PEER REVIEWED

Vaccination with a multivalent modified-live virus vaccine administered one year prior to challenge with bovine viral diarrhea virus type 1b and 2a in pregnant heifers

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Abstract

A total of 85 beef heifers were used to determine if vaccination with a modified-live virus (MLV) vaccine containing bovine viral diarrhea virus types 1 and 2 (BVDV1 and BVDV2) approximately 1 year prior to challenge with virulent BVDV1b and BVDV2a will protect against fetal infection. BVDV1 and BVDV2 seronegative heifers were vaccinated at approximately 15 months of age with a commercially available combination MLV vaccine containing BVDV1a and BVDV2a or placebo; heifers were artificially inseminated approximately 9 months after vaccination. The heifers were challenge-inoculated intranasally at 80 to 90 days of gestation with a virulent BVDV1b or BVDV2a virus (i.e., 368 and 374 days post-vaccination, respectively). Clinical signs of BVDV infection including serologic status, complete blood count, rectal temperature, and viremia were monitored for 14 days following challenge. After challenge, vaccinated heifers in the BVDV1b and BVDV2a challenge groups experienced significantly less leukopenia and viremia than control heifers. Fetuses were harvested approximately 70 days following challenge and tissues were collected for virus isolation. All heifers that received vaccine were protected from fetal infection following challenge with BVDV1b or BVDV2a, whereas 15 of 18 (83.3%) and 21 of 22 (95.5%) control heifers had infected BVDV1b and BVDV2a fetuses, respectively. In this study, a combination MLV vaccine containing the minimum immunizing dose of BVDV1a and BVDV2a, with other fractions at release dose or higher, administered 1 year prior to challenge protected against fetal infection in the face of a substantial challenge infection with either BVDV1b or BVDV2a.

Key words: BVDV, fetal infection, PI, vaccine

Résumé

Un total de 85 taures de boucherie ont été utilisées afin de déterminer si la vaccination avec un vaccin à virus vivants modifiés contenant le virus de la diarrhée virale bovine de type 1 et de type 2 (BVDV1 et BVDV2) approximativement 1 an avant une infection expérimentale avec des souches virulentes de BVDV1a et BVDV2a protégerait les fœtus de l'infection. Des taures séronégatives aux virus BVDV1a et BVDV2a ont été vaccinées vers l'âge de 15 mois approximativement soit avec un vaccin commercial combiné à virus vivants modifiés contenant les virus BVDV1a et BVDV2a ou soit avec un placébo. Les taures ont été inséminées artificiellement approximativement 9 mois suivant la vaccination. Une infection expérimentale de ces taures par voie nasale a pris place entre les jours 80 et 90 de gestation avec des souches virulentes de BVDV1a ou de BVDV2a (i.e. 368 ou 374 jours suivant la vaccination, respectivement). Les signes cliniques compatibles avec une infection au BVDV incluant le statut sérologique, l'hémogramme, la température rectale et la virémie, ont été suivis pendant 14 jours après l'infection expérimentale. Après l'infection expérimentale, la leucopénie était moins prévalente chez les taures des groupes vaccinés avec le vaccin à virus vivants modifiés et infectés expérimentalement avec le BVDV1a ou le BVDV2a que chez les taures du groupe témoin. Les fœtus ont été recueillis approximativement 70 jours suivant l'infection expérimentale et les tissus ont été examinés pour l'isolement viral en culture. Toutes les taures vaccinées étaient protégées contre l'infection fœtale suivant l'infection expérimentale avec

le BVDV1a ou le BVDV2a. Au contraire, des fœtus infectés par le BVDV1a ou le BVDV2a étaient respectivement présents chez 15 taures sur 18 (83.3%) et chez 21 taures sur 22 (95.5%) des groupes témoins. Dans cette étude, l'utilisation d'un vaccin combiné à virus vivants modifiés contenant la dose immunisante minimum de BVDV1a et de BVDV2a, avec d'autres fractions à la dose de libération ou plus élevée, administré 1 an avant l'infection expérimentale, a permis de protéger les fœtus de l'infection en dépit d'une infection expérimentale substantielle avec soit le BVDV1a ou soit le BVDV2a.

Introduction

Bovine viral diarrhea virus (BVDV) is one of the most complex and clinically significant diseases of cattle, affecting livestock worldwide. The disease is endemic in cattle populations throughout the world,[32] and has potential to cause devastating economic impact.[12,14,16,21,37,38] The virus can cause a wide range of clinical signs in adult animals and calves, including subclinical infections, bovine viral diarrhea, immunosuppression, repeat breeding problems, abortion and mummification, congenital defects, immunotolerance and persistent infection (PI), and acute and chronic mucosal disease.[1] Transmission of BVDV can occur vertically (i.e., from infected dam to fetus) or horizontally (i.e., via exposure to the virus in various excretions and secretions such as urine, nasal discharge, milk, and semen, and/or fomites such as milk bottles, balling guns, and re-used needles).[35] Fetuses infected in utero before approximately days 125 to 150 of gestation,[3-5,10,11,13-17,21,22,34] prior to becoming immunocompetent at approximately 150 days of gestation,[1] are born PI and will likely shed BVDV throughout their lives.[11,22] Persistently infected cattle pose a continuous health risk to other cattle, and are themselves also at risk of developing mucosal disease that has a mortality rate of nearly 100%.[11,13] Non-vaccinated pregnant cattle are susceptible to the reproductive effects of BVDV, and infection during pregnancy can result in abortion, congenital abnormalities, and birth of PI animals.[4,17,21,22]

BVDV is typically classified into two specific genotypes (types 1 and 2) that are further divided into multiple subgenotypes. Recent data suggests that types 1a, 1b, and 2a are the most prevalent in the United States.[17,29,30] Up to 14 type 1 and 4 different type 2 subgenotypes have been documented.[25] Of these subtypes, BVDV type 1b is predominant,[22,36] comprising 44%[29] to 75%[30] of recent BVDV cases in the US. BVDV type 1b has also shown to be prevalent in other countries including Italy,[18,30] Germany, Spain, Argentina, Chile, Peru, and Japan.[30] One US-based report[31] shows that of 25 PI cattle tested in 2006, all were infected with type 1b. Although some data are regional in nature, and differences in collection and/or testing methods exist, the high prevalence of type 1b is a significant threat.[22,31,36] In utero infection with BVDV type 1b is capable of causing fetal infection that may lead to the birth of PI calves,[17,18,31] therefore it is essential that current vaccines are capable of protecting from this prevalent subtype.

BVDV is further subdivided into the cytopathic and noncytopathic (most clinically relevant)[36,39] biotypes. Both types 1 and 2 contain cytopathic and noncytopathic biotypes;[13] however, only fetal infection with noncytopathic BVDV can cause calves to be born persistently infected.[1,6-8,11,13-15,17,22,27,32,34,36,37]

Vaccinating dams prior to potential fetal infection is an ideal and easily managed tool to provide protection against BVDV in utero. Vaccination with a modified-live virus (MLV) vaccine prior to breeding, or with a temperature-sensitive MLV vaccine during pregnancy,[1,2] can effectively control BVDV infection in utero. Several BVDV fetal protection studies using MLV vaccines have been reported.[3-5,10-15,21,22,25,32,36,37] Since many different protective options are available, and preventing dams from producing PI calves is vital, it is important for any vaccine to be proven effective in preventing fetal infection and subsequent production of PI calves.

The objectives of this study were to 1) prove a 12-month duration of immunity for prevention of fetal infection with BVDV types 1 and 2 in naive animals vaccinated once prior to breeding, and 2) to demonstrate the effectiveness of this vaccine as a prophylactic treatment against abortion caused by intranasal (IN) challenge with virulent BVDV1b and BVDV2a.

