# Evaluation of skin samples for bovine viral diarrhea virus by use of reverse transcriptase polymerase chain reaction assay after vaccination of cattle with a modified-live bovine viral diarrhea virus vaccine

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**Objective**—To determine whether vaccine virus can be detected by use of reverse transcriptase (RT)-PCR assays for pooled and individual skin samples obtained from cattle after vaccination with a commercially available modified-live bovine viral diarrhea virus (BVDV) vaccine.

**Animals**—12 BVDV-seropositive steer calves and 7 BVDV-seronegative (antibody titer < 1:4) heifers; all cattle were free of persistent infection with BVDV.

**Procedures**—2 experiments were conducted. Cattle were vaccinated on day 0 with a commercially available modified-live BVDV vaccine. Skin samples were collected on days 0, 3 to 14, 16, and 18 for virus detection by use of RT-PCR assay on individual and pooled samples. In addition, blood samples and nasal swab specimens were collected for virus isolation.

**Results**—All cattle, regardless of serologic status, had negative results for BVDV as determined by use of RT-PCR assay of individual and pooled skin samples. Virus was detected via virus isolation in serum or the buffy coat in 5 of 7 heifers that were seronegative when vaccinated.

**Conclusions and Clinical Relevance**—These findings indicated that it would be unlikely to detect BVDV vaccine virus in skin by use of RT-PCR assay of individual or pooled skin samples obtained from cattle after vaccination with a commercially available modified-live BVDV vaccine. Veterinarians and producers should be confident that positive test results for BVDV on skin samples would not likely be caused by the vaccination virus after administration of a modified-live virus vaccine. (*Am J Vet Res* 2012;73:319–324)

Bovine viral diarrhea virus is an economically important pathogen affecting cattle throughout the world. Economic losses associated with BVDV infection have been estimated in the range of $10 million to $40 million/1 million calvings.[1] In 1 report,[2] the predicted economic effects on reproduction and performance in commercial cow herds over a 10-year period indicated the mean return to fixed cost was $20.16 less for farms with at least 1 PI animal in the herd; the decrease in economic return was attributed to effects on reproduction and calf mortality rate. In another study,[3] it was reported that feedlot cattle exposed to PI animals cost producers a mean of $88.26/animal in performance losses.

**Abbreviations**

| | |
|---|---|
| ACE | Antigen-capture ELISA |
| BT | Bovine turbinate |
| BVDV | Bovine viral diarrhea virus |
| PI | Persistently infected |
| RT | Reverse transcriptase |
| VN | Virus neutralization |

Persistently infected cattle are the major reservoir for transmission of BVDV within and between farms. It is estimated that 4% to 9% of cow-calf herds and 15% of dairy herds have at least 1 PI animal.[4,5] Identification and removal of PI cattle from herds is a key component for controlling BVDV transmission. Many methods of virus detection have been used to detect PI cattle. Currently, the most frequently used methods rely on testing of skin samples for BVDV.[6] Methods to detect BVDV in skin samples include immunohistochemical analysis, ACE, and RT-PCR assays.[6] More recently, ACE and RT-PCR assays have become widely used as screening tests because of their low cost, ease of use, adaptation for testing a high volume of samples, and analytic sensitivity. The RT-PCR assay has the potential to detect small concentrations of virus, thereby allowing strate-

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gies that involve the use of pooled samples, which reduce test costs to producers. The high analytic sensitivity of the RT-PCR assay also increases the likelihood of detecting transient infections.

