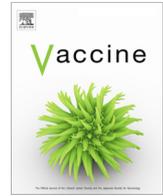




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Changes in circulating lymphocytes and lymphoid tissue associated with vaccination of colostrum deprived calves

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ABSTRACT

The objective of this study was to compare immunological responses and lymphoid depletion in young, colostrum deprived calves following administration of vaccines containing modified-live bovine viral diarrhea virus (BVDV). A group of calves exposed to a typical virulence non-cytopathic (ncp) BVDV-2 field strain (ncp exposed) was included to compare responses of calves receiving vaccine to responses generated against a field strain (mimicking a natural infection). A negative control group administered a placebo was used in all comparisons. All vaccines used in the study were administered per manufacturer recommendations while ncp BVDV exposed calves received 5 ml intranasally (2.5 ml/nare; 4.2×10^6 TCID₅₀/ml) of the BVDV-2 field strain. Samples collected at each time point included nasal swabs for virus detection, blood samples for complete blood counts and detection of viremia, PBMCs for flow cytometric analysis, serum for virus neutralization titers, and thymus tissue at necropsy for evaluation of lymphoid depletion. A measurable neutralizing BVDV titer was observed for all treatment groups excluding the control animals, which remained negative during the study period. Virus shedding was only detected from the ncp vaccinated and ncp exposed calves. A decline from baseline was observed for peripheral lymphocyte and CD4⁺ cells for the groups receiving the adjuvanted cytopathic (cp) vaccine, the double deleted genetically modified (ddGM) vaccine, the ncp vaccine and ncp exposed calves, but not for the control group or groups receiving cp vaccines. Thymus depletion was observed for the ncp vaccine and ncp exposed calves and to a lesser extent for the ddGM vaccine calves. Collectively, these data suggest that the virus biotype, method of attenuation, presentation, and use of adjuvant will influence vaccine impacts on lymphoid tissues and the immune response. As such, multiple variables should be considered when determining costs and benefits of vaccination.

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1. Introduction

Ruminant pestiviruses are globally-distributed pathogens that include bovine viral diarrhea virus-1 (BVDV-1) and BVDV-2 that are grouped in two different species, Pestivirus A and B, respectively. BVDV-1 and 2 are comprised of multiple subgenotypes that belong to the genus *Pestivirus*, family *Flaviviridae*. BVDV infections result in a wide range of clinical manifestations that can affect the

reproductive, digestive and respiratory systems, with the impact ranging from mild to severe [1,2]. The form of clinical disease observed is dependent on the agent, host, and environmental factors, as well as the interaction of these factors. BVDV can exist as two biotypes, cytopathic (cp) or non-cytopathic (ncp), as defined by their effect in cultured cells. Similarities and differences can be observed between the two biotypes with regard to immune evasion mechanisms [3]. The most notable difference is impact of infection on pregnant animals. The ncp biotype is most frequently detected in the field and infection with ncp biotype in pregnant animals may result in embryonic death, resorption, and stillbirth,

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or the birth of persistently infected (PI) calves. Regardless of biotype, a hallmark characteristic associated with BVDV infection is immunosuppression [4,5]. The changes associated with the immunosuppressive actions include, but are not limited to, a decrease in circulating lymphocytes, depletion of lymphoid tissue, and inhibition of metabolic activities of stimulated lymphocytes. Variation in the severity of immunosuppression has been reported between BVDV isolates that differ in virulence [6], as well as biotype [7]. Some immunosuppressive effects of BVDV are thought to be properties specific to each respective isolate and may not necessarily be representative of biotype or genotype [7,8].

The composition of currently available modified live viral (MLV) BVDV vaccines in the US, include cp BVDV isolates and may contain adjuvants. While currently available MLV vaccines contain cp vaccine strains, the strains used in each vaccine can differ. Furthermore, previously available BVDV MLV vaccines have contained ncp BVDV in their composition. In addition, double-deleted, genetically modified (ddGM) attenuated BVDV isolates have been licensed in other countries. Therefore, BVDV MLV vaccine composition can vary in biotype, strain, adjuvant, and attenuation method. Each of these variables could affect the immune response and potentially alter the degree of immune suppression that is observed with each respective BVDV MLV vaccine.

In 2014, the USDA Center for Veterinary Biologics issued a notice (No. 14-06) that outlined additional safety data that may be required for products containing agents considered immunosuppressive, which included MLV BVDV vaccines. The guidance, however, does not specify what additional safety testing should be conducted, and a current benchmark for changes expected with BVDV vaccination has not been established. A better understanding of the outcomes associated with BVDV vaccination as it relates to lymphoid depletion would be beneficial. The various BVDV MLV vaccines could modulate the immune response differently; therefore, as new vaccines are developed, understanding the impact of BVDV MLV vaccines on the bovine immune system will be necessary.

The objective of the current study was to compare immunological changes after vaccination with current and previously available BVDV MLV vaccines in the US, and a ddGM vaccine which is licensed in the EU, Mexico, Brazil and other countries around the world. Comparison of the vaccines was based upon detection of virus from nasal swabs and blood, CBCs to determine lymphocyte numbers, flow cytometric analysis of PBMC subpopulations, development of neutralizing antibodies, and lymphoid depletion in thymus tissue.

2. Materials and methods

2.1. Experimental design

The study was designed as a randomized, controlled study. All animals were handled in accordance with the Animal Welfare Act Amendments (7 U.S. Code §2131 to §2156) and all study procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the National Animal Disease Center (ARS-2017-616).

Cattle enrolled in the study consisted of a sixty-four ($n = 64$) colostrum-deprived Holstein bull calves approximately 3–4 weeks of age at initiation of the study. The study was conducted in two replicates consisting of thirty-two calves each assigned to eight treatment groups with four calves in each respective group. Calves were housed in biocontainment facilities (ABSL2) separated by group for the duration of the study. Prior to the start of the study, all calves were tested to ensure they were negative for BVDV antigen and antibodies as described previously [9,10].

