

# No vaccine interference between bovine coronavirus and bovine herpesvirus-1 in a randomized trial when coadministering two intranasal modified-live viral vaccines to neonatal calves

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## OBJECTIVE

Compare immune responses induced by 2 commercial intranasal (IN) modified-live viral (MLV) vaccines given individually or coadministered and evaluate prevention of infection and lung pathology following bovine herpesvirus-1 (BHV-1) challenge.

## ANIMALS

36 male Holstein calves (ages, 5 to 12 days).

## METHODS

In a randomized complete block design, each calf received an IN injection of either vaccine diluent (Placebo), an MLV vaccine containing bovine herpesvirus-1 (BHV-1; N3), bovine coronavirus vaccine (BC), or both N3 and BC (BC + N3) with a booster 4 weeks later. Nasal secretions and blood were collected weekly. Three weeks after the booster, the calves were challenged with BHV-1, sampled for virus shedding, and euthanized 10 days later to quantify lung pathology. The study period was September 7, 2020, to April 6, 2021.

## RESULTS

Calves were seropositive for BHV-1 and BC before vaccination. No significant difference in BC-specific serum immunoglobulin G and nasal immunoglobulin A antibody responses in the BC versus BC + N3 group or BHV-1-specific serum immunoglobulin G and nasal immunoglobulin A antibody responses in the N3 versus BC + N3 group. Cytokine responses to BHV-1 and BC did not differ among groups. BHV-1 shedding after challenge was significantly reduced in N3 groups versus Placebo and BC. There was a significant reduction in lung pathology in the N3 + BC group versus Placebo.

## CLINICAL RELEVANCE

This study provides evidence an MLV vaccine containing BHV-1 and an MLV BC vaccine can be coadministered to neonatal calves without significantly altering immune responses to the 2 viruses or compromising the prevention of BHV-1 respiratory disease. Calves receiving the BC + N3 vaccine had a significant reduction in lung pathology after BHV-1 aerosol challenge.

**Keywords:** bovine coronavirus, vaccine interference, intranasal vaccine, bovine herpesvirus-1, newborn calves

For more than half a century, modified-live viral (MLV) vaccines have been used to mitigate or prevent infectious disease in both humans and animals. One of the earliest, and most studied, of these was the measles vaccine with the virus isolated in 1954

but another 10 years of serial passage was required before it was safe for vaccination.<sup>1</sup> Similar efforts to culture and attenuate bovine herpesvirus-1 (BHV-1) resulted in the development of the first MLV vaccine for cattle during nearly the same time frame.<sup>2</sup> There are, however, several factors that may inhibit or interfere with the efficacy of MLV vaccines, especially when these vaccines are delivered parenterally. In the very young, the immature systemic immune system may limit the magnitude or duration of immune

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responses.<sup>3</sup> Maternal antibodies that neutralize vaccine viruses may also inhibit vaccine efficacy, blocking the induction of antibody responses but not the induction of T-cell responses.<sup>4,5</sup> A third type of vaccine interference was observed following covaccination with an MLV vaccine and a bacterin. This vaccine interaction varied, however, from a minor effect on antibody responses with no significant impact on disease protection to a significant impact on immune responses and disease protection.<sup>6,7</sup> Thus, vaccine interference must be considered whenever coadministering 2 or more vaccines. While this issue has been addressed for parenteral vaccination, there is little information regarding vaccine interference when coadministering 2 MLV vaccines that replicate at the same or adjacent mucosal sites.<sup>8</sup>

Intranasal (IN) vaccination has been adopted in neonatal calves as an effective strategy to circumvent vaccine interference by maternal antibodies and target the rapidly developing mucosal immune system in the upper respiratory tract (URT).<sup>9,10</sup> Furthermore, IN vaccination of neonatal calves can induce long-term immune memory that responds with rapid induction of protective immunity following a booster vaccination.<sup>11</sup> When few IN vaccines were available for use in young calves, there was no need to investigate potential vaccine interference when delivering multiple IN vaccines. New IN vaccines continue to be developed for use in young calves, however, which raises the question of whether vaccine interference may occur if multiple IN vaccines are coadministered. The current study investigated whether vaccine interference occurs following IN coadministration of 2 MLV vaccines to calves less than 2 weeks of age.

One possible mechanism for vaccine interference is when 2 MLV vaccines replicate at the same or adjacent mucosal sites and replication of one virus interferes with the replication of a second virus.<sup>8</sup> Current MLV vaccines contain both BHV-1 and bovine parainfluenza-3 (PI-3) that induce local interferon (IFN) production in the URT of neonatal calves.<sup>12</sup> The IFN response induced by BHV-1 infection is associated with increased expression of antiviral genes throughout the URT, and within 48 to 72 hours, this innate immune response can reduce viral replication when calves are challenged with virulent BHV-1.<sup>13,14</sup> Thus, MLV vaccines with BHV-1 and PI-3 as components may inhibit the replication of a second vaccine virus if coadministered IN, and this virus also replicates in the URT.

Both enteric and respiratory infections have been associated with bovine coronavirus (BC) in cattle, and modified-live BC vaccines that are administered either orally or IN are commercially available to prevent diarrhea in neonatal calves.<sup>15</sup> Emerging evidence that BC may play a role as a respiratory pathogen in the context of other respiratory viral infections provides a rationale for the IN coadministration of a BC vaccine with multivalent, MLV vaccines targeting viral respiratory pathogens in neonatal calves.<sup>16-18</sup> The replication of BC is inhibited by IFN, and this virus has developed mechanisms to inhibit IFN production and signaling within infected cells.<sup>19</sup> If the

modified-live BC vaccine replicates in the URT, then IFN induced by BHV-1 and PI-3 components of a second IN vaccine may inhibit BC replication and vaccine immunogenicity. Alternatively, the BC vaccine may replicate in the small intestine, and then there may be less vaccine interference due to local IFN production in the URT. The current study determined if coadministering 2 MLV vaccines, one of which could induce IFN, would interfere with either the local immunoglobulin A (IgA) response or the systemic immunoglobulin G (IgG) and T-lymphocyte responses to the BC vaccine. Conversely, if BC inhibited IFN responses in viral infected cells, then this may enhance BHV-1 vaccine replication since IFN- $\beta$  inhibits BHV-1 replication.<sup>13</sup> Therefore, the current study determined whether IN coadministration of a BC vaccine with a multivalent, MLV vaccine, with BHV-1 as a component, would alter immune responses to BHV-1 or immune protection against a BHV-1 respiratory infection.

