Association of Serum Antibody Levels Following Vaccination with A Modified Live BVDV Vaccine and Protection from Clinical Disease upon Challenge

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Abstract

Two studies were conducted to examine the range of virus neutralizing serum antibody (VNSA) response after vaccination with a modified-live viral vaccine in cattle and the level of protection elicited when subsequently challenged with a highly virulent type 2 BVDV. Study 1 examined responses in colostrum deprived (CD) calves with no passively acquired antibodies whereas Study 2 examined responses in conventionally raised (CR) calves which had varying levels of passive antibodies prior to vaccination. Study 1 used CD calves averaging 120 days of age. For Study 2, calves averaged 130 days of age and were stratified into CR-Low, Mid and High response groups based response to vaccination. Grouping was based on standard deviations from the mean value obtained from ELISA assay and reconfirmed by virus neutralization. Twelve calves, from each Rep in Study 2, were selected to represent low, mid and high response groups (n = 4 calves per group). The VNSA values for the three groups were as follows; CR-Low (titer < 1:4), CR-Mid (titer 1:4 to 1:16) and CR-High (titer > 1:16). Calves were challenged with a high virulence BVDV2 strain. Samples were collected for both studies on days -2, 2, 4, 6, 9, 11 and 13 post challenge to determine levels of circulating white blood cells, virus isolation and levels of BVDV VNSA. While, overt respiratory or enteric disease was not observed in any of the vaccinated calves, clinical symptoms were more likely to be observed in calves with lower BVDV VNSA levels following the BVDV challenge demonstrating significant association between VNSA levels and clinical protection. However, the extent of clinical symptoms including decreases in WBC and pyrexia, were more severe in the non-vaccinated animals which indicates vaccination did provide a level of protection.

Keywords: Bovine Viral Diarrhea Virus; Vaccination; Serology

Abbreviations

IBR: Bovine Herpesvirus Type-1;
Introduction

Bovine respiratory disease complex (BRDC) is a major burden to the beef industry. The level of difficulty in control is evidenced by the incidence of disease remaining steady over the past 10 years despite vaccination and other control measures as reported by the National Animal Health Monitoring Service [1]. A common agent included in many of the commercially available vaccines to control viral pathogens associated with the BRDC is bovine viral diarrhea virus (BVDV). Vaccines containing BVDV have been available since 1967 [2] and currently there are over 160 BVDV modified-live vaccines (MLV) and killed vaccines commercially available in the United States [3]. Although vaccines have been in use for over 40 years, losses due to BVDV remain significant.

While vaccination confers a level of herd protection it is not effective at eliciting protection for each individual animal [4]. One potential reason for lack of vaccine efficacy is the presence and variability of maternal antibodies (MAb) at the time of vaccination. The interference between MAb and production of a humoral response to infection or vaccination with modified live or killed vaccination has been previously demonstrated [5]. Therefore, varying levels of MAb at the time of vaccination could reduce efficacy of the vaccine and induce increased variability in response to vaccination, predisposing calves to be more susceptible to infection. Humoral antibodies are widely used to measure the efficiency of vaccines as a correlate to protection. While the absolute level of Ab required to prevent disease is unknown, it has been demonstrated that passively acquired immunity with Ab titers of 1:16 would protect animals against BVDV clinical disease but not viral shedding [6]. Therefore, there is a need to understand if vaccination in the face of MAb impact the level of protection conferred when calves are challenged later in life due to the variation in the humoral Ab response.

The objectives of these studies were to determine the range of humoral response after vaccination with a modified-live vaccine for cattle managed in a conventional beef operation and in age matched colostrum deprived (CD) calves. In addition, the level of protection associated with different humoral response levels was evaluated based on challenge with a high virulent strain (BVDV2-1373) of BVDV. To this end, CD calves (no Ab) managed under controlled settings and conventionally raised (CR) categorized into low, mid and high antibody titer response groups following vaccination were used. Both CD and CR vaccinated calves were subsequently exposed to a high virulence BVDV. Clinical presentation (pyrexia), viremia and immunosuppression (leukopenia) were compared in all cattle following the challenge.

Materials and Methods

Handling and treatment of all cattle utilized for this study complied with the Animal Welfare Act as amended (7 USC, 2131-2156).