2.2. Vaccine and virus administration

All cp vaccines were USDA licensed stock material approved for commercial sale in the US and purchased through a single commercial source with the exception of the ddGM vaccine that was provided directly by Boehringer Ingelheim (BI) Animal Health and the other ncp vaccine provided directly by Elanco Animal Health. All vaccines were received in proper cold chain and were cataloged and refrigerated. Vaccines were administered per manufacture label recommendation, and were administered within 15 min of preparation. Treatment group and vaccine details are listed in Table 1 and described hereafter. Calves in group 1 received monovalent ncp BVDV ddGM vaccine (Bovela[®], Boehringer Ingelheim Vetmedica, Inc. St. Joseph, MO). Calves in group 2 received a 4-way multivalent MLV vaccine containing BoHV-1, BPI3V, BRSV, and ncp BVDV-1 (Arsenal[®] 4, Elanco Animal Health, Larchwood, IA). Calves in group 3 received a 5-way multivalent MLV vaccine containing bovine herpes virus-1 (BoHV-1), bovine parainfluenza-3 virus (BPI3V), bovine respiratory syncytial virus (BRSV), and cp BVDV-1 and 2 fractions (BoviShield GOLD[®] 5, Zoetis, Parsippany, NJ). Calves in group 4 received a 5-way multivalent MLV vaccine containing BoHV-1, BPI3V, BRSV, and cp BVDV-1 and 2 fractions (Titanium[®] 5, Elanco Animal Health, Greenfield, IN). Calves in group 5 received a 5-way multivalent MLV vaccine containing BoHV-1, BPI3V, BRSV, and cp BVDV-1 and 2 fractions (Vista[®] 5, Merck Animal Health, Madison, NJ). Calves in group 6 received a 5-way multivalent adjuvanted MLV vaccine containing BoHV-1, BPI3V, BRSV, and cp BVDV-1 and 2 fractions (Express[®] 5, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO). The adjuvant is a sub-potent dose of Haemophilus Somnus in the Express[®] 5 vaccine. Calves in group 7 received 2 ml of sterile water by subcutaneous (SQ) injection. Calves in group 8 were exposed to ncp BVDV-2 field isolate RS886 via intranasal route (5 ml total/2.5 ml per nostril; 4.2×10^6 TCID₅₀/ml) that has been previously characterized to induce mild clinical signs consisting of pyrexia (>40 °C) early in the infection, moderate decrease in lymphocytes, viremia and viral shedding. Characterization, propagation, and viral titer was determined as previously described [11].

Eight total treatment groups were utilized in the study that included six different vaccines. While different BVDV strains were present in each of the vaccines, the biotype (cp) of the BVDV isolates were the same for three of the vaccines (cp vaccines; Groups 3, 4, and 5) and these three vaccines also lacked an adjuvant. Distinguishing characteristics of the other three vaccines, were presence of an adjuvant (adjuvanted vaccine; Group 6), ncp biotype (ncp vaccine; Group 4), and attenuation by double deleted genetic modification (ddGM vaccine; Group 1). Groups 7 and 8 consisted of the control and ncp exposed BVDV field strain treatments, respectively.

2.3. Sample collection

Sample time points included two baseline samples collected on days -2 and -1 prior to administration of treatments for each respective treatment group (Day 0). A rumen temperature probe given as a bolus to record basal temperatures was administered on day -2 of the study as previously described [12]. After administration of the respective material for each treatment group (Day 0), samples were collected on days 2, 4, 6, 9, 11, 13, and 16 post administration of treatments. Samples collected at each time point included; two nasal swabs for virus detection, blood samples for detection of viremia, blood samples for complete blood counts (CBCs), PBMC isolation for flow cytometric analysis and serum for virus neutralization assays. Calves were euthanized by intravenous (IV) administration of sodium pentobarbital on day 17 for collection of thymic tissue to be evaluated for lymphoid depletion.

Table 1
Treatment groups and vaccine details.

Group	Vaccine composition or type of treatment**	Route*	BVDV biotype	Vaccine characteristics	Company
1	monovalent BVDV	IM	nep	double deleted genetically modified (ddGM)	Bovela, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO
2	BoHV-1, BPI3V, BRSV, and BVDV-1	SQ	nep	MLV	Arsenal 4, Elanco Animal Health, Larchwood, IA
3	BoHV-1, BPI3V, BRSV**, and BVDV -1 and 2	SQ	cp	MLV	BoviShield GOLD 5, Zoetis, Parsippany, NJ
4	BoHV-1, BPI3V, BRSV, and BVDV-1 and 2	SQ	cp	MLV	Titanium 5, Elanco Animal Health, Greenfield, IN
5	BoHV-1, BPI3V, BRSV, and BVDV-1 and 2	SQ	cp	MLV	Vista 5, Merck Animal Health, Madison, New Jersey
6	BoHV-1, BPI3V, BRSV, and BVDV-1 and 2	SQ	cp	MLV (with adjuvant)	Express 5, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO
7	control: 2 ml of sterile water	SQ	na	na	na
8	typical virulent BVDV-2 field isolate RS886	IN	nep	na	na

* IM = Intramuscular; SQ = Subcutaneous; IN = Intranasal.

** bovine herpes virus-1 (BoHV-1), bovine parainfluenza-3 virus (BPI3V), bovine respiratory syncytial virus (BRSV), and bovine viral diarrhea virus (BVDV).

2.4. Virological assays

For virus detection, jugular blood samples were collected in EDTA tubes, and buffy coat (BC) was separated by centrifugation (25 min at 1200 × g). The BC was collected and put through one freeze/thaw cycle (-20 °C/25 °C). An aliquot of 140 µL of BC sample was submitted to RNA extraction using QIAcube® (Viral RNA kit) according to the manufacturer's recommendations (Qiagen, Valencia, CA). Extracted RNA was used for conventional reverse transcriptase (RT) polymerase chain reaction (PCR) for DNA sequencing to confirm that the virus detected was vaccine or virus used for exposure. RT-PCR was performed using two published RT-PCR assays that target the 5' untranslated region of the viral genome (5' UTR). The primer sets used were HCV90-368 and 324-326 with the reactions being performed as previously described [13,14]. Sequences were edited and aligned using Geneious software (Biomatters Inc, Newark, NJ), and comparison of generated sequences to sequences of vaccine strains was performed using Molecular Evolutionary Genetics Analysis software package 7 (MEGA7).

Upon collection, nasal swab (NS) samples were placed in microtubes (1.5 ml) to which 1 ml of sterile PBS was added. NS samples were stored at -20 °C until analysis. After thawing, the NS samples containing the swab were centrifuged for 2 min at 400 × g to separate any debris from the swab itself. Similar to the BC sample, an aliquot of 140 µL of the NS sample was submitted to RNA extraction, and RT-PCR was performed as previously described.