## Methods

### Animals

The study was conducted in accordance with guidelines of the Canadian Council of Animal Care and animal use was approved by the University of Saskatchewan Animal Care Committee (protocol 20200039). Forty male Holstein calves were obtained from a single dairy herd and calves were separated from dams at parturition and fed a commercial colostrum replacement product (Calf's Choice Total Gold; Saskatoon Colostrum Company). Each calf received a total of 200 g IgG within 4 hours of birth. All calves were housed in individual pens until transported (17 miles) within 2 to 5 days postpartum to the study site (University of Saskatchewan).

Before study enrollment, no pharmaceutical or biologic products were administered. All calves were tested for bovine viral diarrhea virus (BVDV) using a PCR test (Prairie Diagnostic Services). On arrival, total serum protein (TP) concentration was determined with a refractometer, a physical examination was performed by a veterinarian, and a nasal swab was collected and submitted for BC testing using a PCR test (Prairie Diagnostic Services).

Calves between 2 and 5 days of age were recruited to the study in cohorts of 4 animals and a total of 10 cohorts were recruited. Calves were housed indoors in 8 X 12-ft individual pens. Ambient temperature was maintained at 18°C, and calves in different treatment groups were separated by a distance of 10 ft. Bottles used to feed milk were washed in a commercial bottle washer following each feeding. Animal care staff began each feeding with the Placebo group and then changed coveralls and latex gloves and disinfected footwear before going to the next treatment group. Calves received no other vaccines and no other procedures were performed throughout the study. Calves were fed twice daily with a 3-L milk replacer/feeder (100 g replacer/L) containing 28% protein and 25% fat (Blueprint 26:18; Masterfeeds). Calves had ad libitum access to water,

mixed grass and alfalfa hay, and 24% protein calf starter (Blue Medallion 20%; Masterfeeds).

## Study design

The study was performed between September 7, 2020, and April 6, 2021, and followed a randomized complete block design with 10 blocks. Blocks of 4 calves (one calf/treatment group) were sequentially enrolled for a total of 10 calves/treatment group. Randomization within each block was done with the Rand function in Excel and calves were assigned to the treatment group based on the ranking of assigned random numbers from lowest to highest. Individual calves were identified by uniquely numbered ear tags. The treatment groups were as follows: 1) IN administration of 2 mL vaccine diluent in both nostrils (Placebo); 2) IN vaccination with 2 mL BOVILIS Coronavirus (BC; SN 00191130; expiration: February 28, 2021) in 1 nostril; 3) IN vaccination with 2 mL Nasalgen 3 (N3; SN 92578014-93060028; expiration: June 1, 2021) in 1 nostril; and 4) IN vaccination with 2 mL BC and 2 mL N3 in separate nostrils (BC + N3). BOVILIS Coronavirus is a univalent, MLV vaccine containing BC. Nasalgen 3 is a multivalent, MLV vaccine containing BHV-1, PI-3, and bovine respiratory syncytial virus (BRSV). The duration of the study for each block was 59 days following the day of primary vaccination (**Figure 1**). Personnel enrolling calves, obtaining samples, and performing laboratory procedures were blinded to the treatment received by each group.

Inclusion criteria on the day of primary IN vaccination (Figure 1) were 5 to 12 days of age; BVDV test negative, TP greater than 5.1 gm/dL and seropositive for BHV-1 and BCV, body temperature below 39.5 °C, and no clinical signs of diarrhea, respiratory disease, or physical abnormalities. Calves were excluded from the study if one or more members of a cohort did not complete the 59-day study period. A single cohort of 4 calves was removed from the study following the death of a calf in the Placebo group, resulting in 9 cohorts being included in the data analyses.

## Treatment administration

A licensed veterinarian blinded to treatment groups supervised all treatments. Calves between 5 to 12 days of age received the first IN vaccination. Single-dose vials were stored, reconstituted, and administered according to label directions. Vaccines were administered immediately after rehydration, and the head of the calf was elevated for 3 seconds after vaccine administration. Vaccine diluent (2 mL) was delivered to both nostrils of calves in the Placebo group. Coadministration of the 2 MLV vaccines was performed by delivering each vaccine in a different nostril. Booster vaccinations were administered 4 weeks after the primary vaccination (Figure 1).

Three weeks after booster vaccination, the calves in each cohort were aerosol challenged with BHV-1 isolate 108 ( $5 \times 10^7$  PFU/animal) as described previously.<sup>20</sup> All calves survived the BHV-1 viral challenge and were euthanized on day 10 postchallenge with an IV injection of pentobarbital sodium (Euthanyl Forte; Bimeda-MTC Animal Health Ltd).

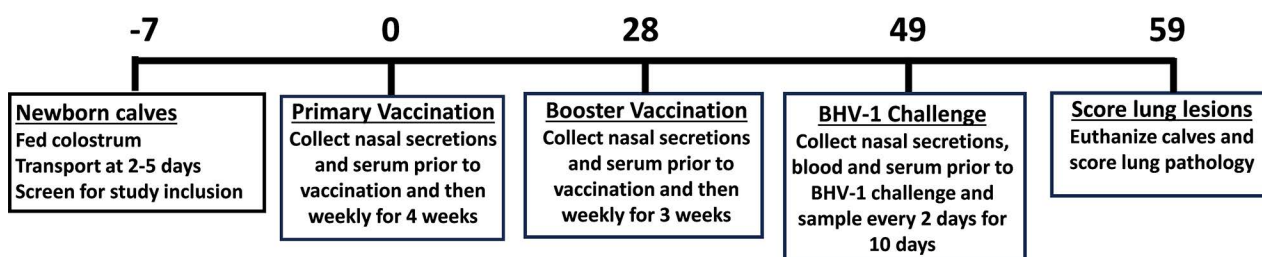
## Sample collection

Calves were monitored for signs of clinical disease twice daily, and an Animal Health Technician recorded any reluctance to rise from bedding, failure to completely empty the milk bottle, and monitored feces consistency for diarrhea. Observations were reported to the attending clinical veterinarian, also blinded to treatment groups, who performed a complete physical exam and instituted treatment if necessary.

