Immune Response in Holstein Calves Vaccinated at Six Months of Age

Camila Costa Baccili¹, Raquel de Sousa Marques¹, Sylvia Marquart Fontes Novo¹, Bruno Toledo Silva¹, Edviges Maristela Pitúco² & Viviani Gomes¹

ABSTRACT

Background: Calves are agammaglobulinemic and immature at birth and their immunological defense must be improved by colostrum, although, maternal antibodies decrease after two months of age and calves are susceptible to Bovine Respiratory Disease Complex (BRDC). Then, this research evaluated the immune response to BVDV and BoHV-1 in young calves to prevent BRDC.

Materials, Methods & Results: Ten male Holstein calves were distributed in two groups with five animals on each: non-vaccinated VAC (-) and vaccinated VAC (+). Calves were vaccinated twice at 180 and 210 days of age. It was selected a commercial multivalent vaccine containing inactivated isolated of BVDV type-1 (strains 5960) and BVDV type-2 (strains 53637), and modified-live BoHV-1 (strains RLB103), with Quil A, cholesterol and Amphigen as adjuvant. The immune response (IR) was evaluated at 180 (T0), 210 (T1) and 240 (T2) days of life by serum neutralization (SN) and immunophenotyping. Specific antibodies to BVDV were detected in 40% (2/5) and 60% (3/5) of vaccinated calves at T1 and T2, respectively. Specific antibodies (Abs) to BoHV-1 were observed in 40% (2/5) at T1 and 100% (5/5) at T2 in the VAC (+) group. Titers of Abs to BoHV-1 increase from T0 to T2 (P = 0.034) in VAC (+). Moreover, geometric mean titer (GMT) to BoHV-1 in VAC (+) group was higher than VAC (-) after secondary IR in T2 (P = 0.006). The proportion of the markers T lymphocytes subpopulation (CD3+, CD4+, CD8+ and WC1+) and B lymphocytes (CD21+) were similar in the two experimental groups, while the expression of the CD25+ marker by gamma-delta (WC1+) was higher in VAC (+) at T1.

Discussion: Low titers of circulating Abs could be observed just for BoHV-1 in the calves from VAC (-) at T0, probably due to remaining Abs from dams transferred by colostrum intake. This fact indicates the need of appropriate vaccination schedules to prevent the virosis in dairy heifers. The low rate of seroconversion to BVDV and BoHV-1 at T1 is compatible with the pattern of the primary immune response, usually weak and slow. The greatest response at the time T2, after booster, was observed due to the presence of memory cells from first vaccination, responsible for the greater intensity and duration of Abs production. The specific immune response to BoHV-1 higher than BVDV could be attributed to the composition of the commercial vaccine that had live attenuated strain of BoHV-1, wich demonstrates greater intensity and duration than inactivated BVDV strain. We could not verify cellular immune response induced by the commercial selected vaccine. The labeling of the blood leukocytes held in this research, without in vitro antigen-stimulation, limited the assessment of IR caused strictly by the immunogen. Thus, environmental pathogens may have contributed to these results, impairing detection of differences between groups. Thus, calves vaccinated at six months old developed humoral immune response, especially for modified-live BoHV-1. In contrast, the animals presented lower intensity of response to the inactivated BVDV. Moreover, cellular immune response after vaccination was not detected under the conditions which this research was conducted.

Keywords: Bovine Herpesvirus type-1, Bovine Respiratory Disease Complex, Bovine Viral Diarrhea, cellular response, serum neutralization.
INTRODUCTION

Calves are susceptible to viral infections attributed to their immature immune system from birth up to eight months of age, and they also show a poor ability to develop a specific immune response [3,4]. Initially, neonates are protected by maternal antibodies (Abs) acquired by colostrum intake, however, these components decrease after two months of age, period of greatest susceptibility to Bovine Respiratory Disease Complex (BRDC) [21,29].

Viral infection is nearly always the primary infection insult, with subsequent infection by opportunistic bacteria exacerbating the resulting lung pathology. The BRDC viral agents most commonly include Bovine Herpesvirus-1 (BoHV-1), Bovine Viral Diarrhea Virus (BVDV), Bovine Respiratory Syncytial Virus (BRSV), Parainfluenza type 3 virus (PI-3V) and Bovine Respiratory Coronavirus (BRCV) [14].