Materials and Methods

Animals

Protocols were reviewed and approved by the Rural Technologies, Incorporated Institutional Animal Care and Use Committee prior to study initiation. Eighty-five 7- to 9-month-old Angus-cross heifers were acquired for the study, with 45 heifers assigned to the BVDV1b challenge group, and 40 heifers assigned to the BVDV2a challenge group. The animals in each challenge group were then randomly assigned to 1 of 2 treatment groups, control or vaccinate. For the BVDV1b challenge, 22 heifers were vaccinates and received the test vaccine,[a] while 23 heifers were controls and received the placebo vaccine (test vaccine without viral fractions). For the BVDV2a challenge, 18 heifers were vaccinates and received the test vaccine, while 22 heifers were controls and received the placebo vaccine. For both challenge groups, control and vaccinates were separated at the time of vaccination to avoid any potential vaccine virus exposure for 29 (BVDV1b challenge group) or 32 days (BVDV2a challenge group), after which the control and vaccinate heifers for each challenge group were commingled for the remainder of the study. All heifers

were managed according to routine animal husbandry procedures and were isolated from other cattle. The BVDV1b and BVDV2a challenge groups were housed separately for the duration of the study.

Pre-vaccination Serologic Assays

Blood was collected from all heifers prior to vaccination. All heifers were seronegative for antibodies against BVDV1 and BVDV2, and were negative to BVDV by ear notch via immunohistochemical (IHC) testing.[b] Serum samples were tested to determine serum neutralizing antibody titers to BVDV1 (Singer)[c] and BVDV2a (A125)[c] by use of the constant virus decreasing serum assay. Briefly, 2-fold serial dilutions (range 1:2 to 1:256) of sera in duplicate were incubated with a constant viral titer (< 500 $TCID_{50}$) before inoculation of BVDV-free bovine turbinate cells[d] in microtiter tissue culture plates.[e] Plates were incubated at 98.6°F (37°C) with 5% CO_2 for 5 days before being evaluated for virus-induced cytopathic effect (CPE) for BVDV1 and for IHC staining[9] for BVDV2. The reciprocal of the last dilution that prevented CPE formation or virus-specific staining was designated the serum neutralizing antibody titer. Geometric mean values were calculated by use of $\log_2$ titers.

Vaccination

All heifers were vaccinated at approximately 15 months of age (day 0). A total of 40 vaccinate heifers, 22 in the BVDV1b challenge group and 18 in the BVDV2a challenge group, were vaccinated once subcutaneously (SC) with a commercially available MLV combination vaccine[a] containing BVDV1a (Singer strain) and BVDV2a (strain 296) at minimum immunizing dose (MID; the amount of antigen necessary to stimulate a protective immune response), as well as infectious bovine rhinotracheitis (IBR), parainfluenza 3 (PI3), bovine respiratory syncytial virus (BRSV), *Campylobacter fetus*, *Leptospira canicola*, *Leptospira grippotyphosa*, *Leptospira hardjo*, *Leptospira icterohemorrhagiae*, and *Leptospira pomona* at release dose or higher, according to manufacturer's recommendations. The 45 control heifers (23 and 22 heifers in the BVDV1b and BVDV2a challenge groups, respectively) were sham-vaccinated once SC with the inactivated bacterin components of the same vaccine without the viral antigens. Heifers were observed daily for 7 days after vaccination for vaccine-related adverse events.

Synchronization and Breeding of Heifers

Estrus cycles were synchronized approximately 9 months following vaccination by use of a vaginal implant[f] containing progesterone, and injections of gonadotrophin-releasing hormone and prostaglandin.[19,28] Briefly, heifers were administered prostaglandin intramuscularly (IM), followed by vaginal implant insertion and gonadorelin diacetate tetrahydrate[g] administration IM 3 days following prostaglandin injection. The implants were removed, estrus detection devices[h] were placed on the tailhead area to aid in estrus detection, and the heifers were administered dinoprost tromethamine[i] IM 6 days later. Heifers were artificially inseminated twice at approximately 60 and 72 hours after implant removal with semen that was BVDV and BHV-1 negative, as determined using a reverse transcription nested PCR (RT-nPCR) assay.[j,24] At initial breeding, an additional dose of gonadorelin diacetate tetrahydrate was administered IM if the heat detector was not activated or missing. A 2-year-old virgin bull vaccinated with BVDV and BHV-1 vaccine (at 4 and 7 months of age with an inactivated vaccine and at 12 months with a MLV vaccine) and negative to BVDV by ear notch testing (via enzyme-linked immunosorbent assay)[j] was used for pasture breeding with the heifers for 2 weeks following artificial insemination. Semen was tested and confirmed negative for BHV-1 and BVDV using RT-nPCR[j,24] prior to pasture breeding. Following breeding, heifers were ultrasounded transrectally prior to challenge to confirm pregnancy status.

Challenge Inoculation

The BVDV1b challenge group heifers (22 vaccinates and 23 controls) were challenged intranasally using an atomizer[k] with 5 mL of BVDV1b (BJ strain,[l] approx 3.7×10^5 $TCID_{50}$/mL) at 83 days of gestation (368 days post-vaccination [DPV]). Further analysis of the BJ strain has revealed that it is a type 1b (Ridpath JF, personal communication), rather than type 1a as previously reported.[4] The BVDV2a challenge group heifers (18 vaccinates and 22 controls) were challenged in the same manner with 5 mL of BVDV2a (PA131 strain,[j] approx 1.3×10^5 $TCID_{50}$/mL) at 90 days of gestation (374 DPV).

Post-challenge Observations

Clinical observations were performed daily by personnel blinded from treatment group assignment, beginning 2 days prior to challenge and continuing through day 14 after challenge. Each heifer was visually examined in the pen prior to handling and scored for clinical signs including anorexia, depression, increased respiration, coughing, nasal discharge, oral ulcers, ocular discharge, and fecal consistency. After the visual assessment, heifers were restrained in a standard cattle chute to measure body temperature using a rectal thermometer,[m] and were visually examined for mucosal ulcers in the oral cavity.

A total of 6 heifers in the BVDV1b challenge group (1 vaccinate and 5 control heifers) and 1 control heifer in the BVDV2a challenge group had fetal loss prior to fetal harvest. Fetal loss in the BVDV1b group occurred some time between challenge and harvest, and no fetuses

were recovered. A single fetus was recovered from the BVDV2a group heifer 1 day prior to the scheduled harvest, and tissues were collected and subsequently tested with the other samples in that group.

Serologic Testing

Blood was collected via jugular venipuncture from the heifers prior to vaccination, prior to challenge, 153 (BVDV1b challenge group) or 125 (BVDV2a challenge group) days following vaccination, at initiation of the synchronization program, at time of challenge, and at the time of fetal harvest. Serum neutralizing antibody titers against BVDV1a (Singer strain) and BVDV2a (A125) were determined by use of the constant virus decreasing serum assay, as described above.

Virus Isolation (Viremia) Testing

Blood was collected via jugular venipuncture from the heifers every other day starting 2 days prior to challenge (i.e., -2, 0, 2, 4, 6, 8, 10, 12, and 14 days post-challenge [DPC]). White blood cells (WBCs) were isolated according to a previously described technique.[9] The isolated WBCs were resuspended in 2 mL of media[n] supplemented with equine serum[o] and tested for BVDV using a modification of an isolation assay previously described.[33] Briefly, 1 10-fold dilution of each sample was made and each diluted sample was added in quadruplicate to BVDV-free bovine turbinate cell monolayers in microtiter tissue culture plates. Culture plates were incubated for 5 days at 98.6°F (37°C) with 5% CO_2. Following incubation, plates were freeze-thawed 3 times and the samples were passaged onto new cell monolayers and incubated for an additional 5 days. This process was repeated for a total of 3 passages before completing IHC staining for BVDV.[33] Samples were considered positive for BVDV if virus-specific staining was observed in inoculated cells.

Hematology Analyses

Blood was collected via jugular venipuncture from all heifers 9 times during the study period from 2 days prior to challenge and every other day through 14 days after challenge (i.e., -2, 0, 2, 4, 6, 8, 10, 12, and 14 DPC). Samples were subjected to hematologic analysis[p] by use of a cytometer. Total WBC and platelet counts were determined for each animal.