An aliquot of 140 µL of each vaccine and the RS886 virus used for intranasal inoculation was subjected to RNA extraction using QIAcube® (Viral RNA kit) according to the manufacturer's recommendations (Qiagen, Valencia, CA). Extracted RNA was subjected to real-time quantitative PCR to quantify the total amount of BVDV in each vaccine being administered to each calf. A 10-fold viral dilution curve starting at 4.2×10^6 TCID₅₀/ml using the RS886 virus used for intranasal inoculation, as a reference to provide a relative quantification of total amount of BVDV RNA in the vaccines. The commercial real-time PCR assay detects a fragment of the BVDV-1 and BVDV-2 5'UTR (BVDV VetMax Gold, ThermoFisher Scientific, Austin, TX). Samples were analyzed using QuantStudio™ 5 System (Applied Biosystems) with the respective software.

2.5. Serological assays

Blood was collected into serum separation tubes with gel and clot activator (BD Vacutainer SST, Franklin Lakes, NJ), centrifuged (25 min at 1200 × g), and serum was collected and frozen at -80C until testing. A comparative virus neutralizing titer (VNT) was determined using cytopathic isolates BVDV-1a-Singer and BVDV-2a-296c as previously described [15]. Briefly, serum was diluted in MEM from 1:2 to 1:256 in 96-well plates, and approxi-

mately 200 TCID of virus was added to each well. After a 1 h incubation at 37 °C with 5% CO₂, 2 × 10⁴ bovine turbinate (BTu) cells were added to each well. After four days of incubation, dilution endpoints were determined by observing cytopathic effect (CPE) in the cell monolayer. Results were expressed as the reciprocal (Log₂ base) of the highest serum dilution able to inhibit the appearance of CPE in cells. Serum samples were run in a 96-well plate with five wells per dilution, and titers were calculated by the Reed-Muench method.

2.6. Evaluation of peripheral lymphocytes

Calves were bled by jugular venipuncture, and blood was collected in EDTA tubes (BD Vacutainer PPT tube, Franklin Lakes, NJ) for submission to the Iowa State Veterinary Diagnostic Laboratory for CBC determination. Specifically, the number of lymphocytes was used to determine fluctuations in total circulating lymphocytes at each respective time point.

A third blood sample was collected in acid citrate dextrose (ACD) (BD Vacutainer ACD Solution A, Franklin Lakes, NJ), and centrifuged at 1200 × g for 25 mins. Buffy coat layers were aspirated, and the contaminating erythrocytes were removed by two rounds of hypotonic lysis. Hypotonic lysis was performed by adding two volumes of buffered water (lysis buffer) for approximately 30 secs and then restoring with suspension to normal osmolality with one volume triple strength PBS (restoring buffer). The resulting cell suspensions were centrifuged at 400 g for 5 min to pellet the cells. The cell pellets were washed with 5 ml of PBS to remove any residual lysis and restoring buffer and centrifuged again at 400 g for 2 min.

For flow cytometry labeling, cells were resuspended at approximately 10⁷ cells/ml in PBS and pelleted prior to resuspension and incubation with fixable viability dye eFluor 450 or 455 UV to identify live/dead (LD) cells as described (eBioscience, San Diego, CA). Cell suspensions were washed twice with stain buffer (BD Biosciences, San Jose, CA) per manufacturer's recommendations prior to labeling. Cells were then resuspended in 100 µL of their respective primary monoclonal antibody mix for 30 min, followed by two wash steps before being resuspended in their respective secondary antibody mix for 30 min as previously described [16,17]. After two wash cycles, cells were resuspended in 200 µL BD™ stabilizing fixative (BD BioSciences, San Diego, CA) and kept in the dark at 4 °C until flow cytometry analysis. Analysis was performed within 24 h of staining. The list of antibodies, panel configuration and reagents used are summarized in Table 2.

2.7. Thymus evaluation

Formalin-fixed tissues were embedded in paraffin, cut in 5 µm sections, stained with hematoxylin and eosin (HE) and examined

by light microscopy and morphometric analysis. Whole tissue slides stained with HE were scanned using an Aperio ScanScope XT workstation (Aperio Technology, Inc., Vista, CA) and analyzed as previously described [6]. The color deconvolution (CD) algorithm had four input threshold values that were modified based on intensity of the hematoxylin stain quantified on the slides analyzed in this study. The positive color channel used was hematoxylin (1), and the threshold values modified from the standard algorithm included: the black threshold (0, clear glass), weak positive threshold (185), medium positive threshold (105) and strong positive threshold (105). The medium and strong threshold parameters are the same only provide weak and strong positive values and no intermediate medium values. Based on these parameters, detection of strong positive staining indicated cortex, and detection of weak positive staining indicated medulla. The quantification of the cortex:medulla ratio was based on the ratio of strong positive to weak positive values.

2.8. Statistical analysis

All statistical analyses were performed using commercially available statistical software (SAS v9.4, SAS Institute, Cary, NC) and p-values of < 0.05 were considered significant. The frequency of labeled cells for the respective PBMC populations (CD2⁺, CD3⁺, CD4⁺, CD8⁺, $\gamma\delta$ ⁺, and CD335⁺) was calculated and this value was used for all subsequent calculations. Post-exposure daily values for CBC lymphocyte data as well as PBMC populations relative to baseline values were determined by calculating the percent change from baseline levels for each calf on each respective day of the study when samples were collected using the formula $((D-B)/B \times 100)$, where B is the baseline value (average the two baseline samples, day -2 and -1) and D is the value for each respective experimental day. The results from the calculation to determine percent decline from baseline for each calf on each day were analyzed using the GLM procedure in SAS (SAS Inst., Cary, NC) with a model that included the fixed effect of treatment at each experimental day. When variables of interest were significant ($P < 0.05$) for the fixed effect of treatment, contrasts among least square (LS) means were evaluated for each experimental day to evaluate differences between treatment groups. Figures were generated in Microsoft[®] Excel for significant variables and the standard error of the mean was included using the standard error function in Microsoft[®] Excel.

Comparisons among LS means, indicated that no differences were observed between the three cp vaccines lacking adjuvant (Table 3). Therefore, for simplicity of presentation for the peripheral lymphocyte, CD4⁺ subpopulation, and thymus data for the vaccines containing the same BVDV cp biotype were combined into one group identified as cp vaccines.

BVDV serum neutralizing titers were calculated as the reciprocal of serum antibody titers log₂-transformed, which were then back-transformed as geometric means for presentation of results.

Table 2
Summary of antibodies used for MP-FCM assays for PBMC comparisons.