Vaccination programs represent an important strategy to prevent virus infection, however, maternal antibodies can block the vaccine immune response in young calves. Abs titers could increase only with three months of age after vaccination to BoHV-1 [10,13,22,24].

In Brazil, vaccination programs do not allow the commercialization of vaccines containing modified-live BVDV virus, then international studies cannot be applied to Brazilian reality. There are some studies about vaccine response to viruses in Brazil [5,6,8,12], although, these researches did not evaluate younger calves.

We hypothesized that young calves could respond to commercial vaccine containing inactivated BVDV antigen and modified-live BoHV-1 at six month of the age. Therefore, this research aimed to evaluate the vaccine immune response to BVDV and BoHV-1 in young calves.

MATERIALS AND METHODS

Animals and blood samples

Ten newborn Holstein calves considered uninfected to BVDV were selected. This procedure was performed before colostrum intake, by serum neutralization test (SN) [27] and Real Time Polymerase Chain Reaction using blood leukocytes [26].

Calves received six liters of fresh colostrum from their dams and were kept isolated in pens up to 240 days of life. At this time, all the calves were seronegative to BVDV (10/10) and only two of them presented low antibody titers to BoHV-1 (8 and 16). The calves were randomly distributed in two experimental groups of five animals each: non-vaccinated VAC (-) and vaccinated VAC (+) calves.

The calves in the VAC (+) group were vaccinated by subcutaneous administration of two doses of the commercial vaccine (5 mL) at 180 and 210 days of life. The commercial multivalent vaccine contain inactivated isolated of BVDV type-1 (strains 5960) from Iowa (USA) and BVDV type-2 (strains 53637) from Ontario (USA), and modified-live BoHV-1 (strains RLB103), with Quil A, cholesterol and Amphigen as adjuvant.

Blood samples were collected in tubes containing ethylenediamine tetraacetic acid (EDTA) and without anticoagulants to obtain serum and titration of specific Abs.

The assessment were realized at 180 days of life (T0) - day of vaccination, at 210 days (T1) - booster moment, and at 240 days (T2) - 30 days after booster. At these moments were also obtained blood samples from the control group non-vaccinated.

Humoral responses - Measurement of specific antibodies

SN test for BVDV was performed using 96-well polystyrene plates; the serum was serially diluted in log₂ steps from 1:5 to 1:5120 by using minimal essential medium (MEM). The diluted samples were added in duplicate to the wells in the plate containing 50 µL stock solution containing 100 TCID₅₀ (50% tissue culture infective doses) of the NADL cytopathogenic strain of BVDV. The plates were then incubated for 1 h at 37°C with 5% CO₂. Subsequently, 50 µL Madin-Darby bovine kidney (MDBK) cell suspension was added to each well at a concentration of 3×10⁶ cells/mL. The plates were again incubated at 37°C with 5% CO₂ for 4 to 5 days.

Neutralizing Abs to BoHV-1 were detected using serum samples serially diluted in log₂ steps from 1:2 to 1:1024 by using MEM containing 1% antibiotics. The diluted samples were added in duplicate to each well containing 50 µL 100 TCID₅₀ of Hbv-1 strain of BoHV-1; the plates were incubated for 24 h at 37°C with 5% CO₂. Subsequently, 100 µL of the MDBK suspension at a concentration of 0.3×10⁶ cells/mL was added to each well, and the plates were further incubated for 4 to 5 days under the same conditions as mentioned before.
Infectivity was measured based on the cytopathic effect (CPE) visible in the cell monolayer on the plates by using an inverted microscope. The antibody titer was expressed as the highest dilution of the serum that completely inhibited the infectivity and therefore the CPE in both the wells of each dilution.

Cellular responses - Phenotyping of lymphocyte subsets

Proportions of blood lymphocytes subsets and their activation (CD25+) were determined using flow cytometry technique.

Blood obtained with EDTA (100 µL) was transferred for flow cytometry tubes and the red blood cells were lysed by adding FACSLyse solution. The phenotype of the cells was evaluated using monoclonal Abs (Table 1). The concentration of 1 µg/mL of these primary antibodies was carried out in Phosphate Buffered (PBS) and the cells were stained for 30 min at 4°C. Cells were washed three times with PBS, and then labeled with the secondary antibodies Fluorescein Isothiocyanate, Phycoerythrin and Peridinin-Chlorophyll Proteins to a final concentration of 1 and 10 µg/mL, respectively, at 4°C during 30 min in the dark. Cells were washed three times and suspended in 0.5 mL of PBS.