Fetal Harvest

Prior to harvest, heifers were palpated transrectally to determine pregnancy status, and a total of 78 heifers were confirmed pregnant. One pregnant heifer from the BVDV2a challenge group had an abscess that prevented acceptance at the packing plant, and was transported to South Dakota State University for necropsy and fetal harvest the day prior to scheduled harvest. The remaining 77 heifers were transported to a commercial packing plant[q] for harvest and fetus collection (BVDV1b and BVDV2a challenge groups were transported and harvested separately). The uteri, complete with cervixes, were collected on the packing plant floor, placed into individual containers, and transported to South Dakota State University[j] for necropsy and fetal tissue collection.

Fetal Tissue Collection and Testing

Samples were collected from the single (recovered) aborted fetus and all harvested fetuses and tested for the presence of BVDV. Heart blood was tested for neutralizing antibody titers against BVDV1 and BVDV2. Thymus, spleen, and brain (cerebellum) were each tested for BVDV by virus isolation.[r] Briefly, dilutions of processed samples were made and each diluted sample was added in quadruplicate to BVDV-free bovine turbinate cell monolayers in microtiter tissue culture plates. Culture plates were incubated for 3 to 4 days at 98.6°F (37°C) with 5% CO_2, followed by two additional passages incubated for 3 to 4 days each. Results were considered positive if BVDV virus-specific staining was observed in inoculated cells.

Statistical Analyses

The Fisher's Exact Test and prevented fractions were used to analyze the presence of BVDV1 or BVDV2 in the fetus, presence of leukopenia, presence of pyrexia, and presence of viremia. Numbers of days leukopenic, pyrexic, and viremic were analyzed with both the Wilcoxon's Rank Sum Test and mitigated fractions. Platelet counts were analyzed using both repeated measures analysis of covariance and mitigated fractions by day. An α-level of 0.05 was used to determine significant treatment group differences. All analyses were performed on data using SAS® System[s] or StatXact®.[t]

Serum neutralizing antibody titer data were analyzed by repeated measures using the Mixed procedure of SAS® as described by Littell et al.[23] All covariance structures were modeled in the initial analysis. The indicated best fit covariance structure, compound symmetry for post-vaccination data and Heterogenous Compound Symmetry for post-challenge data, was used for the final analysis. The model included the independent variables of treatment, time, and treatment x time.

Results

Clinical Observations

No clinical signs or adverse vaccine reactions were observed in any heifers following vaccination (data not shown). Following challenge, body temperature was measured rectally in all heifers every other day starting

2 days prior to challenge through 14 days after challenge, with the mean determined for each group. Generally, no statistical differences were found in mean pyrexia values between vaccinates and controls in either the BVDV1b or BVDV2a groups following challenge (P=0.3141 for BVDV1b and P=1.0 for BVDV2); however, rectal temperatures were significantly higher (P=0.0054) in the BVDV1b challenge group controls when compared to the vaccinates on day 4 post challenge. Mean rectal temperatures ranged between 102.6°F (39.2°C) and 103.3°F (39.6°C) for the BVDV1b challenge group controls, and between 102.2°F (39°C) and 103.2°F (39.6°C) for the vaccinates (data not shown). Mean rectal temperatures ranged between 102.4°F (39.1°C) and 103.7°F (39.8°C), and between 102.9°F (39.4°C) and 103.5°F (39.7°C) for the BVDV2a challenge group controls and vaccinates, respectively (data not shown). There were no elevated clinical scores (i.e., anorexia, depression, nasal discharge, or diarrhea) following challenge for either the BVDV1b or BVDV2a challenge groups (data not shown).

Serum Neutralizing Antibody Titers

None of the heifers in the BVDV1b (Figure 1) and BVDV2a (Figure 2) challenge groups had detectable neutralizing antibody (titers of 0) at the time of the first vaccination. Detectable serum neutralizing antibody activity was significantly higher (P<0.001) in vaccinated heifers in the BVDV1b challenge group ($9.64_{log2} \pm 0.13$ [~907] against BVDV1 and $6.86_{log2} \pm 0.13$ [~153] against BVDV2; Figure 1) and in vaccinated heifers in the BVDV2a challenge group ($9.56_{log2} \pm 0.11$ [~924] against BVDV1 and $6.44_{log2} \pm 0.11$ [~101] against BVDV2; Figure 2) than control heifers (no detectable neutralizing antibody [0] against both viruses for both challenge groups) on 153 (BVDV1b challenge group) and 125 (BVDV2a challenge group) DPV. At the time of initiation of estrus synchronization, detectable neutralizing antibody activity was significantly higher (P<0.001) in vaccinated heifers in the BVDV1b challenge group ($10_{log2} \pm 0.11$ [~1199] against BVDV1 and $6.32_{log2} \pm 0.13$ [~100] against BVDV2; Figure 1) and in the BVDV2a challenge

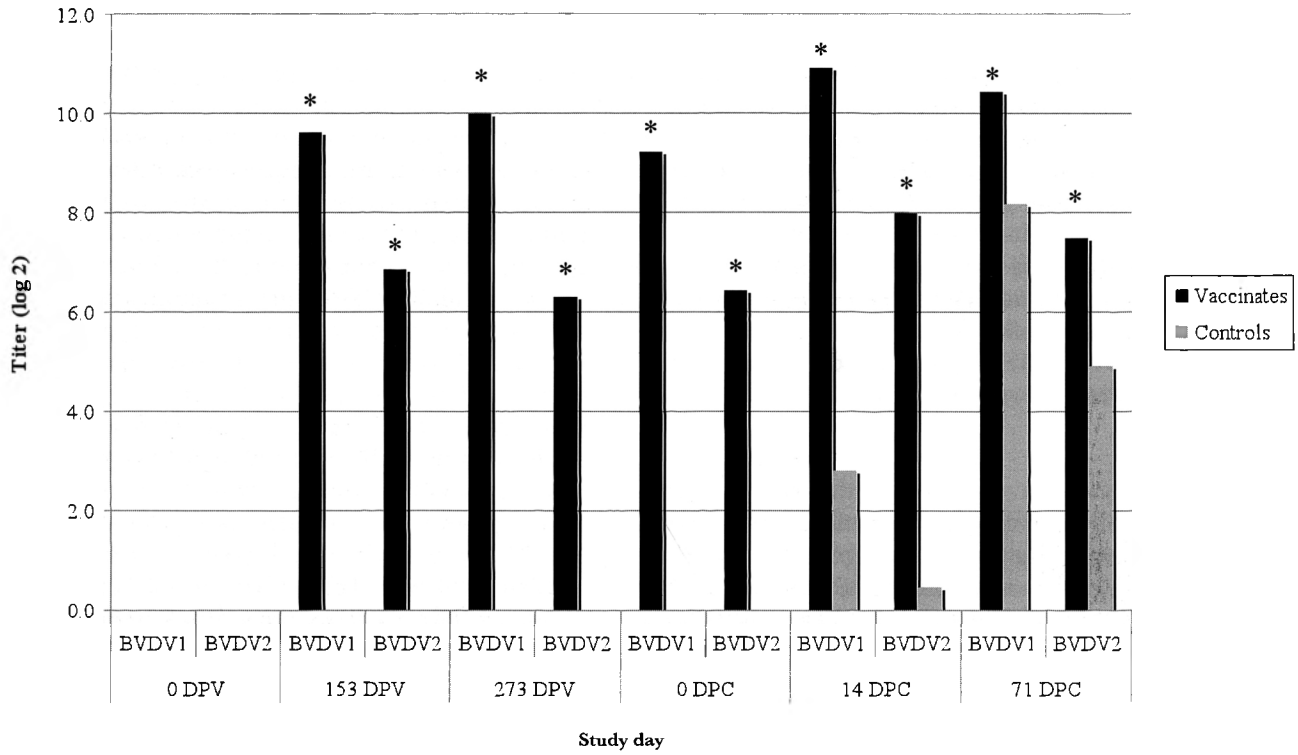

Figure 1. Mean BVDV1b challenge group serology results against BVDV1a (Singer strain) and BVDV2 before and after vaccination (0 DPV) and challenge (0 DPC) with virulent BVDV1b. The vaccinates (black columns; n = 21) consisted of seronegative heifers that received the test vaccine (i.e., multivalent vaccine containing BVDV1a and BVDV2 fractions). The controls (gray columns; n = 18) consisted of seronegative heifers that received placebo vaccine (i.e., multivalent test vaccine without BVDV fractions). Notice that vaccinated heifers had significantly higher (P<0.001) mean titers than control heifers on 153 DPV, 273 DPV, on the day of challenge (0 DPC), and 14 DPC (asterisks). Additionally, vaccinated heifers had significantly higher (P<0.001) mean titers than control heifers against BVDV1 and BVDV2 at the time of fetal harvest (71 DPC) (asterisks).