Panel designation	Primary antibody	Clone	Isotype	Secondary antibody dye
Panel 1	CD3	MM1A	IgG1	BV421
	CD4	IL11A	IgG2a	APC
	CD8 α	BAQ11A	IgM	PE
	GD	GB21A	IgG2b	APC/Cy7
	CD45RO	ILA116A	IgG3	BUV395
	CD31	MCA1097F	IgG2a	FITC (direct conjugation)
	CD62L		IgG1	PE/Cy7 (direct conjugation)
	CD2	MUC2A	IgG2a	FITC
Panel 2	CD335	ASK1	IgG1	BV421
	sIgM	PIG45A	IgG2b	PE
	CD14	CAM66A	IgM	APC

3. Results

3.1. Clinical presentation

Calves were observed daily and no overt clinical disease was observed in any of the study animals throughout the study. Temperature was monitored over the course of the study as previously described [12]. An increase in temperature was observed for calves in the ncp exposed group (Group 8) on approximately day 6 of the study and in the ncp vaccine calves (Group 2) on approximately days 9 and 10. The increase in temperature was associated with virus detection in the calves receiving the ncp vaccine and ncp exposed calves.

3.2. BVDV-specific virus neutralizing antibodies

Antibody titers were determined prior to administration of any treatments and 16 days post administration of treatments. No detectable neutralizing antibody titers to BVDV were observed prior to vaccination or exposure in all calves. A measurable neutralizing BVDV antibody titer at day 16 was observed for all treatment groups excluding the control animals which remained negative for the duration of the study (Table 4). While antibody titers observed at day 16 do not represent the complete immunological response to each respective antigen, these antibody titers do support recognition by the immune system and response to the respective BVDV antigens. These titers are reported to support recognition of the immune system to BVDV, but the titers observed on day 16 are not reflective of the titers that may be observed at a later time point or should be correlated to immunity as these titers do not reflect a complete response.

At 16 days post-vaccination, the BVDV-1 antibody titers for adjuvanted vaccine calves (Group 6) were greater than the antibody titers observed for the ncp exposed calves (Group 8). In general, at 16 days post-vaccination the BVDV-1 response was greater for calves vaccinated with ncp vaccine (Group 2) and calves in cp vaccine Group 5, but the BVDV-2 response was greater for cp vaccinated calves in Groups 3 and 4. In addition, the BVDV-2 response was greater for ddGM vaccine and ncp exposed calves (Groups 1 and 7, respectively).

3.3. BVDV virus detection

For real-time PCR, samples were considered positive if the Ct value was <30. Samples with Ct values >30 were considered suspect or negative. Based on real-time PCR values, all vaccines used in the study contained similar amounts of virus antigen, with the exception of the adjuvanted vaccine that had the least amount of virus as detected by PCR (Table 4). The amount of live virus was not determined. While the real-time PCR results suggested less BVDV virus in the adjuvanted vaccine, the level of neutralizing antibodies elicited by this vaccine was greater than that elicited by other vacci-

Table 3
Significant variables associated with treatment and associated contrasts across treatments.

	% decline day 2	% decline day 4	% decline day 6	% decline day 9	% decline day 11	% decline day 13	% decline day 16	necropsy day 17
CBC Lymphocyte (Lym)	0.71	0.0001	0.0001	0.003	0.22	0.38	0.06	
PBMC CD4 ⁺	0.22	0.03	0.03	0.29	0.68	0.67	0.14	
Gross thymus								0.001
Histo thymus								0.0001
	Group 1 ddGM vaccine	Group 2 ncp vaccine	Group 3 cp vaccine	Group 4 cp vaccine	Group 5 cp vaccine	Group 6 adjuvanted vaccine	Group 7Control	Group 8 ncp exposed
Lym % decline day 4	ab	cd	cde	e	de	bc	e	a
Lym % decline day 6	abc	abc	d	d	d	a	d	ab
Lym % decline day 9	abc	a	d	d	d	abcd	d	ab
Lym % decline day 16	a	ab	c	c	bc	abc	bc	abc
CD4 ⁺ % decline day 4	b	b	b	b	b	b	b	a
CD4 ⁺ % decline day 6	abc	a	c	c	abc	abc	c	ab
Gross thymus	bc	a	c	c	c	c	c	ab
Histo thymus	bc	a	cd	d	d	D	d	ab

*Means with the same letter across each row are statistically similar ($P < 0.05$).

Table 4
Averages from all calves from Rep 1 and 2 for real-time PCR Ct values, virus neutralization log(2) titer, and virus detection by reverse transcriptase PCR for each respective treatment group.

	Group 1 ddGM vaccine	Group 2 ncp vaccine	Group 3 cp vaccine	Group 4 cp vaccine	Group 5 cp vaccine	Group 6 adjuvanted vaccine	Group 7 control	Group 8 ncp exposed
RT-qPCR Ct values for total BVDV in each vaccine	22.2	22.0	22.3	18.5	18.6	30.0	UD*	17.7
Virus neutralization log(2) titer								
BVDV type 1 (Singer)	0.4	1.8	0.0	0.0	0.7	5.4	0.0	0.5
BVDV type 2 (296c)	0.9	0.2	0.1	0.4	0.5	1.5	0.0	5.4
Virus isolation		PBMC and NS day 9						PBMC and NS days 4, 6, 9, and 11

* Undetermined.

nes in the study. Therefore, it is unknown if the adjuvant elicited a greater response with less virus, or if the adjuvant or other components interfered with PCR amplification. The manufacture of the cp adjuvanted vaccine indicated that the vaccine includes a high titer of BVDV, therefore, it would appear the lack of detection was associated with PCR interference.

All study animals were negative for BVDV prior to the start of the study, and all control calves remained negative throughout the study. In the calves that received a vaccine, virus was only detected from the ncp vaccine calves; no virus was detected from any other vaccinated calves (Table 4). Virus was detected from nasal swabs and PBMCs on day 9 in the ncp vaccinated calves. Virus was also detected from nasal swabs and PBMCs in ncp exposed calves on days 4, 6, 9, and 11.

3.4. Complete blood counts and PBMC subpopulation comparisons

As comparisons among LS means indicated that no differences were observed between the three cp vaccines lacking adjuvant (Table 3), presentation for the peripheral lymphocyte and CD4⁺ subpopulation for the three vaccines containing the cp BVDV biotype but lacking adjuvant were combined into one group identified as cp vaccines.

The effect of treatment ($P < 0.05$; Table 3) was observed for two of the peripheral leukocyte variables measured in the study. Percent change from baseline was observed for the circulating lymphocytes on days 4, 6, and 9 and percent change from baseline was also observed in the CD4⁺ subpopulation on days 4 and 6.