Data from fifty thousand events were acquired per sample (CellQuest Pro) using a flow cytometer (FACSCalibur). For all analysis (FlowJo software) lymphocytes were gated based on their forward and scatter characteristics on density plots. Immunofluorescence was expressed as the percentage of positive cells less the negative controls.

Table 1. Primary antibodies used to identify mononuclear cells.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Clone</th>
<th>Isotype</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>MM1A</td>
<td>IgG1</td>
<td>TL</td>
</tr>
<tr>
<td>CD4</td>
<td>IL-A11</td>
<td>IgM</td>
<td>T Helper</td>
</tr>
<tr>
<td>CD8</td>
<td>BAQ111A</td>
<td>IgM</td>
<td>T cytotoxic</td>
</tr>
<tr>
<td>WC1</td>
<td>B7A1</td>
<td>IgM</td>
<td>T gama-delta</td>
</tr>
<tr>
<td>CD21</td>
<td>GB25A</td>
<td>IgG1</td>
<td>BL</td>
</tr>
<tr>
<td>CD25</td>
<td>CACT108A</td>
<td>IgG2a</td>
<td>High affinity</td>
</tr>
</tbody>
</table>

*T: T lymphocytes; BL: B lymphocytes.

Statistical analysis

Statistical analysis was performed using SPSS 20.0 program. Firstly, all variables were tested using the Kolmogorov-Smirnov test, opting for non-parametric tests due to abnormal distribution of some variables and the small number of samples in the experimental groups.

Qualitative and quantitative analyzes of antibody titers were made due to the large number of negative results for BVDV in the SN. The cutoff values used to determine positive results for BVDV and BoHV-1 were 10 and 2, respectively. The comparison between the frequencies of results across experimental groups was performed by Chi-square test.

Antibody titers were transformed to log2, then the arithmetic means were calculated to compare the differences between groups by Mann-Whitney U test. Finally, the geometric mean titers could be expressed by GMT = 2a; where “a” is the arithmetic mean of the log2 antibody titers [31].

The relative values (%) of lymphocyte subpopulations between groups were also evaluated with the U test of Mann-Whitney. The differences were considered significantly when P < 0.05.

RESULTS

Neutralizing Abs titers to BVDV were not detected in VAC (-) at 180 days (T0) up to 240 days of age (T2), except for a calf that showed titer 10 at T1 (210 days). In the vaccinated group (VAC +), seroconversion to BVDV was observed in 40% of the calves (2/5) at T1 and 60% (3/5) at T2 (Table 2). At this point, the observed variation range was between 20 to 40 at T1 and 10 to 1280 at T2 (Figure 1). The geometric mean titer (GMT) for the BVDV was not calculated due to the prevalence of negative results after vaccination. For BoHV-1 was possible to detect seroconversion of 40% (2/5) and 100% (5/5), such as the variation range of 0 to 1024 and 128 to 1024 at T1 and T2, respectively (Table 2 and Figure 1).

The comparison of the frequencies obtained between the groups revealed no differences for both viruses, although there was a trend (P = 0.083) in Chi-square test at T2 to BoHV-1 (Table 2). The GMT values to BoHV-1 observed for VAC (+) and VAC (-) groups were 0 and 11.3 at T0 (P = 0.906), 90.5 and 11.3 at T1 (P = 0.136), 675.6 and 4.0 at T2 (P = 0.006). The increase in the antibody titers to BoHV-1 for VAC (+) was verified by the Friedman and Wilcoxon test, showing differences in the values obtained between T0 and T2 (P = 0.034).
The median of relative values (%) of B lymphocytes (BL), T lymphocytes (TL) and their subpopulations CD4⁺, CD8⁺ and WC1⁺ are shown in Table 3.

The lymphocyte proportions found for VAC (-) group at T0, T1 and T2 were, 24, 23 and 23% of CD21⁺; 53, 55 and 48% of CD3⁺; 22, 29 and 24% of CD3⁺ CD4⁺; 11, 15 and 17% of CD3⁺ CD8⁺; 38, 39 and 20% of CD3⁺ WC1⁺ at T0, T1 and T2, respectively. The lymphocyte ratios did not show significant difference according to vaccination.