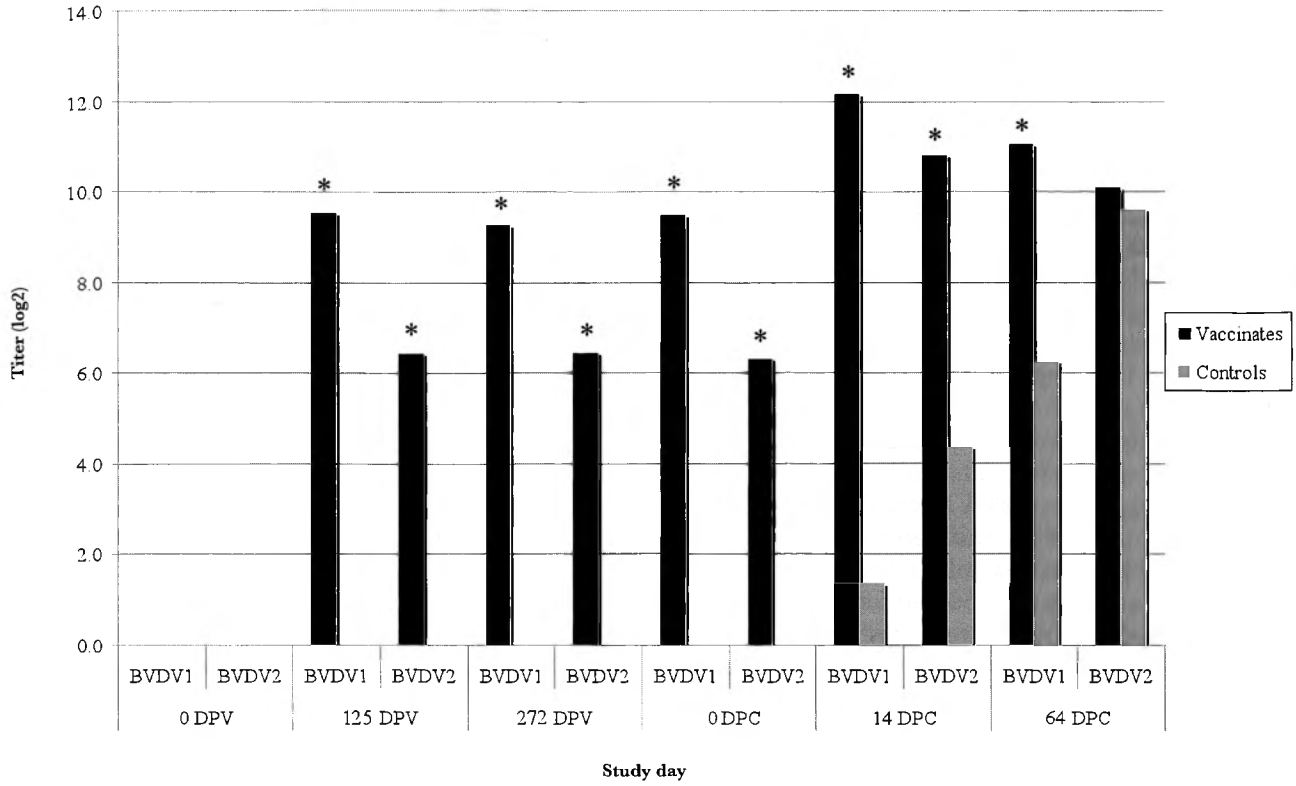

Figure 2. Mean BVDV2 challenge group serology results against BVDV1a (Singer strain) and BVDV2 before and after vaccination (0 DPV) and challenge (0 DPC) with virulent BVDV2. The vaccinates (black columns; n=18) consisted of seronegative heifers that received the test vaccine (i.e., multivalent vaccine containing BVDV1a and BVDV2 fractions). The controls (gray columns; n=22) consisted of seronegative heifers that received placebo vaccine (i.e., multivalent test vaccine without BVDV fractions). Notice that vaccinated heifers had significantly higher (P<0.001) mean titers than control heifers on 125 DPV, 272 DPV, on the day of challenge (0 DPC), and 14 DPC (asterisks). Additionally, vaccinated heifers had significantly higher (P<0.001) mean titers than control heifers against BVDV1 at the time of fetal harvest (64 DPC) (asterisks); however, titers were not significantly higher against BVDV2 (P=0.10).

group ($9.28_{log2} \pm 0.11$ [~697] against BVDV1 and $6.44_{log2} \pm 0.11$ [~96] against BVDV2; Figure 2) than control heifers (no detectable neutralizing antibody [0] against both viruses for each challenge group). On the day of challenge, vaccinated heifers in the BVDV1b challenge group (368 DPV) had a mean titer of $9.23_{log2} \pm 0.13$ (~734) against BVDV1 and $6.45_{log2} \pm 0.16$ (~112) against BVDV2 (Figure 1), and vaccinated heifers in the BVDV2a challenge group (374 DPV) had a mean titer of $9.5_{log2} \pm 0.13$ (~882) against BVDV1 and $6.33_{log2} \pm 0.13$ (~93) against BVDV2 (Figure 2). For both groups, antibody levels (i.e., titers) were significantly higher (P<0.001) than control heifers, which had no detectable neutralizing antibody (0) against both viruses for each challenge group. Vaccinated heifers in the BVDV1b challenge group had a mean titer of $10.91_{log2} \pm 0.24$ (~2048) against BVDV1 and $8.00_{log2} \pm 0.25$ (~383) against BVDV2 (Figure 1), and vaccinated heifers in the BVDV2a challenge group had a mean titer of $12.17_{log2} \pm 0.35$ (~6201) against BVDV1 and $10.83_{log2} \pm 0.40$ (~2876) against BVDV2 (Figure 2) at 14 DPC. These antibody levels (titers) were significantly higher (P<0.001) than the controls in the BVDV1b challenge group (mean titers of $2.83_{log2} \pm 0.23$ [~7.1] against BVDV1 and $0.48_{log2} \pm 0.24$ [~1.4]; Figure 1) and the controls in the BVDV2a challenge group (mean titers of $1.36_{log2} \pm 0.32$ [~2.6] against BVDV1b and $4.36_{log2} \pm 0.36$ [~20.5] against BVDV2; Figure 2). At the time of fetal harvest, 72 DPC for the BVDV1b challenge group and 65 DPC for the BVDV2a challenge group, vaccinated heifers in the BVDV1b challenge group had a mean titer of $10.45_{log2} \pm 0.16$ (~1512) against BVDV1 and $7.50_{log2} \pm 0.25$ (~124) against BVDV2 (Figure 1), and vaccinated heifers in the BVDV2a challenge group had mean titers of $11.06_{log2} \pm 0.21$ (~2844) against BVDV1 and $10.11_{log2} \pm 0.21$ (~1252) against BVDV2 (Figure 2). The control heifers in the BVDV1b challenge group had a mean antibody titer of $8.17_{log2} \pm 0.15$ (~345; P<0.001) against BVDV1 and $4.91_{log2} \pm 0.25$ (~30.1; P<0.001) against

BVDV2 (Figure 1), and the BVDV2a challenge group had a mean antibody titer of $6.27_{log2} \pm 0.19$ (~103; $P<0.001$) against BVDV1 and $9.64_{log2} \pm 0.19$ (~989; $P=0.10$) against BVDV2 (Figure 2).