No statistically significant differences ($P < 0.05$) were observed in percent decline of peripheral lymphocytes between the control calves and calves that received the cp vaccines at any of the sample

time points (Table 3; Fig. 1). On days 4 and 6, a difference in percent decline for peripheral lymphocytes was observed for calves receiving the ncp, ddGM, and adjuvanted vaccines, as well as the ncp exposed calves, as compared to the control and cp vaccinated calves (Table 3; Fig. 1). On day 9, a difference in percent decline for peripheral lymphocytes was observed for the ncp and ddGM vaccines, and the ncp exposed calves, as compared to the control and cp vaccinated calves (Table 3; Fig. 1). In general, calves administered ncp BVDV either via vaccination or intranasal exposure had a greater decrease in circulating peripheral lymphocytes as determined by percent decline from baseline compared to control calves and calves receiving cp vaccines. While a difference in percent decline for peripheral lymphocytes was observed for calves receiving the adjuvanted vaccine, the duration of decline was only observed on days 4 and 6 and was not as prolonged as the calves exposed to ncp BVDV.

No difference was observed in percent decline of CD4⁺ lymphocytes between the control calves and calves that received the cp vaccines at any of the sample time points (Table 3; Fig. 2). On day 4, a difference in percent decline for CD4⁺ lymphocytes was observed for the ncp exposed calves, as compared to all other treatment groups (Table 3; Fig. 2). On day 6, a difference in percent decline for CD4⁺ lymphocytes was observed for ncp vaccine and ncp exposed calves, as compared to the control and cp vaccinated calves (Table 3; Fig. 2). While a slight decline in the CD4⁺ cells on day 6 for the ddGM and adjuvanted vaccinated calves, the percent decline was not as substantial as the decline seen with the ncp vaccine and ncp exposed calves. The percent decline for the CD4⁺ cells on day 6 for the ddGM and adjuvanted vaccinated calves was between that of the control and cp vaccinated calves and the ncp vaccine and ncp exposed calves.

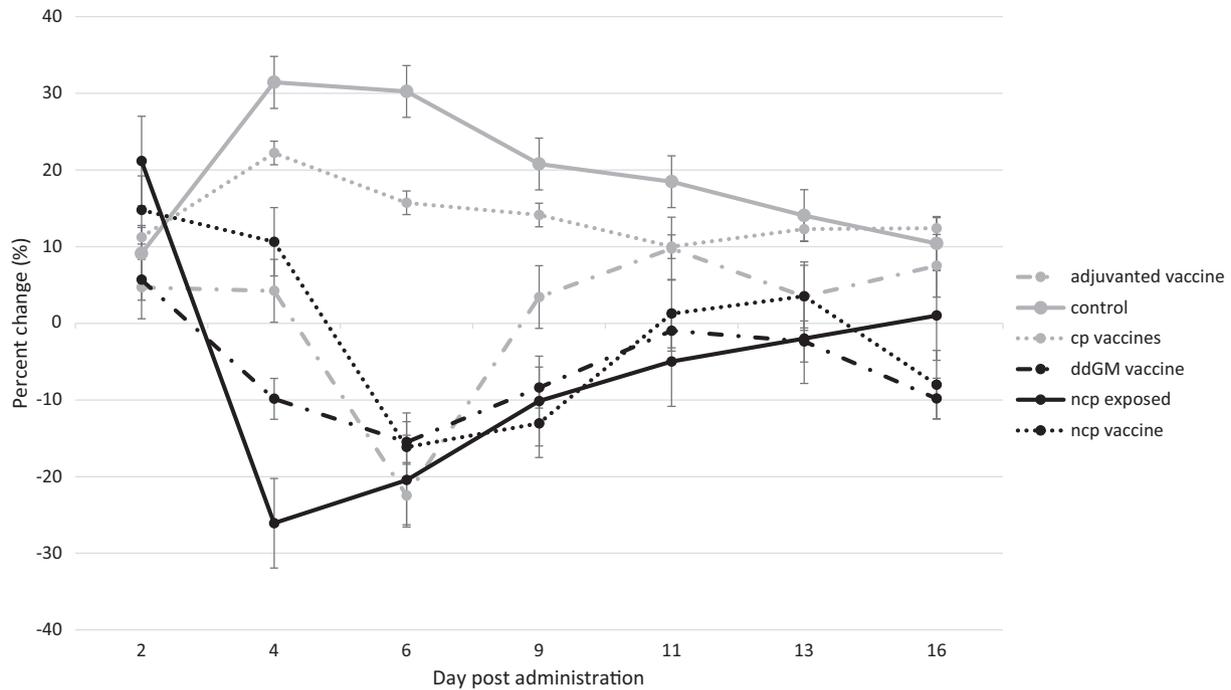


Fig. 1. Percent change from baseline values for peripheral circulating lymphocytes values as determined by complete blood counts. Data represents means from the respective treatment groups with the bars representing the standard error for each respective treatment group at each day post administration.

While there was not an effect of treatment ($P < 0.05$) observed on day 16 for the percent change from baseline for the CD4⁺ population, a rebound effect after ncp exposure prompted a retrospective examination of the day 16 data. In short, the percent decline from baseline at each respective time point should not be the only variable of interest, and the rebound effect for each vaccination or exposure should be considered. The percent decline is temporary, and an emphasis on a greater rebound should be considered which may be associated with a more robust memory response. The

subsequent rebound effect in the CD4⁺ population can be seen in Figs. 2 and 3. There was no change from baseline in the control calves, which is expected, but of the vaccines that induced a significant decline in the CD4⁺ population on days 4 and 6 post-exposure, ddGM and the adjuvanted vaccines had a positive rebound effect (Figs. 2 and 3). The ncp vaccine induced a significant percent decline from baseline on days 4 and 6 (Table 3; Fig. 2) for the CD4⁺ population, and by day 16 post-vaccination, the CD4⁺ population had not rebounded to previous baseline values. This