Blood was collected once weekly using 10-mL evacuated serum separation tubes (Becton Dickinson). Blood remained at room temperature for 3 to 4 hours before centrifuging at 1,400 X g for 20 minutes at 22 °C. Separated serum was archived as duplicate 0.8-mL aliquots at -15 °C. Blood samples were also collected in 10-mL evacuated blood collection tubes containing EDTA (Becton Dickinson) 3 weeks after the booster vaccination and before BHV-1 challenge.

Nasal secretions were collected weekly throughout the trial and every second day during the week

## Experimental Schedule (Days)



**Figure 1**—Study design for analyzing vaccine immunogenicity and prevention of bovine herpesvirus-1 (BHV-1) infection and respiratory disease protection following c-administration of 2 modified-live viral (MLV) intranasal (IN) vaccines. Newborn calves received colostrum and were transported to research facility when 2 to 5 days old. One week later calves were vaccinated IN, receiving either a single MLV vaccine or both MLV vaccines delivered in separate nostrils. The placebo group received vaccine diluent IN. Nasal secretions and serum were collected immediately before each vaccination and at weekly intervals. Three weeks after booster vaccination blood was collected for in vitro analysis of cytokine responses and calves then aerosol challenged with BHV-1. Calves were monitored every second day for 10 days before being euthanized and removing lungs to quantify visible pathology.

following primary and booster vaccinations. The collection of nasal secretions was performed as described previously.<sup>9</sup> Secretions were clarified by centrifugation at 2,200 X *g* for 8 min at 4°C, and 500- $\mu$ L aliquots, containing 5  $\mu$ L protease inhibitor (Sigma-Aldrich), were stored at -20°C. Separate aliquots of each sample were used for antibody and IFN ELISAs to minimize protein degradation that may occur with repeated freezing and thawing of samples. Nasal secretions for analysis of virus shedding were collected before BHV-1 challenge on day 0 and on days 2, 4, 6, and 9 postchallenge. Nasal secretions were collected using sterile cotton swabs and swabs were immersed in 1 mL minimum essential medium and transported on ice to the lab. Swabs were stored in minimum essential medium at -20°C before viral plaque assays were performed to quantify infectious BHV-1 particles.

Calves were euthanized 10 days after BHV-1 aerosol challenge; the trachea, lungs, and heart were removed; and the lung was examined within 15 minutes of euthanasia.

### Sample analysis

The ELISA for quantification of BHV-1-specific antibodies used recombinant BHV-1-truncated glycoprotein D (tgD; VIDO-Intervac) to coat ELISA plates for antibody capture. The protocol followed was previously described for the detection of serum IgG antibodies.<sup>21</sup> This protocol was modified for the detection of IgA antibodies in nasal secretions reacting with BHV-1.<sup>9</sup> The ELISAs to quantify IgG and IgA antibodies reacting with BC followed similar protocols with the exception that 0.1  $\mu$ g of cesium chloride gradient purified BC was used to coat each well of the ELISA plates for antibody capture. The protein concentration of tgD and purified BC was measured with the Pierce bicinchoninic protein assay (ThermoFisher Scientific). Antibody titers are expressed as the reciprocal of the highest serum or nasal secretion dilution with an OD that exceeded the OD value for the negative control by 2 SDs. Negative controls were 3 replicate wells incubated with a serum or nasal secretion sample previously determined to not react with the coating antigen.

IFN- $\alpha$  and IFN- $\gamma$  concentrations in nasal secretion and culture supernatant samples were quantified using protein capture ELISAs.<sup>22</sup> The IL-10 concentration in culture supernatants was quantified using a commercial ELISA with capture and detection antibodies (product DIY2157B-004; Kingfisher Biotech). The IFN and IL-10 ELISAs included serial dilutions of recombinant bovine IFN- $\alpha$  (Ciba-Geigy), recombinant bovine IFN- $\gamma$  (Ciba-Geigy), or recombinant bovine IL-10 (product RP2148B-005; Kingfisher Biotech) from which standard curves were generated to convert OD values from test samples to cytokine concentration (pg/mL).

Mononuclear leukocytes were isolated from blood using the protocol described previously.<sup>17</sup> Blood mononuclear cells were cultured in 96-well culture plates with 3 X 10<sup>5</sup> viable cells added to each well. For each antigen-specific cytokine secretion

assay, triplicate wells were stimulated with the following antigens: culture medium alone (unstimulated cells); 0.2  $\mu$ g/well tgD (BHV-1), 0.2  $\mu$ g/well-purified BC, and 0.1  $\mu$ g polyclonal T-cell mitogen concanavalin A (Con A)/well (Sigma-Aldrich). Culture supernatants were collected 24 hours after cell stimulation to assay cytokine production.

Percent pathology for the entire lung of each calf was calculated by quantifying grossly visible lung lesions characterized by tissue consolidation and congestion.<sup>23</sup> Scoring was performed by a clinical veterinarian blinded to treatment groups. Each lobe was visually examined with the lung positioned dorsal surface up and palpated before estimating the percentage of each lobe affected. Lung lobes were given a weighted value representing that lobe's percentage of total lung volume (value A). The percentage of each lobe affected (value B) was multiplied by value A and the sum of these products (A X B) for all lung lobes was recorded as total lung score (out of 100%).

Quantification of infectious BHV-1 recovered from nasal swabs was performed using a plaque assay with Madin-Darby bovine kidney cells as described previously.<sup>24</sup>

### Statistical analysis

All analyses were performed using general (normal distribution) and generalized (nonnormal distributions) linear mixed models in PROC GLIMMIX (SAS, version 9.4; SAS Institute Inc). Individual animals (within the cohort) were the experimental unit for all analyses. The treatment structure was 2 by 2 (BC x N3) with a study day for repeated measures. An unstructured covariance structure was used to account for repeated measures over time. Cohort was included as a random intercept term when models allowed. Final models were fitted with a restricted maximum likelihood estimation, Kenward-Roger degrees of freedom approximation, and Newton-Raphson and Ridging optimization procedures. Pair-wise comparisons were adjusted for multiple comparisons using Tukey methods. Model-adjusted means and corresponding SE or 95% CI are reported for all outcome variables. *P* < .05 was used for statistical significance.