Heifer Viremia Results

There were no viremic heifers in the BVDV1b challenge group vaccinates (0/22; 0.0%) following challenge; however, a total of 19/23 (82.6%) of controls were viremic from days 4 to 10 following challenge, with the greatest number of positive virus isolations occurring on 8 DPC ($P<0.0001$; Table 1); these numbers include heifers that experienced fetal loss. On days 6 and 8 post challenge, viremia results for controls were significantly higher ($P\leq0.0054$) than vaccinates. The calculated prevented fraction for viremia in the BVDV1b challenge group was 1.0 (100.0%) with exact 95% confidence limits of (0.831, 1.0). There was 1 positive viremia in the BVDV2a challenge group vaccinates (1/18; 5.6%) on a single day, while 20/22 (90.9%) of the controls were viremic ($P<0.0001$; Table 1). Viremias were detected in the BVDV2a challenge group from days 4 to 14 following challenge, with the highest number of virus isolations occurring from 16 animals on 6 DPC and 19 animals on 8 DPC (Table 1). On days 6, 8, and 10 post challenge, viremia results for controls were significantly higher ($P\leq0.0042$) than vaccinates. The calculated prevented fraction for viremia in the BVDV2a challenge group was 0.94 (94.0%) with exact 95% confidence limits of (0.716, 0.998).

Hematology Results

There was a statistical difference for leukopenia in the BVDV1b challenge group between vaccinates (8/22; 36.4%) and controls (21/23; 91.3%) ($P\leq0.0001$; Figure 3). This resulted in a calculated prevented fraction for leukopenia of 0.60 (60.0%) with exact 95% confidence limits of (0.322, 0.804). Initially (i.e., -2, 0, and 2 DPC), the vaccinates had lower mean WBC counts compared to the controls; however, on days 4 to 8 post challenge, the WBC counts for the vaccinates were higher than the controls, and were significantly higher on days 4 and 6 ($P<0.001$) post challenge. On days 10 to 12 post challenge, WBC counts for the controls were again higher than the vaccinates (Figure 3); however, the difference ranged from 0.2 to 0.4 cells/uL on those days and was not significant. The mean WBC counts in control heifers ranged from 3.3 to 8.5 cells/uL, while the mean WBC counts in vaccinated heifers ranged from 5.6 to 7.6 cells/uL on days -2 to 14 post challenge (Figure 3).

There was a statistical difference for leukopenia in the BVDV2a challenge group between vaccinates (1/18; 5.6%) and controls (11/22; 50.0%) ($P\leq0.0001$; Figure 4). The calculated prevention of leukopenia in vaccinates was supported by prevented fraction of 0.889 (88.9%) with exact 95% confidence limits of (0.426, 0.996). On days -2 and 0 DPC, mean WBC counts in the vaccinates were higher by approximately 0.2 to 0.5 cells/uL. On day 2 post challenge, the mean WBC counts for the controls were 0.4 cells/uL higher than the vaccinates; however, on day 4 post challenge, the mean WBC counts for the controls dropped below the mean WBC counts for the vaccinates, and remained lower for the remainder of the study period. The mean WBC counts in control heifers ranged from 3.6 to 6.0 cells/uL, while mean WBC counts in vaccinated heifers ranged from 5.4 to 7.6 cells/uL on days -2 to 14 post challenge (Figure 4). Mean WBC counts in vaccinated heifers were significantly higher ($P=0.0353$) when compared to control heifers on days

Table 1. Proportion of positive viremias in vaccinated and nonvaccinated heifers after challenge with BVDV type 1b or type 2 as determined by buffy coat virus isolation after challenge.

	BVDV type 1b challenge		BVDV type 2 challenge	
Day after challenge	**Nonvaccinated**	**Vaccinated**	**Nonvaccinated**	**Vaccinated**
-2	0/23	0/22	0/22	0/18
0	0/23	0/22	0/22	0/18
2	0/23	0/22	0/22	0/18
4	1/23	0/22	2/18	0/18
6	7/23	0/22	16/22	1/18
8	17/23	0/22	19/22	0/18
10	1/23	0/22	8/22	0/18
12	0/23	0/22	1/22	0/18
14	0/23	0/22	1/22	0/18
Total viremic	**19/23†**	**0/22**	**20/22†**	**1/18**

†Significantly ($P<0.001$) different from proportion of total numbers of vaccinated heifers from which virus was isolated.

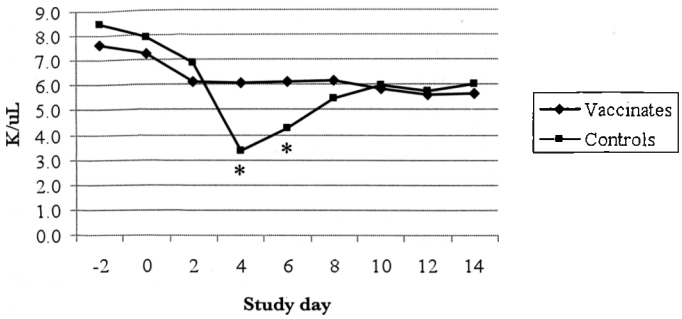

Figure 3. Mean BVDV1b challenge group WBC results before and after challenge (368 DPV, 0 DPC) with virulent BVDV1b. The vaccinates (black diamonds; n=21) consisted of heifers that received the test vaccine (i.e., multivalent vaccine containing BVDV1a and BVDV2 fractions). The controls (black squares; n=18) consisted of heifers that received placebo vaccine (i.e., multivalent vaccine without BVDV fractions). Notice that the WBC were significantly higher ($P<0.001$) on days 4 and 6 post challenge in the vaccinates, when compared to the controls (asterisks).

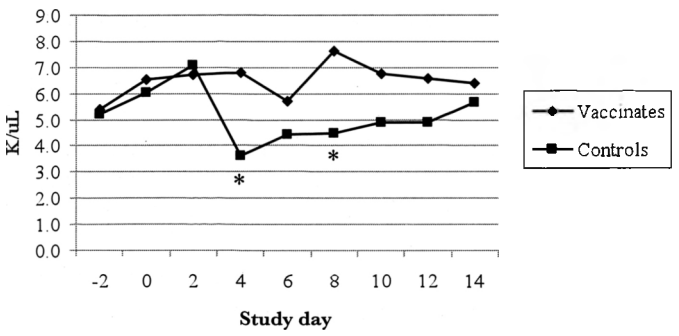

Figure 4. Mean BVDV2 challenge group WBC results before and after challenge (374 DPV, 0 DPC) with virulent BVDV2. The vaccinates (black diamonds; n = 18) consisted of heifers that received the test vaccine (i.e., multivalent vaccine containing BVDV1a and BVDV2 fractions). The controls (black squares; n=22) consisted of heifers that received placebo vaccine (i.e., multivalent vaccine without BVDV fractions). Notice that the WBC were higher in the vaccinated heifers versus the control heifers on all days except 2 DPV. The vaccinates had significantly higher ($P<0.0353$) WBC on days 4 and 8 post challenge when compared to the controls (asterisks).

4 and 8 and had a tendency to be higher ($P=0.0800$) on days 10 and 12 after challenge.

