of the disease (Sasindran & Torrellas, 2011). It has been demonstrated that TB infection becomes uncontrolled whereby granuloma formation migrates away from the infection site to other tissues causing subsequent damage (Capuano et al., 2003). VitD has been identified within such granulomas in *M. bovis*-infected cattle (Rhodes et al., 2003). VitD has demonstrated immunomodulatory effects, including the induction of T-reg lymphocytes which limit Th1 activity via modulation of cytokine expression without the reduction of antitubercular activity, and the modification of co-stimulatory molecules on accessory cells (Fabri et al., 2011; Rhodes et al., 2003). This potentially explains how 25(OH)D concentrations may contribute to reduction of pathology, as the reduction of the inflammatory state will in turn reduce damage to the LN and lung tissues pi. Increased local production of 25(OH)D by these immune cells during infection could also potentially influence dendritic cell migration to the LNs whereby further 25(OH)D production could continue, which would explain why the effect of 25(OH)D on pathology score becomes significant in the LNs before the lungs (Sigmundsdottir & Butter, 2008).

While this study contributes to our understanding of the complex interactions between vaccination, infection and vitD status in cattle, suggesting a role for vitD in the outcomes of TB infection, further research is essential to elucidate the underlying mechanisms responsible. Specifically, investigations into the pathways involved in the interplay between vaccination efficacy, infection susceptibility, and vitD modulation are warranted. The findings from such studies could have significant implications e.g., in the use of vitD supplementation to improve TB vaccination or control strategies. Furthermore, understanding the role of vitD in modulating immune responses could facilitate the development of novel therapeutic interventions.

References


Notes

Rachel L. Lee https://orcid.org/0009-0004-0731-8473 Kieran G. Meade https://orcid.org/0000-0002-3051-8586 Shelley G. Rhodes https://orcid.org/0000-0002-6389-5265 Ilias Kyriazakis https://orcid.org/0000-0001-7703-3626

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