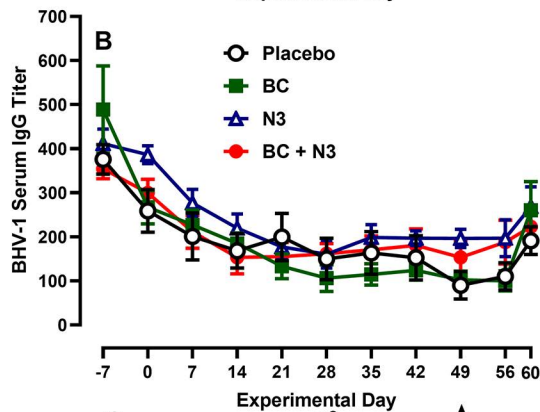
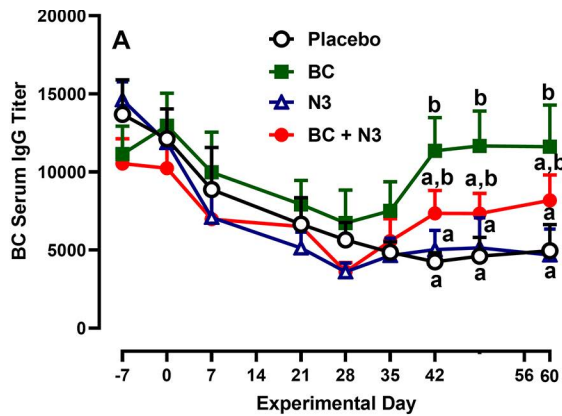
## Results

### Clinical results during IN vaccination period

For the 36 calves completing the study, TP values ranged from 5.1 to 7.3 g/dL, and the mean and SD for all calves included in the study group was 5.9  $\pm$  0.5 g/dL. Therefore, maternal antibody transfer ranged from fair (5.1 to 5.7 g/dL) to excellent (> 6.2 g/dL).<sup>25</sup> All calves entering the study were seropositive for both BHV-1 and BCV with no significant differences in serum antibody titers when comparing among the treatment groups (**Figure 2**).

All calves entering the study tested negative for BVDV and PCR testing of nasal swabs collected from calves on arrival at the study site revealed approximately one-third (36%; 13/36 calves) of calves had positive PCR reactions (cycle threshold



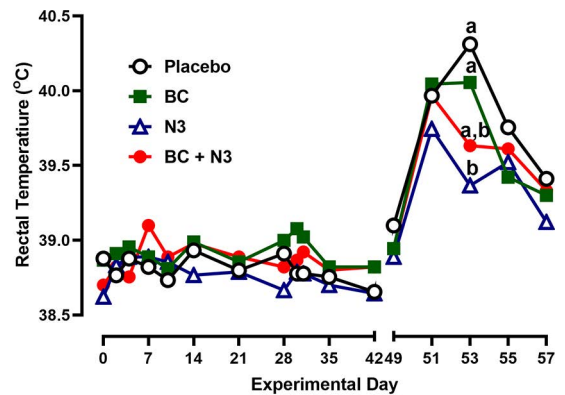


Vaccination 1° 2° ↑ BHV-1

**Figure 2**—Serum immunoglobulin G antibody titers specific for bovine coronavirus (BC; A) and bovine herpesvirus-1 (BHV-1; B) before and following vaccination. Treatment groups ( $n = 9$  calves/group) were as follows: diluent (Placebo); modified-live BC vaccine (BC); modified-live vaccine containing BHV-1 (N3); and concurrent N3 and BC vaccines (BC + N3). Calves received primary (1°) vaccination on day 0 and a booster vaccination (2°) 4 weeks later. Calves were challenged with BHV-1 (arrow) on Day 49. Data at each time point are presented as mean values, and vertical line represents SEM. <sup>a,b</sup>Results on days 42, 49, and 60 with different letters differed significantly ( $P < .01$ ).

[Ct]  $< 39.0$ ) for BCV. Four calves were strong positive (Ct  $< 30$ ), 5 calves were weak positive (Ct = 30 to 36), and 4 calves suspect (Ct  $> 36$  to 39). Seven of the 13 calves with positive PCR results for BCV were allocated to either the BC or BC + N3 groups. Among these 7 calves, 4 were allocated to the BC group and 3 were allocated to the BC + N3 group.

One cohort of 4 calves was removed from the study when a calf in the Placebo group died 3 weeks after recruitment to the study. The calf was diagnosed with bacterial pneumonia and *Mycoplasma* spp and *Mannheimia haemolytica* were isolated from the lung. There were no reportable adverse events throughout the 7-week vaccine period (Figure 1; days 0 to 49), and calves did not develop fever (temperature  $> 39.5^\circ\text{C}$ ) following primary and booster vaccination (Figure 3). There were no significant differences in body temperature when comparing among treatment groups throughout the 7-week postvaccination period.



Vaccination 1° 2° ↑ BHV-1

**Figure 3**—Body temperature throughout the vaccination and after bovine herpesvirus-1 (BHV-1) challenge period. Data presented at each time point are mean values for each treatment group. Treatment groups ( $n = 9$  calves/group) were as follows: diluent (Placebo); modified-live bovine coronavirus vaccine (BC); modified-live vaccine containing BHV-1 (N3); and concurrent N3 and BC vaccines (BC + N3). <sup>a,b</sup>Results on day 53 with different letters differed significantly ( $P < .01$ ).

### Serum IgG responses following vaccination and BHV-1 challenge

All calves were seropositive for BC before the first vaccination, and antibody titers were then decreased following primary vaccination (Figure 2). There was no significant difference in maternal antibody decay when comparing BC titers among the 4 vaccine treatment groups. Following booster vaccination, however, a significant ( $P < .01$ ) increase in BC serum antibody titers was observed in the BC Group when compared to the Placebo and the N3 groups, but there was no significant difference between the BC and BC + N3. Thus, N3 vaccination had no significant effect on the serum IgG response induced by the BC vaccine. Pooling data from all calves receiving the BC vaccine confirmed a significant ( $P = .04$ ) vaccine effect on serum IgG when compared to groups not receiving the BC vaccine (Placebo and N3).