There was no statistical difference for platelet counts between vaccinates and controls for the BVDV1b challenge group ($P=0.5051$). Initially (i.e., 2 DPC), the vaccinates had lower mean platelet counts than the controls; however, on days 4 to 8 post challenge, the platelet counts for the vaccinates were higher than the controls (data not shown). On days 10 to 14 post challenge, the controls had higher platelet counts than the vaccinates. There was a significant difference between vaccinates and controls for the BVDV2a challenge group on days 10, 12, and 14 post challenge (i.e., $P=0.0367$, $P=0.0050$, and $P=0.0202$, respectively; data not shown). On each of those days, the vaccinates had significantly higher platelet counts when compared to controls.

Fetal Tissue Results

There were no fetal infections in the vaccinated heifers from the BVDV1b challenge group (0/21; 0.0%), while 15/18 (83.3%) of fetuses from the control group heifers were BVDV-positive ($P<0.0001$; Table 2). However, this analysis did not include fetal loss, as 5 control heifers and 1 vaccinate previously confirmed pregnant were found to be open upon rectal palpation prior to fetal harvest. Since the fetuses were not recovered, the animals were not included in the statistical analysis, as no definitive diagnosis could be made. Virus was consistently isolated from the brain, spleen, thymus, and heart blood of the infected fetuses in both of the control groups (Table 2). The BVDV1b challenge group had a calculated prevented fraction of 1.0 (100.0%) with exact 95% confidence limits of (0.825, 1.0) when comparing the treatment groups. Similar results were seen in the vaccinated heifers from the BVDV2a challenge group, where there were no fetal infections (0/18; 0.0%), while 21/22 (95.5%) of fetuses from the control heifers were infected ($P<0.0001$; Table 2). The BVDV2a challenge group had a calculated prevented fraction of 1.0 (100.0%) with exact 95% confidence limits of (0.814, 1.0) when comparing the treatment groups.

Discussion

This study demonstrated that a single dose of a multivalent MLV vaccine containing the MID of BVDV types 1a and 2a, administered SC to heifers at approximately 15 months of age before breeding, protected against BVDV1b or BVDV2a fetal infection 1 year post-vaccination. Additionally, vaccinated heifers had fewer viremia events (i.e., no heifers in the BVDV1b and 1 heifer in the BVDV2a challenge groups) and fewer vaccinated animals were leukopenic (i.e., 8 and 1 heifer[s] in the BVDV1b and BVDV2a challenged groups, respectively). Among vaccinated heifers, 100% of fetuses in the BVDV1b and BVDV2a challenge groups were protected against infection, whereas 83.3% and 95.5% of fetuses in non-vaccinated control heifers were infected. Fetal loss occurred in the BVDV1b challenge group, as 6 previously confirmed pregnant heifers (5 control and

Table 2. Total numbers of BVDV1 and BVDV2 positive fetal tissues for vaccinates and controls in both challenge groups. Notice that 0 out of 21 and 0 out of 18 fetal tissues were positive for BVDV from vaccinated dams in their respective challenge groups. In contrast, 13 and 15 out of 18 control fetuses were positive in 3 and 4 tissues, respectively, following challenge with virulent BVDV1b; while 20 and 21 out of 22 control fetuses were positive in 2 and 4 tissues, respectively, following challenge with virulent BVDV2. Fetal tissue positive results for controls were significantly higher ($P<0.0001$) when compared to the vaccinates (all fetal tissues negative in both challenge groups).

		Fetal tissue virus isolation results			
		Fetal tissue			
Treatment group	Number of infected fetuses	Spleen	Thymus	Heart blood	Brain
BVDV1b group vaccinates	0/21	0/21	0/21	0/21	0/21
BVDV1b group controls	15/18	13/18	15/18	15/18	15/18
BVDV2 group vaccinates	0/18	0/18	0/18	0/18	0/18
BVDV2 group controls	21/22	20/22	20/22	21/22	21/22

1 vaccinate heifer[s]) were no longer pregnant by rectal palpation prior to fetal harvest. Since the fetuses were not recovered and the cause of fetal death could not be determined, the animals were not included in the statistical analysis.[14,15,21,22,32]

The ability of a vaccine to protect heifers from producing PI calves is important because PI calves serve as reservoirs for this devastating disease and continually shed virus.[4,5,11,13,14,16,17,20-22,29,36,37] Previously published BVDV fetal protection studies have been performed involving the use of MLV vaccines administered ≤148 days prior to challenge.[3-5,10,11,13,15,16,21,22,25,36] Since various types of challenge models were employed, such as direct challenge or challenge by contact with PI animal(s), use of monovalent or bivalent vaccines, heterologous or homologous challenge, and differing gestational ages at challenge, it is difficult to accurately compare results from those studies to the present study. In previous studies, efficacy was variable; however, 1 general trend was vaccines administered IM appeared to be moderately more effective than vaccines administered SC at preventing production of PI offspring. Three monovalent vaccines (containing BVDV1 only) administered via a single IM injection prior to breeding resulted in 100% protection from production of PI offspring when dams were challenged with a BVDV1 strain.[11,16,25] Conversely, monovalent vaccines containing BVDV1 administered IM prior to breeding protected only 58%[3] to 67% (1 injection)[15] and 63% (2 injections)[15] of fetuses from infection when challenged with BVDV2; however, when vaccinated with a bivalent vaccine (containing BVDV1 and BVDV2), protection reached 100%.[15] In a similar study, Brock et al reported 100% and 87.5% protection from production of PI offspring with a BVDV1 vaccine administered IM or SC, respectively, prior to breeding, when challenged with BVDV2.[4] Additional studies performed by Dean et al[11] and Brock et al[5] that utilized monovalent BVDV1 vaccines administered SC prior to breeding reported 85% and 93% protection from fetal infection and subsequent production of PI animals when challenged with BVDV2, respectively.

The BVDV subgenotype 1b has recently been shown to be the most common isolate of BVDV in the United States.[17,22,29,30,36] Since very few vaccines exist that contain a BVDV1b vaccinal strain, it is essential that currently available vaccines are effective at cross-protection against this strain. Previous studies that tested the cross-protective efficacy of vaccines containing BVDV1a against BVDV1b challenge showed varying results.[4,10,13,21,22,36,37] Xue et al reported 82% and 75% protection from fetal infection when a vaccine containing BVDV1a and BVDV2 was administered SC approximately 198 days prior to challenge with BVDV1b and BVDV2, respectively.[37] In contrast to the present study, however, the aim was to prevent fetal infection and the subsequent birth of congenitally infected (i.e., transiently infected, with infection eventually cleared by the animal's immune system, similar to acute infection) calves, rather than persistently infected calves, as the dams were challenged at 163 to 177 days gestation when fetuses are likely immunocompetent.[1,3-5,10,11,13-17,21,22,34] Fairbanks et al reported 100% and 95% prevention of the production of PI calves with a MLV vaccine containing BVDV1a (Singer) and BVDV2 (296) strains administered SC 129 days prior to breeding in the face of challenge with BVDV1b or BVDV2 at 76 to 79 days gestation, respectively.[13] These are the same vaccinal strains utilized in the present study, which showed comparable results. Similar studies utilizing vaccines containing either BVDV1a alone or in combination with BVDV2 have been shown to be 83 to 91% effective at prevention of production of PI animals in the face of challenge with

BVDV1b.[4,10,21,22] These studies show that vaccines containing BVDV1a are effective in preventing production of PI calves when given up to 137 days prior to challenge with BVDV1b and/or BVDV2, while the present study showed 100% protection from fetal infection with a vaccine containing BVDV1a and BVDV2a when challenged with BVDV1b and BVDV2a.