Studies were conducted to evaluate the response to vaccination in CD calves not allowed colostrum, and CR calves that were left with their dams and allowed colostrum. The purpose of the CD calf study was to determine vaccination response in the complete absence of maternal antibodies. The responses in the CD calves were compared to the responses in typical production CR calves that can have variability in the timing, quality and amount of colostrum they receive impacting levels of circulating MAb present at vaccination. The CD calves provided a clear response to the vaccination without interference from passive immunity while the CR calf study reflected vaccination response under conventional production practices. The same commercially available 5-way MLV vaccine was used for both studies.

Study #1 (CD calves)

Eight CD Holstein bull calves were procured at approximately 120 days of age and allowed to acclimate to the facility for approximately 1 week. Calves were group housed based on experimental group; CD non-vaccinated/non-challenged (CD-NN; n=2), CD non-vaccinated/challenge (CD-NC; n=2) and CD vaccinated/challenged (CD-VC; n=4). Vaccinated calves were given 2 ml of a 5-way commercially available MLV containing strains of BVDV1, BVDV2, bovine herpesvirus type-1 (IBR), parainfluenzavirus3 (PI3) and bovine respiratory syncytial vi-
Praisals for signs of clinical illness were monitored each day and body temperatures were recorded daily beginning at -2 days post-exposure. Clinical illness scores were based on the University of Wisconsin-Madison calf health scoring criteria for calves [8].

Samples were collected on days -2, 0, 2, 4, 6, 9, 11 and 13 post-inoculation in both studies for determination of circulating white blood cell (WBC) counts, VNSA titers and VI. Blood samples for WBC counts were collected in buffered sodium citrate and WBC counts were determined using an HV 1500 cytometer (CDC Technologies, Inc., Oxford, CT) following the manufacturer’s directions. Results from days -2 and 0 for WBC counts were used to generate baseline values.

Serum samples were collected in serum separation tubes with gel and clot activator. Titers for VNSA were determined, using BVDV2-296c and BVDV1-Singer strain as described previously [7].

Nasal swabs and buffy coat samples were collected on the same days and used for VI. For VI from buffy coat, blood was collected in sodium heparin tubes and BC was separated by centrifugation (800 x g, 20 min) and put through one freeze-thaw cycle (-20 °C/25 °C). A 250 µl aliquot of BC freeze/thaw lysate was mixed with 250 µl media. The resulting mixture was used to inoculate a 10 cm2, 60-70% confluent, flask of Madin Darby bovine kidney (MDBK) cells. After rocking at 37 °C for 1 h, the inoculum was removed from the cells and replaced with 3 ml of cell culture media. Four days later, the cell culture (including media) was frozen at -80 °C. Upon thawing to 25 °C, 500 µl of the resulting lysate was added to a fresh 10 cm2 flask of MDBK cells. Flasks were rocked for 1 h at 37 °C and 3 ml of cell culture media was added. After incubating for 4 days, total ribonucleic acid (RNA) was extracted from the culture and tested for BVDV as described [9].

Nasal swabs were collected via nylon flocked swabs by swiping the mucosal surface. Swabs were placed in micro tubes and stored at -20 °C. For reconstitution 1 ml of cell culture media was added to the tube with the swab, vortexed for 10-15 s then allowed to sit for 1 h prior centrifuging at 800 x g for 5 min. Of the resulting supernatant 500 µl was used to inoculate a 10 cm2, 60-70% confluent, flask of MDBK cells. Flasks were treated in the same manner from this point forward as described in the previous paragraph for BC description of VI.

Study #2 (CR calves)

Two replicates of this study, Rep 1 and Rep 2, were conducted using fall and spring born calves raised under typical production practices. Calves in Rep 1 (216 spring born beef calves, 177.03 ± 30.05 kg body weight), were given an initial vaccination with the same commercially available 5-way MLV at an average age of 150 days. Calves in Rep 2 (120 fall born beef calves, 141.06 ± 29.43 kg BW), were given the initial vaccination, with the same vaccine, at an average age of 107 days. A booster vaccination was administered with the same product 20 days after the initial vaccination in both replicates.

A serum sample was collected to characterize the response to vaccination 20 days after booster vaccination (40 days after initial vaccination). For initial screening level of serum antibodies against BVDV was initially evaluated using a commercial ELISA (BioX ELISA; BioX Diagnostics, Belgium, Europe) for preliminary classification into vaccination response groups. Criteria for high and low titer response were values one standard deviation above and below the mean by replicate, respectively. Criteria for the mid response group comprised levels within one standard deviation of the mean by replicate. Sera from selected animals in each response group were tested by virus neutralization, as described previously [7] using reference strains BVDV2-296c and BVDV1-Singer, to determine the titer of VNSA present. Serial log2 base dilutions were performed from 1:2 to 1:256. Samples were considered positive with VNSA titer ≥1:2 and expressed as geometric mean titer (GMT) with dilution of 1:256. Samples were collected on days -2, 0, 2, 4, 6, 9, 11 and 13 post-inoculation in both studies for determination of circulating white blood cell (WBC) counts, VNSA titers and VI. Blood samples for WBC counts were collected in buffered sodium citrate and WBC counts were determined using an HV 1500 cytometer (CDC Technologies, Inc., Oxford, CT) following the manufacturer’s directions. Results from days -2 and 0 for WBC counts were used to generate baseline values.