Calves were also seropositive for BHV-1 before first vaccination and BHV-1 serum antibody titers then declined following primary and booster vaccinations (Figure 2). Throughout this interval, there were no significant differences in BHV-1 titers among the 4 treatment groups. Pooling data from groups receiving the N3 vaccine (N3 and BC + N3) did, however, reveal a significant ( $P = .05$ ) difference when compared with groups not receiving the N3 vaccine (Placebo and BC). Following BHV-1 challenge there was a significant ( $P < .05$ ) increase in BHV-1 serum IgG titers on day 60 within the Placebo and BC groups when compared to preinfection titers on day 49.

### IgA in nasal secretions following vaccination and BHV-1 challenge

Newborn calves had low to undetectable BC IgA antibody titers in nasal secretions at the time of primary vaccination, and IgA antibody titers remained

unchanged in Placebo and N3 groups throughout the study period (**Figure 4**). In contrast, BC and BC + N3 groups had significant ( $P < .01$ ) time-dependent increases in BC-specific IgA titers following primary and booster vaccinations when compared to Placebo and N3 groups. Newborn calves also had low to undetectable levels of BHV-1 IgA antibody in nasal secretions during the first week of life and IgA antibody titers remained unchanged in the Placebo and BC groups before BHV-1 challenge. Both N3 and BC + N3 groups displayed significant ( $P < .01$ ) increases in BHV-1-specific IgA antibody titers following primary and booster vaccinations when compared to Placebo and BC groups. Pooling data from the 2 groups receiving the N3 vaccine (N3 and BC + N3) confirmed the N3 vaccine had a significant ( $P < .01$ ) effect on nasal IgA when compared to groups

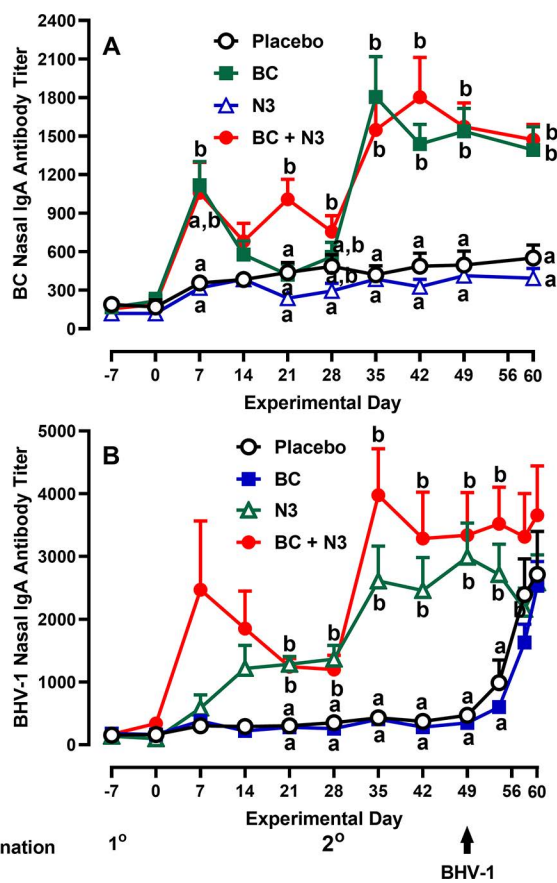
not receiving the N3 vaccine (Placebo and BC). Following BHV-1 challenge, the IgA antibody titers in nasal secretions remained relatively constant in the N3 and BC + N3 groups. In contrast, there was a significant ( $P < .05$ ) increase in the BHV-1 IgA titers in nasal secretions collected from calves in both the Placebo and BC groups on days 8 and 10 postinfection when compared to preinfection levels on day 49.

## T-cell responses to BHV-1 and BC

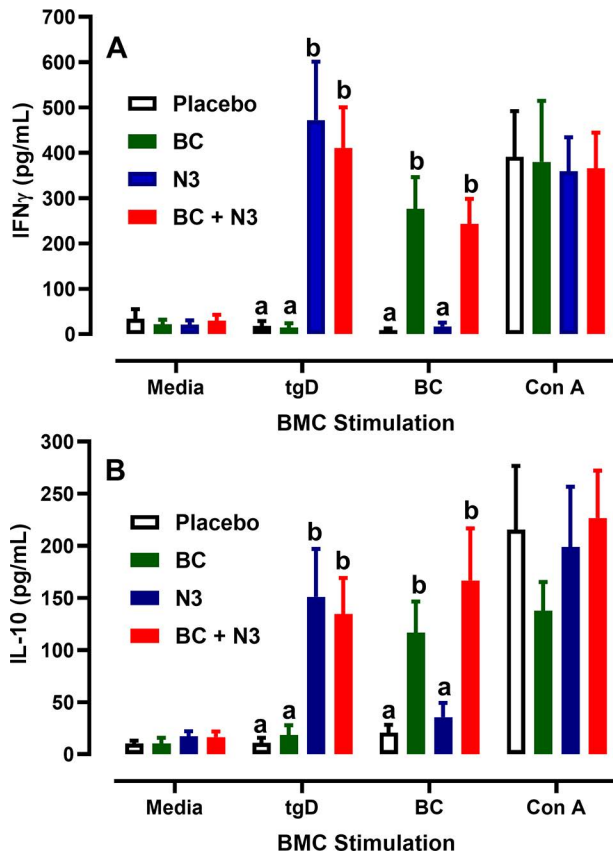
Production of IgG and IgA antibodies is dependent on T-lymphocyte activation and the type of T-lymphocyte responses impacts both antibody production and cell-mediated immune responses.<sup>26</sup> Antigen-specific IFN- $\gamma$  and IL-10 secretion was measured with lymphocytes isolated from blood and stimulated in vitro with tgD protein or purified BC to monitor T-cell memory or effector responses induced by the N3 vaccine and BC vaccines, respectively.

To determine whether coadministering N3 and BC vaccines altered T-lymphocyte responses the level of IFN- $\gamma$  secretion was measured 3 weeks after booster vaccination and before BHV-1 challenge. A low level of spontaneous IFN- $\gamma$  secretion ( $< 50$  pg/mL) was observed in the absence of antigen stimulation, and the polyclonal T-cell mitogen Con A induced high levels (mean  $> 350$  pg/mL) of IFN- $\gamma$  secretion in peripheral blood mononuclear cells isolated from calves in all groups (**Figure 5**). The response to Con A confirmed isolated lymphocytes were functional in all groups. When compared to Placebo and BC groups, a 24-h stimulation with BHV-1 tgD protein induced significantly ( $P < .01$ ) greater IFN- $\gamma$  secretion for calves vaccinated with N3, either alone (N3) or coadministered with BC (BC + N3). Stimulation with purified BCV induced significantly ( $P < .01$ ) elevated levels of IFN- $\gamma$  secretion by blood lymphocytes isolated from calves vaccinated with BC, either alone (BC) or coadministered with N3 (BC + N3) when compared to groups not receiving the BC vaccine (Placebo and N3).