Activation of T cells was assessed by CD25⁺ marker expression, obtaining the following proportions: 10, 5 and 12% of CD4⁺ CD25⁺; 9, 2 and 6% of CD8⁺ CD25⁺; 7, 1 and 7% of WC1⁺CD25⁺ from T0 to T2, in the VAC (-) group, respectively; 15, 15 and 16% of CD4⁺ CD25⁺; 4, 7 and 5% of CD8⁺ CD25⁺; 24, 20 and 12% of WC1⁺CD25⁺ from T0 to T2, in the VAC (+) group, respectively. The expression of CD25⁺ marker by gamma-delta (WC1⁺) was higher in the group of vaccinated animals (20%) compared to the non-vaccinated group (1%) (P = 0.002).

Table 3. Medians of relative values (%) of lymphocytes subsets from blood of non-vaccinated VAC (-) and vaccinated VAC (+) calves.

<table>
<thead>
<tr>
<th>Cell Marker</th>
<th>Time</th>
<th>VAC (-)</th>
<th>VAC (+)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD21⁺</td>
<td>T0</td>
<td>24</td>
<td>23</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>23</td>
<td>35</td>
<td>0.63</td>
</tr>
<tr>
<td>TL (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3⁺</td>
<td>T0</td>
<td>53</td>
<td>53</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>44</td>
<td>48</td>
<td>0.84</td>
</tr>
<tr>
<td>CD3⁺CD4⁺</td>
<td>T0</td>
<td>27</td>
<td>31</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>32</td>
<td>24</td>
<td>0.31</td>
</tr>
<tr>
<td>CD3⁺CD8⁺</td>
<td>T0</td>
<td>20</td>
<td>19</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>12</td>
<td>17</td>
<td>0.90</td>
</tr>
<tr>
<td>CD3⁺WC1⁺</td>
<td>T0</td>
<td>30</td>
<td>35</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>39</td>
<td>20</td>
<td>0.19</td>
</tr>
<tr>
<td>CD4⁺CD25⁺</td>
<td>T0</td>
<td>10</td>
<td>15</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>12</td>
<td>16</td>
<td>0.31</td>
</tr>
<tr>
<td>CD8⁺CD25⁺</td>
<td>T0</td>
<td>9</td>
<td>4</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>6</td>
<td>5</td>
<td>0.90</td>
</tr>
<tr>
<td>WC1⁺CD25⁺</td>
<td>T0</td>
<td>7</td>
<td>24</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>7</td>
<td>12</td>
<td>0.41</td>
</tr>
</tbody>
</table>

*Values of gamma-delta lymphocyte were higher in the animals of VAC (+) group at T1.
DISCUSSION

This research evaluated the vaccine response in young calves at six month of age using vaccine containing killed BVDV and modified-live BoHV-1 strains.

At the beginning of this study low titers of circulating Abs could be observed just for BoHV-1 in the calves from control group (VAC-). Probably, it represents the remaining Abs from dams transferred by colostrum intake, although levels of Abs lower than 32 to BoHV-1 and 16 to BVDV represent a risk factor to Bovine Respiratory Disease at this period of life [15,16,22]. This fact indicates the need of appropriate vaccination schedules to prevent the virosis in dairy heifers [20].

The low rate of seroconversion to BVDV and BoHV-1 at T1 is compatible with the pattern of the primary immune response, usually weak and slow. After development of the secondary immune response, seroconversion was observed in 60% and 100% of calves to BVDV and BoHV-1, respectively. The greatest response at the time T2, after booster, was observed due to the presence of memory cells from first vaccination, responsible for the greater intensity and duration of Abs production. In addition, there is a predominance of immunoglobulin G in secondary immune response, and this immune component has higher neutralizing activity than immunoglobulin M found in the primary immune response [32].

The specific immune response to BoHV-1 was higher compared to BVDV, considering the frequency of seroconversion and titers of neutralizing Abs obtained. This fact can be attributed to the composition of the commercial vaccine that had live attenuated strain of BoHV-1, which demonstrates greater intensity and duration of response compared to the inactivated immunogens of BVDV [1,2,17].