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Conventionally raised (CR) calves, used in this study, were transported to the containment barn approximately 1 week prior to the start of the study to acclimate to the facility. All CR calves, regardless of vaccination response, were group housed during the course of the study.

Challenge studies and sampling

Prior to the start of both studies all calves were tested negative for BVDV in buffy coat (BC) and ear notches samples by virus isolation (VI) and enzyme-linked immunosorbent assay (ELISA; Herdcheck BVD-PI, IDEXX Laboratories Inc., Westbrook, Maine), respectively. All calves were inoculated with 5 ml of BVDV2-1373 inoculum [2.5 ml / nostril; titer of 1 X 106 ml-1 tissue culture infectious dose (TCID)] intranasally. Visual appraisals for signs of clinical illness were monitored each day and body temperatures were recorded daily beginning at -2 days post-exposure. Clinical illness scores were based on the University of Wisconsin-Madison calf health scoring criteria for calves [8].

The virulent strain BVDV2-1373, isolated from a severe acute BVDV outbreak in Ontario, Canada [10] was used as the challenge strain in both studies. The virus was determined non-cytopathic by amplification characteristics in culture epithelial cells [11] and no evidence of cleavage of the viral protein, NS2/3 [12]. This strain reproducibly causes severe acute dis-
ease in calves seronegative to BVDV [13]. Viral stocks were propagated as previously described [14] with the exception that the MDBK cell line was used rather than bovine turbinate cells. Fetal bovine serum used to supplement the culture medium was tested free of BVDV antigen and antibodies [15].

Statistical analysis

Mean WBC measures were compared between antibody response groups using GLM model in SAS (SAS Institute Inc., Cary NC; v 9.2). Means were separated and tested using Fishers least significance difference test and differences were considered significant for p values <0.05. The correlation procedure (PROC CORR) in SAS was utilized to test correlations between variables of interest and significance was assumed at the p <0.05 level.

Because no significant differences were detected between Rep 1 and 2 in Study 2 for all measurements in the CR calves, Reps 1 and 2 results were combined.

Results

Titers prior to challenge

Only BVDV Type 2 titers will be reported since the challenge was a Type 2 BVDV and the same trends were observed for Type 1 BVDV titers. On d -2 prior to challenge non-vaccinated CD calves (CD-NN and CD-NC treatment groups) had no detectable VNSA titer to Type 2 BVDV (Figure 1). The CD vaccinated calves (CD-VC) had a titer as measured by virus neutralization at 7.8 (log base 2 scale; Figure 1).

Contrasts between the 2 studies would highlight that while all vaccinated calves, regardless of CD or CR groups, did have detectable titers; calves in the CD-VC group did have the highest titer at 7.8 versus 5.65 in the CR-High group (Figure 1). This would suggest that MAb interference could have played a role in the vaccination responses observed in the CR calves.

Post challenge measures

Post challenge measures consist of; pyrexia defined as a 1°C above baseline temperature, viral shedding, viremia, and immunosuppression (measured by a 20% decline in total circulating WBC). For calves in the CD-NN and CD-VC groups pyrexia was not detected, virus was not in nasal secretions or in BC samples (viremia) and total WBC counts fluctuated minimally. WBC counts did not drop below 20% of the baseline values (Table 1 and Figure 2). Conversely, calves in the CD-NC group pyrexia was observed, virus was isolated from both nasal secretions and BC samples. On day 2 post challenge a greater than 20% decline for total circulating WBC was detected (Table 1 and Figure 2). Pyrexia, viral shedding in nasal secretions, and viremia was sustained over the course of the study as well as a continual decline with no recovery for WBC with a 68% observed decline from baseline value on day 13 post challenge. Regardless of titer response group in the CR calves, all cattle exhibited pyrexia averaging 2.75, 5 and 3.8 days for the CR-High, Mid and Low groups respectively. Similarly, virus was isolated from both BC and nasal swab for calves in all response groups following challenge. There was no trend associated with vaccination response groups and day of viral shedding or viremia in the CR calves. Calves in the CR-High and Mid groups had a total circulating WBC decline of 21% at one sampling date, days 9 and 4, respectively. The CR-High calves returned to baseline values on day 11 and the CR-Mid returned to baseline values on day 6 post-challenge. In contrast, calves characterized as CR-Low had an initial decline for total circulating WBC of 36% on day 4 with sustained WBC suppression greater than 20% through day 11 (27%) returning to baseline values by day 13 post-challenge.