Very few challenge studies have been done that have demonstrated comparable duration of immunity. One study[14] similar to the present study (i.e., duration of immunity for ≥1 year) involved the use of a single IM injection of a combination (BHV-1, PI3, BRSV, BVDV types 1a and 2 [cytopathic], as well as *Campylobacter fetus* and *Leptospira canicola*, *icterohaemorrhagiae*, *pomona*, *hardjo*, and *grippotyphosa*) vaccine given 370 days prior to IN challenge with BVDV1a (816317b) or BVDV2 (94B-5359a) strains. No clinical signs were noted in any animals following challenge, and all vaccinated heifers challenged with BVDV1a delivered live, non-PI calves. Eighteen vaccinated heifers challenged with BVDV2 delivered live, non-PI calves, while 2 vaccinated heifers aborted, one possibly as a result of amniocentesis performed earlier, and another from an undetermined cause. Eight of 9 (i.e., 2 fetuses and 6 live calves) and all progeny (i.e., 1 fetus and 9 live calves) delivered from non-vaccinated BVDV1a or BVDV2-challenged control animals were determined to be PI, respectively. Similarly, the present study utilized a SC administered combination vaccine also containing BVDV1a and BVDV2a; however, animals in the present study were challenged with different strains (BVDV1b [BJ] and BVDV2a [PA131]). No significant clinical signs were noted in the present study following challenge with either BVDV1b or BVDV2a; however, challenge was determined to be effective, as the fetuses of 83.3% and 95.5% of control animals, respectively, were found to be infected. The significance of the present study is that it is the first vaccine to report 100% fetal protection against BVDV1b challenge when administered as a single SC dose 1 year prior to challenge. Additionally, fetal protection conferred by vaccination was 100% for the BVDV2a challenge group, which is similar to previous studies.[13-15,21] Another study[12] in which heifers were administered a single IM vaccination 490 days prior to challenge resulted in 92% fetal protection when challenged using a 16-day period of continual exposure to BVDV2a PI calves at 149 through 217 days of gestation, when fetuses are likely immunocompetent.[1,3-5,10,11,13-17,21,22,34] The PI exposure challenge resulted in a cumulative 80% challenge efficacy (i.e., 8 out of 10 control heifers tested positive for BVDV following challenge); whereas, the current study resulted in 82.6% (i.e., 19 out of 23 control heifers tested positive) and 90.9% (i.e., 20 out of 22 control heifers tested positive) challenge efficacy in heifers challenged intranasally with BVDV1b and BVDV2a, respectively (Table 1), as determined by virus isolation. The PI exposure challenge resulted in 9 of 10 calves and 1 calf from control and vaccinated heifers, respectively, being considered born as congenitally infected,[12] whereas the current study showed 15/18 and 21/22 fetuses from the control heifers in the BVDV1b and BVDV2a challenge groups, respectively, and no fetuses from either group of vaccinated heifers were infected.

Conclusion

A vaccine effective against the most prevalent subtype(s) of BVDV is an essential part of animal production. This study showed that 1 dose of a multivalent MLV vaccine containing the MID of BVDV types 1a and 2a, administered 1 year prior to challenge, was effective in preventing fetal infection in bred heifers challenged with BVDV types 1b and 2a. Additionally, there was no evidence of interference from other fractions within the vaccine. Consequently, initial vaccination followed by yearly booster vaccinations administered prior to or during gestation can provide significant fetal protection from the most prevalent BVDV infections to a majority of cattle and prevent fetal infection and subsequent production of PI calves.

Endnotes

[a]Express® FP5-VL5, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO
[b]University of Nebraska-Lincoln Veterinary Diagnostic Laboratory, Lincoln, NE
[c]National Veterinary Services Laboratory, Ames, IA
[d]American Type Culture Collection, Manassas, VA
[e]Greiner Bio-One, Frickenhausen, Germany
[f]EAZI-BREED™ CIDR® implants, Pharmacia & Upjohn Company, a Division of Pfizer, New York, NY
[g]Cystorelin®, Merial Limited, Duluth, GA
[h]Estrotect™, Rockway, Inc., Spring Valley, WI
[i]Lutalyse®, Pharmacia & Upjohn Company, a Division of Pfizer, New York, NY
[j]Animal Disease Research and Diagnostic Laboratory, South Dakota State University, Brookings, SD
[k]Chromist™, Gelman Sciences, Ann Arbor, MI
[l]BVDV1 BJ Strain (Lot Number BVD1 BJ 28Jun05) and BVDV2 PA131 Strain (Lot Number BVD2 PA131, 19Apr10), Rural Technologies, Inc., Brookings, SD. Strains originally obtained from Dr. Kenny Brock.
[m]GLA Thermometer, GLA Agricultural Electronics, San Luis Obispo, CA
[n]Cellgro, Mediatech Inc, Herndon, VA
[o]Hyclone® Donor Equine Serum, HyClone Laboratories Inc., Logan, UT
[p]Sanford Health, Sioux Falls, SD
[q]Cimpl Meats, Yankton, SD

[r]Rural Technologies Inc., Brookings, SD
[s]SAS® System, Version 9.2, SAS Institute, Cary, NC
[t]StatXact®, Version 8.0, Cytel Incorporated, Cambridge, MA

Acknowledgement

The authors thank Tanya Triebwasser, George Perry, Justin Fruechte, Devan Schomp, and Kayla Behrens for technical assistance, and Boehringer Ingelheim Vetmedica for funding the study.