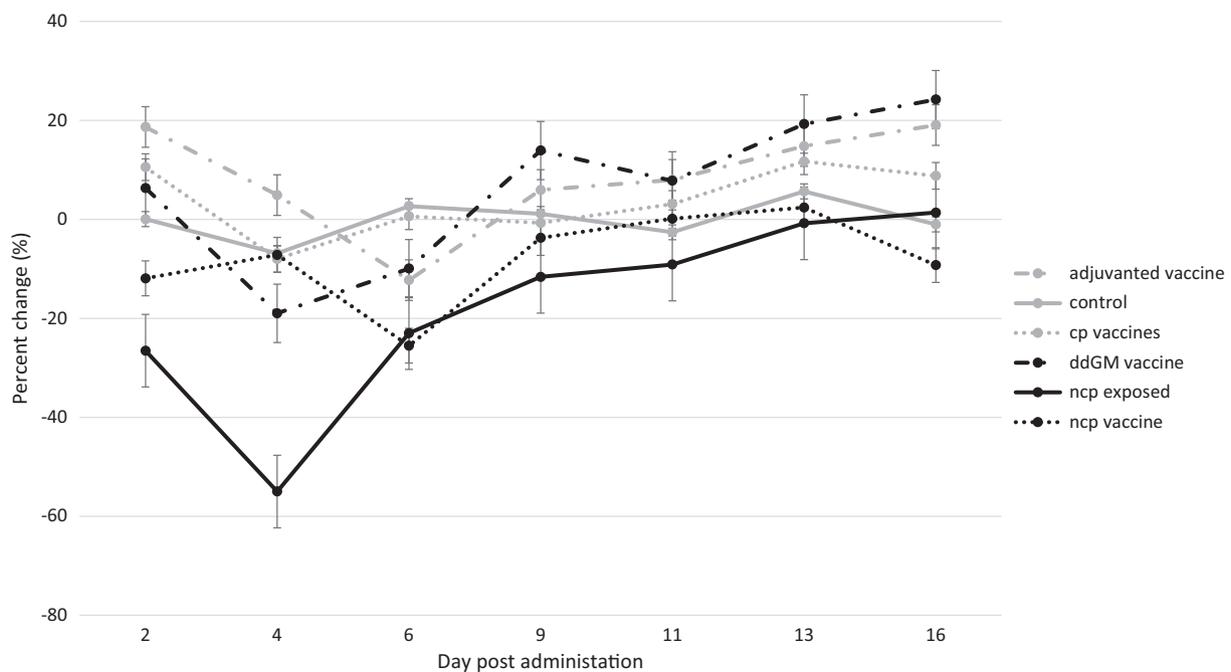


Fig. 2. Percent change from baseline values for CD4⁺ cells as determined by multi-parameter flow cytometric analysis. Data represents means from the respective treatment groups with the bars representing the standard error for each respective treatment group at each day post administration.

is interesting given that the ncp exposed calves that had the greatest percent decline in the CD4⁺ population on day 4 had returned to just above baseline values on day 16 (Fig. 2). Calves administered cp vaccines did not have a significant percent decline from baseline in the CD4⁺ population after vaccination, however, a positive increase from baseline on day 16 was observed, although it was not as remarkable as the rebound in the ddGM and adjuvanted vaccinated calves (Fig. 3).

No statistical differences ($P > 0.05$) were observed between treatment groups for percent change in the following PBMC subpopulations; the monocyte (CD14⁺) cell population, $\gamma\delta$ T cells, CD8⁺ T cells, B cells (sIgM⁺), CD2⁺ cells, natural killer cells (CD335⁺), and CD31⁺ cells. Minimal changes may have been observed ($0.05 > P < 0.1$), but no significant trends existed.

3.5. Thymus histopathology analysis

As comparisons among LS means indicated that no differences were observed between the three cp vaccines lacking adjuvant (Table 3), presentation for thymus measures for the three vaccines containing the BVDV cp biotype but lacking adjuvant were combined into one group identified as cp vaccines.

Histological observations of thymus depletion correlated with reduced gross thymus weights. Three treatments resulted in significant changes ($P < 0.05$) in thymus histology and weight: ncp exposure, ncp vaccine, and ddGM vaccine. There was a treatment effect ($P < 0.05$; Table 3; Figs. 4 and 5) with the ncp vaccinated calves having the greatest depletion of the thymus as measured by gross thymus weight (reported as a ratio of thymus:kidney weight) and depletion of the cortex:medulla ratio defined by strong:weak staining of H&E stained slides (Figs. 4 and 5). The ncp exposed calves had similar depletion of the thymus to that of the ncp vaccinated calves, but the depletion in the ddGM vaccine calves was not as extensive as the ncp vaccine calves (Figs. 4 and 5). The depletion pattern in the ddGM vaccine calves was between that of the ncp exposed calves and the cp vaccine and control calves,

signifying that the depletion it was not as severe as in the ncp exposed calves (Figs. 4 and 5). In general, both gross thymus weight and histological observations were similar among treatment groups, with the control and cp vaccine calves having the largest thymus with the greatest amount of strong positive staining suggestive of less depletion of the cortex:medulla ratio.

4. Discussion

Colostrum-deprived calves were utilized in this study as this model allows comparison of immune responses with fewer variabilities than would be observed with conventional calves. The immune systems of these calves may not be representative of neonatal calves under standard management that have received passive immunity via colostrum. However, as colostrum uptake and/or quality may not always be ideal, this data provides insight into responses under undesirable field/management conditions.

This study demonstrates differences in responses among the commercial BVDV MLV vaccines utilized in this study. Some of the vaccines also contained MLV strains of BoHV-1, BRSV, and BPI-3. These viruses may have contributed to some of the effects of the vaccines, but our goal was to evaluate the commercially available vaccines as used in the field. One commonality between all the vaccines used in this study was the inclusion of BVDV, but the presentation of the BVDV within the vaccines was different; cp or ncp BVDV, adjuvanted, or genetically modified. For this reason, ncp exposed calves were included to compare the response to commercially available vaccines containing BVDV to the response generated by exposure to a field strain of virus. In addition, control calves administered a placebo, were used for comparisons.