Contrasts between the two studies for post challenge measures revealed interesting differences between the CD-VC and the CR-Low, Mid and High groups (Table 1). While a positive sample for either viremia or viral shedding was detected on at least 1 day during the 13 day post challenge period for all CR calves, no positive samples were detected in the CD-VC calves. The differences in vaccination response between the 2 studies were also reflected in the percent decline from baseline values.
for WBC counts as the CD-VC calves had the least decline at only 15.78 percent compared to 36.27, 21.6 and 20.94 percent for the CR-Low, Mid and High groups, respectively (Figure 2). Calves in the CD-NN group which served as the control only had a decline of 12.11 percent over the course of the post challenge period.

<table>
<thead>
<tr>
<th>Response groups</th>
<th>CR-High</th>
<th>CR-Mid</th>
<th>CR-Low</th>
</tr>
</thead>
<tbody>
<tr>
<td># calves with pyrexia</td>
<td>4/7†</td>
<td>5/8</td>
<td>5/7†</td>
</tr>
<tr>
<td>Average days of pyrexia</td>
<td>2.75</td>
<td>5</td>
<td>3.8</td>
</tr>
<tr>
<td>Highest temperature</td>
<td>40.3</td>
<td>41.0</td>
<td>41.1</td>
</tr>
<tr>
<td>Viremia and shedding</td>
<td>8/8</td>
<td>8/8</td>
<td>8/8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CD-NN</th>
<th>CD-VC</th>
<th>CD-NC</th>
</tr>
</thead>
<tbody>
<tr>
<td># calves with pyrexia</td>
<td>0/2</td>
<td>0/4</td>
</tr>
<tr>
<td>Average days of pyrexia</td>
<td>0/2</td>
<td>0/4</td>
</tr>
<tr>
<td>Highest temperature</td>
<td>39.1</td>
<td>39.3</td>
</tr>
<tr>
<td>Viremia and shedding</td>
<td>n/a</td>
<td>0/4</td>
</tr>
</tbody>
</table>

*Pyrexia defined as 1 °C above average baseline temperatures
†No temperature data for 1 calf

**Table 1.** Temperature and viral shedding evaluation during the challenge period for colostrum deprived (CD) and conventionally raised (CR) calves.

**Anamnestic response post challenge**

The CD-NN had no detectable VNSA titers throughout the study period. Calves in the CD-NC group did have a titer of 3.7 13 days post-challenge compared to no detectable titer prior to the challenge and the CD-VC calves had a slight anamnestic response with the increase in titer from 7.8 on day -2 to 8.5 13 days after the challenge (Figure 3).

An anamnestic response was observed for all calves in the CR-Low, Mid and High titer response groups with all 3 groups having comparable titers 7.8, 8.48 and 8.1 respectively, on day 13 post challenge (Figure 3). The initial increase associated with the anamnestic response was first observed on day 6 with the majority of calves in the CR-Mid and High groups reaching peak titers by day 9 and calves in the CR-Low reaching similar peak titer on day 13 post challenge (Figure 3).

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No major differences for titers post challenge were observed between the two studies for the vaccinated calves in the CD or CR groups (Figure 1).

Discussion

Previous studies have evaluated protective immunity for cattle vaccinated against BVDV [16-18] as well as the effect of maternal antibodies on achieving passive immunity [17,18], and have concluded that circulating antibodies are protective. Maternal derived antibodies elicit passive immunity that serves as the first line of defense until a fully functional immune system is present, but circulating maternal antibodies can vary at the time of calfhood vaccination and contribute to the variability in response to vaccination.

In these studies we used two approaches using CR and CD calves to understand the level of protection elicited by vaccination. Regardless the level of VNSA in response to vaccination, calves that were vaccinated in either the CR or CD study experienced a shorter length of pyrexia, decreased number of days with viremia and viral shedding, and less of a WBC count decline compared to CD-NC calves. The greatest percent decline regardless of day post challenge was averaged for all vaccinated calves (CD and CR) and was 23.65% as compared to 72.51% for the CD-NC calves. This suggests that vaccination, in the face of or lacking circulating MAb at the time of vaccination, did decrease clinical disease and agree with studies comparing protected and unprotected calves [16,18].