While IFN- $\gamma$  is a potent immune stimulatory cytokine, IL-10 inhibits proinflammatory responses and down-regulates acquired immune responses.<sup>27</sup> The analysis of antigen-specific IL-10 secretion revealed a low level of spontaneous IL-10 secretion ( $< 48$  pg/mL), and Con A induced high levels (mean  $> 138$  pg/mL) of IL-10 secretion in lymphocytes isolated from calves in all treatment groups (Figure 5). Con A induced similar levels of IL-10 secretion among all treatment groups, confirming blood mononuclear cells in all groups had similar capacities to secrete IL-10. When compared to the Placebo and BC groups, the BHV-1 tgD protein induced significantly ( $P < .01$ ) greater levels of IL-10 secretion following booster vaccination with N3, either alone (N3) or coadministered with BC (BC + N3). Stimulation with purified BC also induced significantly ( $P < .01$ ) greater IL-10 secretion following the booster vaccination with BC, either alone (BC) or coadministered with N3 (BC + N3) when compared to the Placebo and N3 groups.



**Figure 4**—IgA antibody titers in nasal secretions specific for bovine coronavirus (BC; A) and bovine herpesvirus-1 (BHV-1; B). Treatment groups ( $n = 9$  calves/group) were as follows: Treatment groups ( $n = 9$  calves/group) were as follows: diluent (Placebo); modified-live BC vaccine (BC); modified-live vaccine containing BHV-1 (N3); and concurrent N3 and BC vaccines (BC + N3). Calves received primary ( $1^{\circ}$ ) vaccination on Day 0 and a booster vaccination ( $2^{\circ}$ ) 4 weeks later. All calves were challenged with BHV-1 (arrow) 3 weeks after booster vaccination. Data presented at each time point are mean values, and vertical lines represent SEM of values for each group. <sup>a,b</sup>Results on days 7, 21, 28, 35, 42, 49, 54, 57, and 59 with different letters differed significantly ( $P < .01$ ).



**Figure 5**—Cytokine responses following in vitro restimulation of blood lymphocytes isolated 3 weeks after booster vaccination. Blood mononuclear cells (BMCs) cultured for 24 hours in either medium alone (no stimulus [Media]) or stimulated with 0.1  $\mu$ g recombinant bovine herpesvirus-1 (BHV-1) glycoprotein D (tgD)/mL, 0.1  $\mu$ g purified bovine coronavirus (BC)/mL, or 0.1  $\mu$ g of the T-cell mitogen concanavalin A (Con A)/mL. IFN- $\gamma$  (A) and IL-10 (B) secreted into culture supernatants were measured with antibody-capture ELISAS. Cytokine secretion was compared among the 4 treatment groups. Treatment groups (n = 9 calves/group) were as follows: diluent (Placebo); modified-live BC vaccine (BC); modified-live vaccine containing BHV-1 (N3); and concurrent N3 and BC vaccines (BC + N3). <sup>a,b</sup>Results with different letters differed significantly ( $P < .01$ ).

### Local IFN response following vaccination and BHV-1 infection

The IN immunization of neonatal calves with MLV vaccines containing BHV-1 and PI-3 can induce IFN secretion in nasal secretion that peaks 5 days postvaccination.<sup>12</sup> This IFN response includes both IFN- $\alpha$  and IFN- $\gamma$ , but the percentage of calves producing detectable levels of either type of IFN varies among vaccines.<sup>12</sup> In the current study, IFN- $\alpha$  and IFN- $\gamma$  concentrations in nasal secretions were measured every second day throughout the week following primary and booster vaccinations. In Placebo and BC groups, only a single animal had a low level of IFN- $\alpha$  (70 pg/mL) in nasal secretions collected on the second day after primary vaccination, and no animals had detectable IFN- $\alpha$  levels in nasal secretions following the booster vaccination. Three calves in the N3 group

had detectable IFN- $\alpha$  following primary vaccination (calf 5, 50 pg/mL on day 7 postvaccination; calf 21, 20 pg/mL on day 2 and 140 pg on day 4 postvaccination; calf 22, 20 pg/mL on day 2 and 30 pg on day 4 postvaccination) and following the booster vaccination only one calf had detectable IFN- $\alpha$  in nasal secretions (calf 1, 10 pg/mL on day 2 postvaccination). In the BC + N3 group, a single calf (calf 20, 20 pg/mL on day 4 postvaccination) had detectable IFN- $\alpha$  in nasal secretion following primary vaccination and no calves had detectable IFN- $\alpha$  following booster vaccination. Following primary vaccination, IFN- $\alpha$  was detected in nasal secretions in 33% (3/9 calves) of calves vaccinated with N3 alone and 11% (1/9) when BC and N3 vaccines were coadministered. The low frequency of animals secreting IFN- $\alpha$  in groups receiving the N3 vaccine precludes any conclusion regarding potential interference when BC and N3 vaccines are coadministered.

A similar response was observed when measuring IFN- $\gamma$ , which is also secreted following IN vaccination of neonatal calves with MLV vaccines containing BHV-1 and PI-3.<sup>12</sup> In the Placebo group, a single animal had a low level of IFN- $\gamma$  (50 pg/mL) in the second day after primary vaccination and subsequently no animals had detectable IFN- $\gamma$  on any of the days monitored following primary and booster vaccination. None of the calves in the BC group had detectable IFN- $\gamma$  levels during the week following primary and booster vaccination. Only 2 calves in the N3 group had detectable levels of IFN- $\gamma$  following primary vaccination (calf 5, 20 pg/mL on day 7 postvaccination; calf 21, 1,180 pg/mL on day 4 postvaccination) and no calves had detectable IFN- $\gamma$  following booster vaccination. In the BC + N3 group a single calf had detectable IFN- $\gamma$  in nasal secretion collected following primary vaccination (calf 20, 130 pg/mL on day 7 postvaccination) and following the booster vaccination (calf 45, 1,280 pg/mL on day 4 postvaccination). The low frequency of responders in the groups receiving the N3 vaccine precludes any conclusion regarding potential interference when coadministering BC and N3 vaccines.