As expected, calves in the ncp vaccine and ncp exposed groups had a greater decline in circulating peripheral lymphocytes and CD4⁺ cells, in addition to viral shedding, viremia, and pyrexia, as is characteristic of ncp BVDV infections [6]. While, the ddGM and adjuvanted vaccinated calves had a decline in circulating periph-

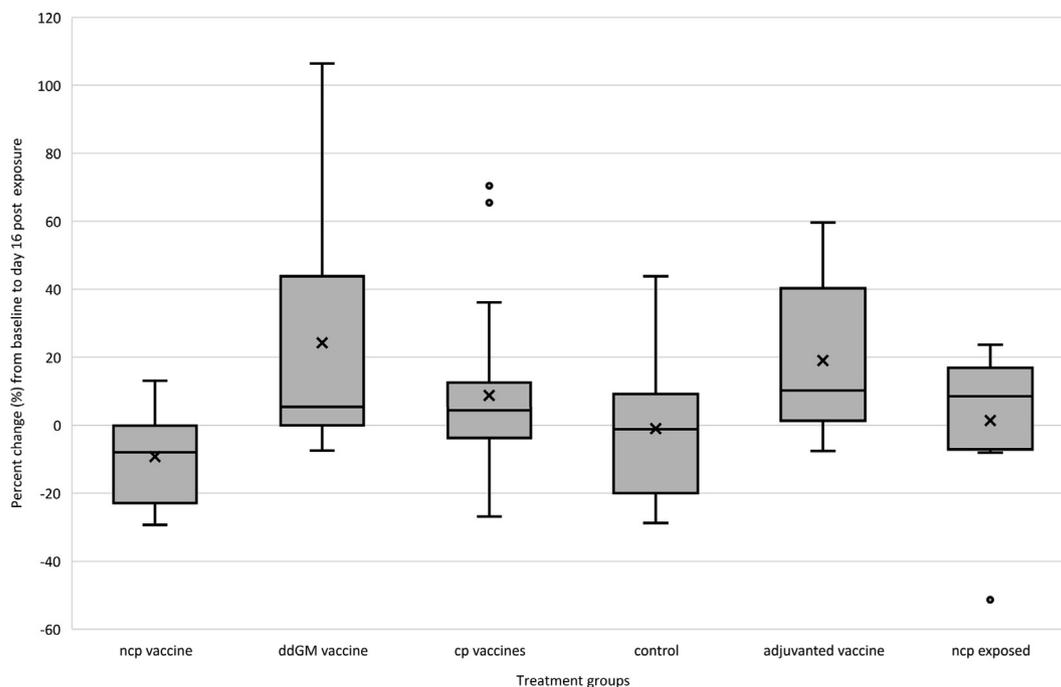


Fig. 3. Percent change on day 16 from baseline values for CD4⁺ cells as determined by multi-parameter flow cytometric analysis. Data represents means from the respective treatment groups.

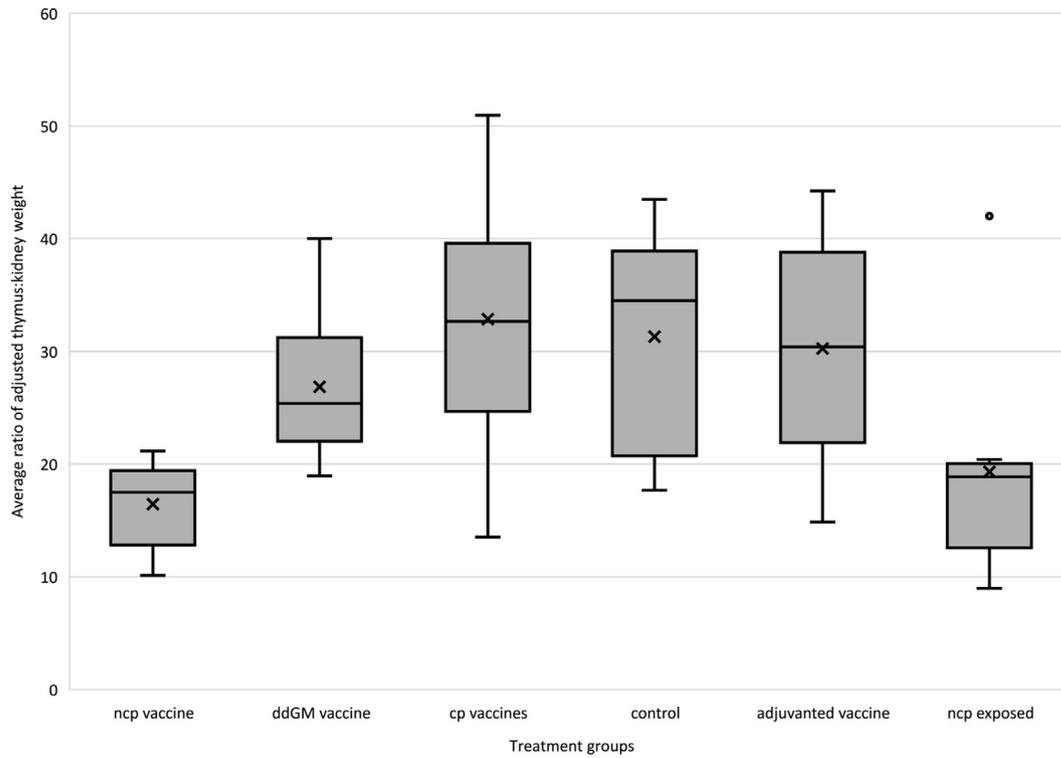


Fig. 4. Average ratio of adjusted gross thymus:kidney weight at necropsy on day 17. Data represents means from the respective treatment groups.

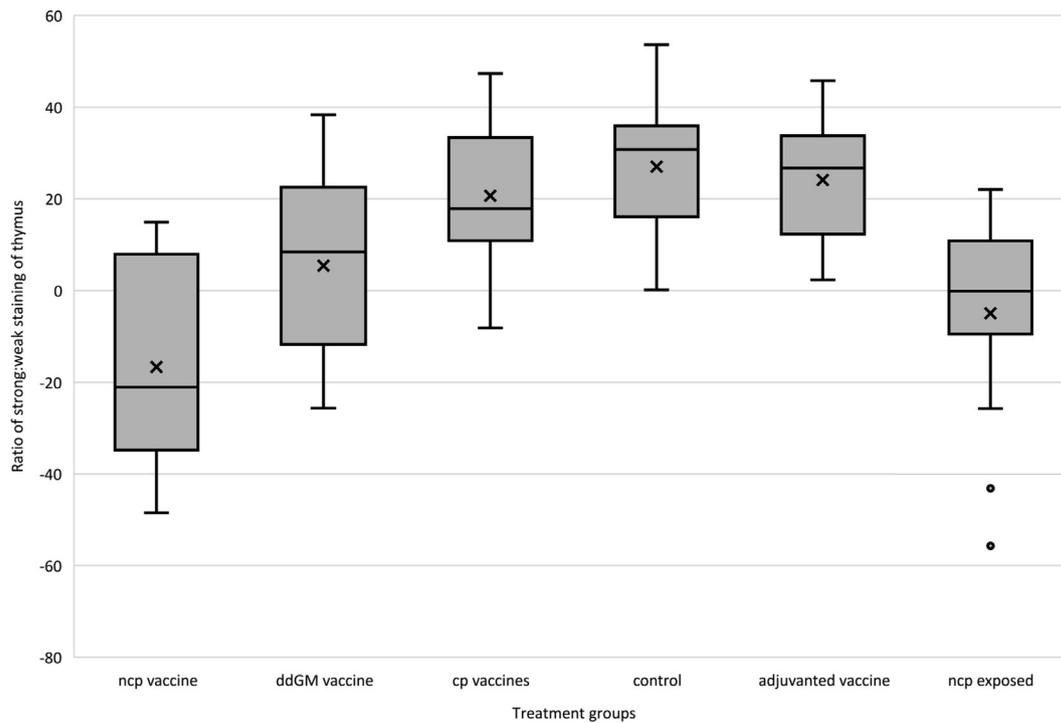


Fig. 5. Average ratio of strong:weak staining, representative of the cortex:medulla ratio of the thymus at necropsy on day 17. Data represents means from the respective treatment groups.