While vaccination did reduce clinical presentation, it did not eliminate viral replication. Viremia and viral shedding was detected in all vaccinated CR calves, based on isolation from nasal swabs and BC samples, up to day 9 post challenge. There were no significant differences (p>0.05) noted for clinical presentation between titer response groups (Low, Mid and High), general trends for the low response group were a greater decrease in WBC (36.27% decline for the CR-Low compared to 21.6 and 20.94 for the CR-Mid and High groups, respectively), when compared to baseline values, and throughout the course of the experiment and highest body temperature was recorded for this group. Suggesting while Ab titers are in the protective range calves with a weaker vaccination response are more susceptible.

Cattle were initially categorized based on titer response to booster vaccination, but a trend observed was the onset of anamnestic response in the low response group with animals not reaching peak titer until later during the trial period. Cattle were subsequently re-classified based on the anamnestic response into groups that reached peak of VNSA level by day 6, 9, or 13 post-challenge (Table 2). Only cattle from study 2 were included in this analysis. In both Rep 1 and Rep 2, in study 2, the earliest anamnestic response group (day 6 compared to day 13) correlated with less of a decline in WBC. The average maximum percent decrease in circulating WBC for day 6, 9 and 13 was -22.9%, -38.2%, and -44.9%, respectively. The cattle that had higher antibody titers by day 6 had significantly less (p=0.005) WBC decline compared to calves with peak titers on day 9 and 13 (Table 2). While WBC decline in the vaccinated calves was not as drastic as decline observed in the CD-NC, vaccinated calves did have a decline in WBC due to the BVDV challenge and a correlation can be observed in WBC decline and peak titer day. Cattle in this study that had earlier anamnestic response (day 6) also demonstrated reduced severity of clinical signs based on the evaluated criteria (Table 2). While this is a broad snapshot of anamnestic responsiveness within this group of cattle, data from this study suggests potential relationships with humoral response profiles and severity of disease upon challenge.

<table>
<thead>
<tr>
<th>Anamnestic response groups</th>
<th>Number of calves per group</th>
<th>Max % Decrease in WBC</th>
<th>Day Max Decrease in WBC</th>
<th>Max Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rep 1†</td>
<td>Day 6 1</td>
<td>-15.9a</td>
<td>9.0</td>
<td>39.5</td>
</tr>
<tr>
<td></td>
<td>Day 9 7</td>
<td>-34.1a</td>
<td>6.4</td>
<td>40.0</td>
</tr>
<tr>
<td></td>
<td>Day 13 4</td>
<td>-40.2a</td>
<td>10.5</td>
<td>40.4</td>
</tr>
<tr>
<td>Rep 2‡</td>
<td>Day 6 4</td>
<td>-24.6a</td>
<td>12.5</td>
<td>39.1</td>
</tr>
<tr>
<td></td>
<td>Day 9 4</td>
<td>-45.4b</td>
<td>3.0</td>
<td>40.2</td>
</tr>
<tr>
<td></td>
<td>Day 13 4</td>
<td>-49.6b</td>
<td>4.5</td>
<td>40.2</td>
</tr>
<tr>
<td>Total§</td>
<td>Day 6 5</td>
<td>-22.9a</td>
<td>11.8</td>
<td>39.2</td>
</tr>
<tr>
<td></td>
<td>Day 9 11</td>
<td>-38.2b</td>
<td>5.2</td>
<td>40.0</td>
</tr>
<tr>
<td></td>
<td>Day 13 8</td>
<td>-44.9b</td>
<td>7.5</td>
<td>40.3</td>
</tr>
</tbody>
</table>

*Average day the maximum decrease was observed for WBC
†p=0.24
‡p=0.002
§All vaccinated cattle included from Rep 1 and Rep 2 (p=0.005)
ab Group means differ in each column for each replicate or total

Table 2. Groups redefined by day of anamnestic response following BVDV challenge.

Data from this study exhibits the variability and extremes or outliers in regard to titers that can be expected in response to vaccination. Understanding variation in response to vaccination, while broadly categorized in this study, is a step in characterizing the level of protection that can be expected to be conferred after vaccination.
Conflict of Interest Statement

The authors of this manuscript have no financial or other relationship with other people or organizations that may appropriately influence the work that has been reported in this manuscript.

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References