Challenging seronegative, 5- to 6-month-old calves with virulent BHV-1 (isolate 108) induces IFN- $\alpha$  and IFN- $\gamma$  secretion in nasal secretions for 7 to 10 days postinfection.<sup>11</sup> Calves in the current study, however, were approximately 2 months old when challenged with BHV-1, and BHV-1-specific maternal antibody was present at the time of viral challenge (Figure 3). Consequently, not all calves in the Placebo and BC groups shed detectable levels of the virus in nasal secretions following BHV-1 challenge, with 7/9 calves shedding the virus in the Placebo group and 8/9 calves shedding the virus in the BC group. In contrast, only 4/9 calves in the N3 and BC + N3 groups shed detectable levels of virus in nasal secretions. Furthermore, not all calves had detectable levels of IFN in nasal secretions following BHV-1 challenge. IFN was detected in nasal secretions of approximately 60% of calves in the Placebo (6/9 calves with detectable IFN- $\alpha$  and 4/9 with detectable IFN- $\gamma$ ) and BC group (5/9 calves with



detectable IFN- $\alpha$  and 6/9 calves with detectable IFN- $\gamma$ ). In contrast, 33% of the N3 group (3/9 calves with detectable IFN- $\alpha$  and IFN- $\gamma$ ) and 22% of the BC + N3 group (2/9 calves with detectable IFN- $\alpha$  and IFN- $\gamma$ ) had detectable IFN levels in their nasal secretions.

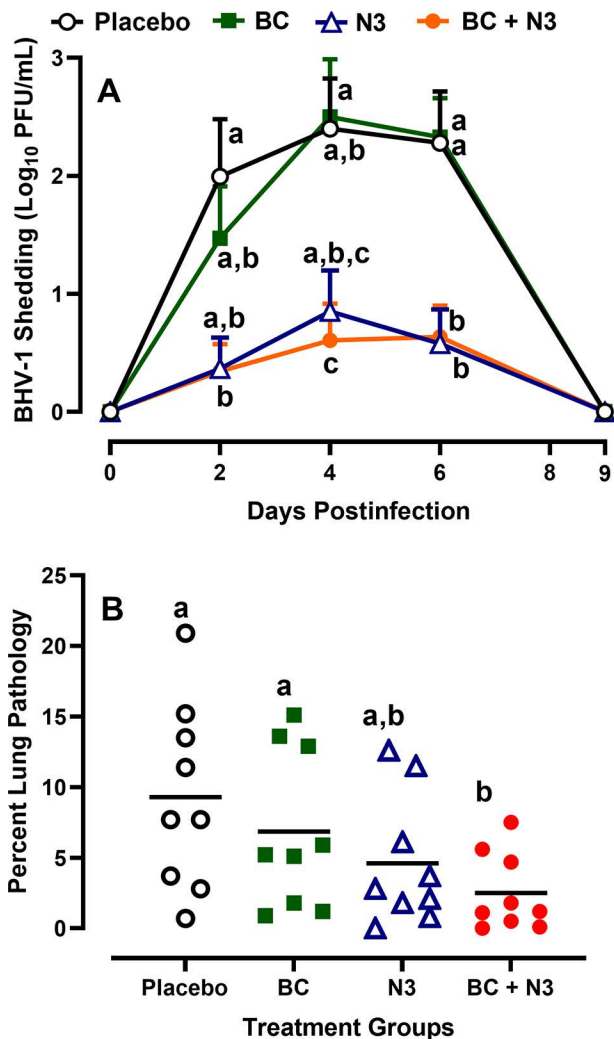
A transient increase in body temperature was observed in all treatments following BHV-1 challenge (Figure 2). This fever response was most pronounced in the Placebo and BC groups and rectal temperatures

were significantly ( $P < .01$ ) greater in these 2 treatment groups on day 5 postinfection when compared to the N3 group. Rectal temperatures in the BC + N3 group were also numerically lower on day 5 postinfection but were not significantly different from any of the other 3 treatment groups.

### Viral shedding and lung pathology following BHV-1 challenge

Significant differences ( $P < .001$ ) in BHV-1 shedding were observed when comparing among the 4 treatment groups (Figure 6). Over 75% of the calves in the Placebo and BC groups shed infectious BHV-1 in their nasal secretions but peak virus shedding was less than  $10^4$  PFU/mL. There was, however, a significant ( $P < .01$ ) reduction in BHV-1 shedding when both groups receiving the N3 vaccine were compared to the Placebo and BC groups. No significant difference was observed when comparing the N3 and BC + N3 groups.

Lung pathology was evaluated 10 days after BHV-1 challenge and the percent visible lung lesions varied broadly within treatment groups (Figure 6). Within the Placebo group lung pathology varied between 0.7 and 20.9% and a similar broad range of visible lung pathology was also observed for the BC group (range, 0.9% to 15.1%). A reduced range of lung pathology was observed in both the N3 (range, 0% to 12.6%) and BC + N3 groups (range, 0% to 7.5%). Coadministration of BC and N3 vaccines did, however, result in a significant ( $P = .02$ ) reduction in lung pathology when compared to the Placebo and BC groups. Pooling data from all calves receiving the N3 vaccine (N3 and BC + N3 groups) revealed a significant ( $P = .003$ ) reduction in lung pathology when compared to calves not receiving the N3 vaccine (Placebo and BC groups). Pooling data from all calves receiving the BC vaccine (BC and BC + N3) also revealed a significant ( $P = .03$ ) reduction in lung pathology relative to calves not receiving the BC vaccine (Placebo and N3).



**Figure 6**—Virus shedding in nasal secretions and lung pathology following bovine herpesvirus-1 (BHV-1) challenge 3 weeks after booster vaccination. Treatment groups ( $n = 9$  calves/group) were as follows: diluent (Placebo); modified-live bovine coronavirus vaccine (BC); modified-live vaccine containing BHV-1 (N3); and concurrent N3 and BC vaccines (BC + N3). A—Shedding of infectious BHV-1 in nasal secretions was quantified PFU/mL nasal secretion, and data were normalized by  $\log_{10}$  transformation. Data at each time point are presented as mean and SEM values for each group. B—Visible lung pathology summated for all lobes within the lung and values presented for individual animals within each treatment group. Horizontal line represents mean value for each group. <sup>a,b,c</sup>Results with different letters differed significantly ( $P < .01$ ).