The major difference in the mechanism of immune response induced by modified-live and inactivated antigen, is the inability of this second replicate in the host cells and display cell-mediated immune response by cytotoxic T lymphocytes (CD8) [23]. In contrast, inactivated antigens could induce predominantly humoral response due to the recruitment of B lymphocytes which differentiate themselves into plasma cells to secrete neutralizing antibodies. They are responsible for combating the infection agent from natural exposure after vaccination. The limitations related to the cytotoxic T cells activation require the inclusion of large amounts of antigen associated with immune response potentiators, included in the adjuvants [11].

The vaccine selected in this research contained a saponin known as Quillaja Saponaria Molina (Quil A) associated with an immunostimulatory (Amphigen) and oily substance (Cholesterol). Immunostimulatory are very efficient since they are able to induce both two types of immune response, producing specific antibodies for the antigen (IgG1 and IgG2) as well as the response of T cells (Th1 and Th2). In addition, the type of emulsion helps to stabilize the concentration of components. Since it cannot be metabolized, the antigen maintains a slow release injected and remains in the tissue for a long period [7].

Some authors [19] evaluated four vaccines, which three of them were commercial and inactivated, and the other was a trial containing attenuated strains. The geometric mean titers of neutralizing antibodies for BVD-1 were higher in the serum of animals that received the experimental vaccine (GMT = 1612.7) compared to the other groups that received inactivated commercial vaccines (GMT = 14.3 - 40.0). Similar results were observed when specific antibodies to BVDV-2 were measured. Thus, could be noted higher intensity of humoral immune response for the attenuated vaccine.

In Brazil, commercialization of only inactivated BVDV vaccines is permitted to ensure biosafety since live vaccines have the ability to promote fetal infection in pregnant cows and disease in immunosuppressed animals [18]. Vaccines for the Herpesvirus also have side effects such as immunosuppression and high virulence for calves and pregnant females [18].

To minimize the side-effects promoted by modified-live vaccine for BoHV-1, heat sensitive vaccine strains were selected to limit the period of their replication. The commercial vaccine selected for this research has BoHV-1 antigen that replicate at lower temperatures (30-33°C) than body (37°C), avoiding the development of systemic infections [25].

The vaccine immune response is generally assessed by measurement of neutralizing antibodies, however the cellular immune response also has protective effect [29]. The cellular immune response evaluation are based on cultures of antigen-stimulated peripheral blood mononuclear cells, however present high complexity and requires skilled labor [9]. Recently, flow cytometry technique has been used
to assess cell activation after immunization by enabling the expression of the CD25+ marker, which has high affinity for the alpha chain of the interleukin-2 receptor, expressed exclusively on the surface of T lymphocytes after antigen-specific recognition [9,28]. Following this aspect, this study evaluated the cellular immune response after vaccination by CD25+ marker expression.

We could not verify cellular immune response induced by the selected vaccine under the conditions that this survey was conducted. Higher proportions of the activation marker CD25+ could be observed just for gamma-delta T lymphocytes (WC1+) at T1.

A similar study was developed [23] with the same vaccine containing inactivated isolated of BVDV type-1 (strains 5960) from Iowa (USA) and BVDV type-2 (strains 53637) from Ontario (USA), and modified-live BoHV-1 (strains RLB103), with Quil A, cholesterol and Amphigen as adjuvant. These authors assessed the expression of CD25+ on cultures of antigen-stimulated peripheral blood mononuclear cells. The induction of memory CD8+ T cells response was not detected. The authors expected that the viral particle could be carried into the cytoplasm by the adjuvant [23].

The labeling of the blood leukocytes held in this research, without in vitro antigen-stimulation, limited the assessment of immune response caused strictly by the immunogen. Thus, environment pathogens may have contributed to these results, impairing detection of differences between experimental groups.

The measurement of neutralizing antibodies were an indicative of humoral immune response induction after vaccination, however, further studies assessing the cellular immune response through stimulation of immune cells in vitro or challenge infection of vaccinated animals is critical to detect the specific cellular immune response.

CONCLUSION

Calves vaccinated at six months old developed humoral immune response, especially for modified-live BoHV-1. In contrast, the animals presented lower intensity of response to the inactivated BVDV. Moreover, cellular immune response after vaccination was not detected under the conditions which this research was conducted.

MANUFACTURERS

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Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content of the paper.

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