eral lymphocytes and CD4⁺ lymphocytes, calves in these groups did not exhibit significant changes in temperature or shed virus. Furthermore, the highest VN titers observed were in the adjuvanted vaccine and ncp exposed calves, followed by the ddGM vaccine and ncp vaccine calves. While neutralizing antibody titers were

observed in all vaccinated calves, higher antibody titers were associated with a greater temporary decline in circulating peripheral lymphocytes following exposure. Thus, the adjuvanted, ddGM, and ncp vaccine and the ncp exposed calves, had both the highest neutralizing antibody titers and the greatest temporary decline in

circulating lymphocytes. Furthermore, the comparatively higher neutralizing antibody titers for the adjuvanted vaccine calves could be due to the adjuvant contained in the vaccine, as none of the other vaccines contained an adjuvant. This is also noteworthy as the adjuvanted vaccine had the greatest PCR Ct value suggestive of less total BVDV administered to the calves, but it is unknown if components of the vaccine interfered with the results obtained by PCR.

Peripheral circulating lymphocytes are constantly recirculating in the blood after their reentry from lymphoid tissue to ensure a full repertoire of antigen specificities is represented. Naïve lymphocytes are able to circulate through secondary lymphoid tissues since they have not been exposed to antigen they recognize. After entering secondary lymphoid tissues and exposure to antigen they recognize, naïve lymphocytes can differentiate and contribute to the adaptive immune response. This trafficking of lymphocytes to lymphoid tissues is a coordinated and deliberate process and may, in part, be responsible for the decline in peripheral circulating lymphocytes observed after exposure to antigen. In addition to lymphocytes trafficking to lymphoid tissues, viruses can also infect lymphocytes leading to apoptosis which can also contribute to a decline in circulating lymphocytes. Both trafficking of lymphocytes and apoptosis can cause a temporary decrease in circulating lymphocytes, but determining if the decline in lymphocytes is associated with a contribution to a robust response or immune suppression is important. Therefore, understanding the cause of the decline in circulating lymphocytes or lymphocyte subpopulations is important to determine if the decline in the lymphocyte population will result in a beneficial or detrimental effect on the immune response. Furthermore, the repopulation or rebound of specific lymphocyte populations in the periphery should also be considered as it relates to efficacy of antigen specific responses. This can be observed in the rebound of CD4⁺ lymphocytes numbers after antigen exposure in the current study, specifically in the ddGM and adjuvanted vaccine calves.

Besides circulating lymphocytes, another site of lymphoid depletion occurs in lymphoid organs such as the thymus. Depletion of thymus tissue in BVDV infected animals correlates with virulence of the virus [6,8,18]. While peripheral decline in circulating lymphocytes has been previously reported to be associated with depletion of the thymus [6], data from this study demonstrates that these events are positively correlated. This is seen in the response of calves to adjuvanted vaccine, as no difference was observed in thymus gross weight or cortex:medulla ratio in this group of calves as compared to control calves, despite the decline in the peripheral blood lymphocyte population. However, significant depletion of the thymus both grossly and histologically was observed for the ncp vaccine and ncp exposed calves which also had a significant decline in circulating lymphocytes. The ddGM vaccine calves exhibited moderate signs of lymphoid depletion, compared to ncp exposed and ncp vaccine, as all induced a decline in circulating lymphocytes, but lymphoid depletion of the thymus was less in the ddGM vaccine calves. The decline in the peripheral blood lymphocyte population could be more of a consequence of trafficking to the lymphoid tissue in adjuvanted vaccine calves as demonstrated by the decline in the periphery, and the lack of thymus depletion. A more detrimental immunological impact may be observed in the ncp exposed and ncp vaccine calves as observed by both peripheral lymphocyte decline and thymus depletion. It is generally considered that the thymus does not regenerate, but regeneration of the thymus has been suggested under the influence of growth hormone [19–21], therefore, it is unknown to what extent thymic depletion may be tolerated in animals that have not reach maturity and are accumulating muscle mass.

Results from this study would suggest that different characteristics of the BVDV within the vaccines used in the current study can

alter immunological measures. Therefore, selection of vaccines for different classification of cattle such as stressed, immune compromised, age, pregnancy status, etc., should consider the collective data rather than focusing on one or two variables. These data highlight the importance and the need for BVDV antigens to be presented in multiple platforms to provide the industry with vaccine choices that can be tailored to the unique needs of each respective cattle operation. Mounting an immune response comes at a physiological cost. When the physiological cost of the protective immune response is less than the physiological cost of contracting the disease, there is a benefit to the animal. However, a more robust immune response with a broader, longer response may come with a higher physiological cost. The key is to effectively manage or strategically induce responses when animals can expend the physiological cost with minimal impact on general health and productivity. Different life stages and environmental stressors leave animals with varying levels of physiological reserves that can be expended in order to mount an effective immune response, therefore impacting long term physiological and immunological consequences.

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Author contributions

Conceived and designed the experiment: SMF, JFR, EG, CG.

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Analyzed the data: SMF, MVP, EC, EG, TJK, CG.

Contributed reagents/materials/analysis tools: JR, JDN, JFR, MVP, EG, TJK, CG.

Wrote the paper: SMF.

Reviewed the paper: RPD, MVP, SS, JR, EG, TJK, CG, JDN, JFR.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [The authors have the following interests: this work received financial support from Boehringer Ingelheim Inc., Saint Joseph, MO, USA. The authors EG, TJK, and CG were employees of Boehringer Ingelheim Inc. at time of study performance. Two of the vaccines utilized in this research are products developed and marketed by Boehringer Ingelheim Inc.].

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