### Discussion

The current vaccine trial is the first to address vaccine interference when 2 MLV vaccines are coadministered IN to neonatal calves. Vaccine interference was investigated by monitoring both innate and acquired immune responses and determining whether protection against BHV-1 respiratory disease was compromised. The vaccines of interest target important viral pathogens in neonatal calves that result in either respiratory or enteric infections. Finally, the study was performed using calves that had maternal IgG antibodies present in blood for both BHV-1 and BC at the time of primary and booster vaccination.

A major finding was that coadministering the N3 vaccine did not significantly alter either serum IgG responses, IgA antibody responses in the URT, or T-lymphocyte responses induced by the BC vaccine. No significant differences in BC-specific IgG titers were observed when comparing the BC and BC + N3 groups and pooling data from these 2 groups



and comparing them to calves not receiving the BC vaccine (Placebo and N3) confirmed the BC vaccine induced a significant increase in serum IgG titers when administering the BC vaccine either alone or with the N3 vaccine. Analysis of BC-specific IgA titers in nasal secretions provided further evidence the local antibody response to the BC vaccine was not altered by coadministering the N3 vaccine. Both the kinetics and magnitude of the IgA response in nasal secretions were similar when the BC vaccine was delivered with or without the N3 vaccine. Finally, an analysis of BC-specific T-lymphocyte responses confirmed that coadministering the BC and N3 vaccines did not alter BC-specific cytokine secretion by blood lymphocytes.

The BC vaccine did not significantly interfere with acquired immune responses induced by the BHV-1 component of the N3 vaccine. The decline in maternal IgG specific for BHV-1 was not significantly altered by vaccination with the N3 vaccine, with and without BC vaccine coadministration. There was, however, a significant increase in BHV-1-specific IgA antibody titers in nasal secretions following both primary and booster vaccinations with the N3 vaccine, both with and without coadministration of the BC vaccine. The kinetics of this IgA response was similar when comparing these 2 vaccine groups, and there was no significant difference when comparing the 2 groups receiving the N3 vaccine. Finally, coadministering the BC vaccine did not significantly alter the blood lymphocyte cytokine responses induced by the BHV-1 tGD protein. Therefore, there was no evidence the BC vaccine altered the immunogenicity of the BHV-1 component of the N3 vaccine, but the effect of the BC vaccine on the PI-3 and BRSV components of the N3 vaccine was not evaluated. The extent to which PI-3 and BRSV replicate in both the URT and lung is not known, but it cannot be assumed the BC vaccine did not alter the immunogenicity of these 2 vaccine components. Further research would be required to confirm that the BC vaccine does not interfere with other viral components of the N3 vaccine.

The possibility that the BC and N3 vaccines may induce markedly different innate immune responses was one reason vaccine interference was investigated with these 2 vaccines. Modified-live viral vaccines containing BHV-1 and PI-3 are known to induce type 1 (IFN- $\alpha$ ) and type 2 (IFN- $\gamma$ ) production in the URT of neonatal calves.<sup>12,14</sup> In contrast, transcriptome analyses demonstrated that enteric BC is associated with an inhibition of IFN signaling response and BC replication is inhibited by IFN- $\alpha$ .<sup>19</sup> Inhibition of BC replication by IFN is one potential mechanism by which the N3 vaccine could interfere with the induction of an acquired immune response by the BC vaccine. The absence of detectable IFN- $\alpha$  or IFN- $\gamma$  in nasal secretions following primary and booster vaccinations with the BC vaccine is consistent with the known innate immune response to BCV infection.<sup>19</sup> It was surprising, however, to observe that the first IN vaccination with the N3 vaccine induced detectable IFN in the nasal secretions of approximately 30% of calves. Furthermore, the IFN- $\alpha$

and IFN- $\gamma$  concentrations detected in these calves were relatively low when compared to levels previously reported when neonatal calves and older calves were vaccinated IN with MLV vaccines containing BHV-1.<sup>11,12</sup> Limited induction of an IFN response by the N3 vaccine may explain why this vaccine did not interfere with acquired immune responses induced by the BC vaccine. Thus, it may not be possible to extrapolate the current observations regarding vaccine interference to other MLV vaccines containing BHV-1 and PI-3 that induce greater IFN responses in the URT of neonatal calves.<sup>12</sup>

The current study also investigated whether coadministering the BC vaccine interfered with N3 vaccine protection against BHV-1 infection and clinical disease. When comparing clinical parameters, such as body temperature postchallenge and virus shedding, there was no significant difference between the N3 and BC + N3 groups. Pooling data from these 2 groups confirmed the N3 vaccine significantly reduced clinical disease, virus shedding, and lung lesions when compared to groups not receiving the N3 vaccine. Thus, there was no significant interference with protection against both BHV-1 infection and disease when coadministering the 2 IN vaccines. A surprising result was that when pooling data from the 2 groups receiving the BC vaccine, there was a significant reduction in lung lesion scores for calves receiving the BC vaccine versus calves not receiving the BC vaccine. This difference suggests an immune response to BC may have reduced lung pathology following the BHV-1 challenge. Approximately one-third of calves had BC present in nasal swabs at the beginning of the study, but calves were not sampled for BC at the time of the BHV-1 challenge. Lung pathology was, however, compared between calves that were PCR positive ( $n = 13$ ) and negative (23) when recruited to the study and there was no statistical difference. Further work will be required to determine if coinfection by BHV-1 and BC exacerbates lung pathology as was observed for coinfection with BVDV and BC.<sup>16</sup> The prevention of BC coinfection during a BHV-1 respiratory infection is one possible mechanism by which the BC vaccine may have significantly reduced lung lesions in the present study. This would be consistent with growing evidence that BCV is a respiratory pathogen in cattle.<sup>16-18</sup> Alternatively, the BC vaccine may have enhanced immune responses to the BHV-1 component of the N3 vaccine in a way that enhanced disease protection but was not apparent in the immune assays performed in this study.

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