References

1. Baker JC. Bovine viral diarrhea: A review. *J Am Vet Med Assoc* 1987; 190:1449-1458.
2. Bolin SR. Control of bovine viral diarrhea infection by use of vaccination. *Vet Clin North Am Food Anim Pract* 1995; 11:615-625.
3. Brock KV, Cortese VS. Experimental fetal challenge using type II bovine viral diarrhea virus in cattle vaccinated with modified-live virus vaccine. *Vet Ther* 2001; 2:354-360.
4. Brock KV, McCarty K, Chase CC, Harland R. Protection against fetal infection with either bovine viral diarrhea virus type 1 or type 2 using a noncytopathic type 1 modified-live virus vaccine. *Vet Ther* 2006; 7:27-34.
5. Brock KV, McCarty K, Harland R. Fetal protection against a type 2 bovine viral diarrhea virus (BVDV) challenge in cattle vaccinated with a type I modified live BVDV vaccine. *Proceedings* 38th Annu Conf Am Assoc Bov Pract 2005; 38:205.
6. Brownlie J, Clarke MC, Hooper LB, Bell GD. Protection of the bovine fetus from bovine viral diarrhoea virus by means of a new inactivated vaccine. *Vet Rec* 1995; 137:58-62.
7. Bruschke CJM, Moormann RJM, van Oirschot JT, van Rijn PA. A subunit vaccine based on glycoprotein E2 of bovine virus diarrhea virus induces fetal protection in sheep against homologous challenge. *Vaccine* 1997; 15:1940-1945.
8. Bruschke CJM, van Oirschot JT, van Rijn PA. An experimental multivalent bovine virus diarrhea virus E2 subunit vaccine and two experimental conventionally inactivated vaccines induce partial fetal protection in sheep. *Vaccine* 1999; 17:1983-1991.
9. Chin AC, Lee WD, Murrin KA, Morck DW, Merrill JK, Dick P, Buret AG. Tilmicosin induces apoptosis in bovine peripheral neutrophils in the presence or in the absence of *Pasteurella haemolytica* and promotes neutrophil phagocytosis by macrophages. *Antimicrob Agents Chemother* 2000; 44:2465-2470.
10. Cortese VS, Grooms DL, Ellis J, Bolin SR, Ridpath JF, Brock KV. Protection of pregnant cattle and their fetuses against infection with bovine vial diarrhea virus type1 by use of a modified-live virus vaccine. *Am J Vet Res* 1998; 59:1409-1413.
11. Dean HJ, Hunsacker BD, Bailey OD, Wasmoen T. Prevention of persistent infection in calves by vaccination of dams with noncytopathic type-1 modified-live bovine viral diarrhea virus prior to breeding. *Am J Vet Res* 2003; 64:530-537.
12. Ellsworth MA, Fairbanks KK, Behan S, Jackson JA. Fetal protection following exposure to calves persistently infected with bovine viral diarrhea virus type 2 sixteen months after primary vaccination of the dams. *Vet Ther* 2006; 7:295-304.
13. Fairbanks KK, Rinehart CL, Ohnesorge WC, Loughin MM, Chase CCL. Evaluation of fetal protection against experimental infection with type 1 and type 2 bovine viral diarrhea virus after vaccination of the dam with a bivalent modified-live virus vaccine. *J Am Vet Med Assoc* 2004; 225:1898-1904.
14. Ficken MD, Ellsworth MA, Tucker CM. Evaluation of the efficacy of a modified-live combination vaccine against bovine viral diarrhea virus types 1 and 2 challenge exposures in a one-year duration-of-immunity fetal protection study. *Vet Ther* 2006; 7:283-294.
15. Ficken MD, Ellsworth MA, Tucker CM, Cortese VS. Effects of modified-live bovine viral diarrhea virus vaccines containing either type 1 or types 1 and 2 BVDV on heifers and their offspring after challenge with noncytopathic type 2 BVDV during gestation. *J Am Vet Med Assoc* 2006; 228:1559-1564.
16. Frey HR, Eicken K, Grummer B, Kenklies S, Oguzoglu TC, Moennig V. Foetal protection against bovine virus diarrhoea virus after two-step vaccination. *J Vet Med B* 2002; 49:489-493.
17. Fulton RW, Ridpath JF, Ore S, Confer AW, Saliki JT, Burge LJ, Payton ME. Bovine viral diarrhoea virus (BVDV) subgenotypes in diagnostic laboratory accessions: Distribution of BVDV1a, 1b, and 2a subgenotypes. *Vet Microbiol* 2005; 111:35-40.
18. Giammarioli M, Pellegrini C, Casciari C, Rossi E, De Mia GM. Genetic diversity of bovine viral diarrhea virus 1: Italian isolates clustered in at least seven subgenotypes. *J Vet Diagn Invest* 2008; 20:783-788.
19. Grant JK, Abreu FM, Hojer NL, Fields SD, Perry BL, Perry GA. Influence of inducing luteal regression before a modified controlled internal drug-releasing device treatment on control of follicular development. *J Anim Sci* 2001; 89:3531-3541.
20. Grooms DL, Bolin SR, Coe PH, Borges RJ, Coutu CE. Fetal protection against continual exposure to bovine viral diarrhea virus following administration of a vaccine containing an inactivated bovine viral diarrhea virus fraction to cattle. *Am J Vet Res* 2007; 68:1417-1422.
21. Kovács F, Magyar T, Rinehart C, Elbers K, Schlesinger K, Ohnesorge WC. The live attenuated bovine viral diarrhea virus components of a multi-valent vaccine confer protection against fetal infection. *Vet Microbiol* 2003; 96:117-131.
22. Leyh RD, Fulton RW, Stegner JE, Goodyear MD, Witte SB, Taylor LP, Johnson BJ, Step DL, Ridpath JF, Holland BP. Fetal protection in heifers vaccinated with a modified-live virus vaccine containing bovine viral diarrhea virus subtypes 1a and 2a and exposed during gestation to cattle persistently infected with bovine viral diarrhea virus subtype 1b. *Am J Vet Res* 2011; 72:367-375.
23. Littell RC, Henry PR, Ammerman CB. Statistical analysis of repeated measures data using SAS procedures. *J Anim Sci* 1998; 76:1216-1231.
24. Mahlum CE, Haugerud S, Shivers JL, Rossow KD, Goyal SM, Collins JE, Faaberg KS. Detection of bovine viral diarrhea virus by TaqMan® reverse transcription polymerase chain reaction. *J Vet Diagn Invest* 2002; 14:120-125.
25. Meyer G, Deplanche M, Roux D, Moulignie M, Picard-Hagen N, Lyazrhi F, Raboisson D, Mathevet P, Schelcher F. Fetal protection against bovine viral diarrhoea type 1 virus infection after one administration of a live-attenuated vaccine. *Vet J* 2011 [e-pub ahead of print]; doi:10.1016/j.tvjl.2011.05.011.
26. Minami F, Nagai M, Ito M, Matsuda T, Takai H, Jinkawa Y, Shimano T, Hayashi M, Seki Y, Sakoda Y, Sugiura K, Akashi H. Reactivity and prevalence of neutralizing antibodies against Japanese strains of bovine viral diarrhea virus subgenotypes. *Comp Immunol Microbiol Infect Dis* 2011; 34:35-39.
27. Patel JR, Shilleto RW, Williams J, Alexander DCS. Prevention of transplacental infection of bovine foetus by bovine viral diarrhoea virus through vaccination. *Arch Virol* 2002; 147:2453-2463.
28. Perry GA, Perry BL, Roberts CA. Estrus response following the PG 6-d CIDR protocol for heifers that do and do not exhibit estrus prior to CIDR insertion and its usefulness as a fixed-time AI protocol. *Proceedings.* Western Section, Am Society Anim Sci 2011; 62:264-267.
29. Pogranichniy RM, Schnur ME, Raizman EA, Murphy DA, Negron M, Thacker HL. Isolation and genetic analysis of bovine viral diarrhea virus from infected cattle in Indiana. *Vet Med International* 2011 [e-pub ahead of print]; doi:10.4061/2011/925910.
30. Ridpath JF, Fulton RW, Kirkland PD, Neill JD. Prevalence and antigenic differences observed between bovine viral diarrhea virus subgenotypes isolated from cattle in Australia and feedlots in the southwestern United States. *J Vet Diagn Invest* 2010; 22:184-191.
31. Ridpath JF, Lovell G, Neill JD, Hairgrove TB, Velayudhan B, Mock R. Change in predominance of bovine viral diarrhea virus subgenotypes among samples submitted to a diagnostic laboratory over a 20-year time span. *J Vet Diagn Invest* 2011; 23:185-193.

32. Rodning SP, Marley MS, Zhang Y, Eason AB, Nunley CL, Walz PH, Riddell KP, Galik PK, Broderson BW, Givens MD. Comparison of three commercial vaccines for preventing persistent infection with bovine viral diarrhea virus. *Therio* 2010; 73:1154-1163.
33. Saliki JT, Fulton RW, Hull SR, Dubovi EJ. Microtiter virus isolation and enzyme immunoassays for detection of bovine viral diarrhea virus in cattle serum. *J Clinical Microbiol* 1997; 35:803-807.
34. Stevens ET, Zimmerman AD, Buterbaugh RE, Barling K, Scholz D, Rhoades J, Chase CCL. The induction of a cell-mediated immune response to bovine viral diarrhea virus with an adjuvanted inactivated vaccine. *Vet Ther* 2009; 10:E1-E8.
35. Unknown. *Bovine Viral Diarrhea Virus*. APHIS Veterinary Services, Centers for Epidemiology and Animal Health. December 2007. http://www.aphis.usda.gov/animal_health/emergingissues/downloads/bvdinfosheet.pdf. Accessed Dec 2011.
36. Xue W, Mattick D, Smith L. Protection from persistent infection with a bovine viral diarrhea virus (BVDV) type 1b strain by a modified-live vaccine containing BVDV types 1a and 2, infectious bovine rhinotracheitis virus, parainfluenza 3 virus and bovine respiratory syncytial virus. *Vaccine* 2011; 29:4657-4662.
37. Xue W, Mattick D, Smith L, Maxwell J. Fetal protection against bovine viral diarrhea virus types 1 and 2 after the use of a modified-live virus vaccine. *Can J Vet Res* 2009; 73:292-297.
38. Zimmer GM, Wentink GH, Bruschke C, Westenbrink FJ, Brinkhof J, de Goey I. Failure of foetal protection after vaccination against an experimental infection with bovine viral diarrhea virus. *Vet Microbiol* 2002; 89:255-265.
39. Zimmerman AD, Buterbaugh RE, Schnackel JA, Chase CCL. Efficacy of a modified-live virus vaccine administered to calves with maternal antibodies and challenged seven months later with a virulent bovine viral diarrhea type 2 virus. *Bov Pract* 2009; 43:35-43.