Vaccination programs are a major component of control and prevention strategies for BVDV. In the United States, modified-live virus vaccines have become the preferred choice for vaccination against BVDV. With this evolution, questions have arisen as to whether use of modified-live virus vaccines could lead to false-positive results on virus detection assays. Several studies have been conducted to evaluate the temporal persistence of vaccine virus in various clinical specimens obtained after cattle have been vaccinated with a modified-live virus vaccine. The BVDV vaccine virus can remain in the ovaries for up to 12 days after vaccination,[7] and it can be shed in semen for up to 10 days after vaccination.[8] In other studies,[9,10] investigators found that when an animal is acutely infected with BVDV, the likelihood of detection of viral antigen in a skin biopsy specimen tested via immunohistochemical analysis or ACE is extremely low. Other investigators have used RT-PCR assays to test serum samples and nasal swab specimens obtained from calves vaccinated with a modified-live BVDV vaccine. In 1 study,[11] serum samples obtained from 78% of vaccinated calves between 3 and 10 days after vaccination and tested by use of RT-PCR assay had positive results for BVDV. However, in that same report,[11] investigators found that none of the calves had positive results for BVDV for nasal swab specimens tested by use of RT-PCR assay. In other studies,[10,12] investigators have reported that BVDV vaccine virus can be detected in blood and certain tissues; however, to our knowledge, BVDV vaccine virus has not been found in skin samples tested by use of immunohistochemical analysis or ACE. It is important to determine whether vaccine virus can be detected in skin samples because a false-positive result for BVDV can lead to confirmatory follow-up testing and more costs to producers or to inappropriate culling of healthy cattle if confirmatory testing is not pursued.

The objective of the study reported here was to determine whether vaccine virus could be detected by use of RT-PCR assay in skin samples obtained from cattle after vaccination with a commercially available modified-live BVDV vaccine. Furthermore, if the vaccine virus was detectable, we intended to determine the amount of time it could be detected in the skin samples.

## Materials and Methods

**Animals**—Calves known to be free of persistent infection with BVDV were used. Twelve BVDV-seropositive steer calves (age, 4 to 5 months) and 7 BVDV-seronegative (antibody titer < 1:4) heifers (age, 11 months to 2.5 years) were included in the study. Seropositive steer calves were obtained from the Michigan State University Purebred Beef Center, and seronegative heifers were obtained from a private cow-calf herd in northeast Michigan. All cattle had negative results for BVDV via RT-PCR assay of skin samples obtained prior to inclusion in the study. Each group was housed in an isolated pen at least 150 yards from other cattle on the premises of the Michigan State University Beef Cattle Teaching and Research Center. The study was approved by the Michigan State University Institutional Care and Animal Use Committee.

**Experimental procedures**—All cattle were administered a commercially available modified-live cytopathic BVDV vaccine[a] on day 0. Skin and blood samples and nasal swab specimens were collected from each animal before vaccination on day 0 and then on days 3 through 14, 16, and 18 after vaccination. Days for sample collection were chosen to correspond to the time frame (days 3 to 14 after vaccination) when there would be an expected viremia attributable to the vaccine virus, as determined on the basis of data from experimental infection trials.[13–15] Two additional samples were obtained on days 16 and 18, which were outside the window of expected viremia. These were obtained to broaden the window without subjecting the cattle to more extensive sample collection.

Skin samples were collected from an ear of each animal by use of a 5/16-inch ear notcher and then placed in separate individually labeled bags. Blood samples from all cattle were collected by jugular venipuncture into a serum separator tube. Nasal swab specimens were collected and placed in tubes containing 1 mL of Dulbecco modified Eagle medium. For the seronegative heifers, whole blood samples were collected into tubes containing EDTA as an anticoagulant. Samples were placed on ice and transported to our laboratory.

At our laboratory, each skin sample was divided into 2 halves, and each half was placed in separate labeled bags. Skin and serum samples and nasal swab specimens were stored briefly at –20°C until testing was performed.

White blood cells were isolated from whole blood samples collected from the seronegative heifers. Samples were stored at 23° to 25°C for 1 hour and then centrifuged at 1,200 × *g* for 15 minutes. After centrifugation, the buffy coat was harvested from each sample by use of individual sterile 1-mL pipettes and placed into labeled 15-mL polypropylene centrifuge tubes. The buffy coat with contaminating RBCs was suspended in 3 mL of sterile water (cell culture grade) for 30 to 45 seconds to lyse the RBCs, and then 1.5 mL of 2× concentrated physiologic PBS solution was added to each tube. Cells were vortexed and then pelleted via centrifugation at 270 × *g* for 12 minutes. A second exposure to 1.5 mL of sterile water was followed by the addition of 0.75 mL of 2× concentrated physiologic PBS solution. Cells were pelleted via centrifugation at 270 × *g* for 12 minutes, suspended in 0.5 mL of Bovarnick solution, and frozen at –70°C.

**RT-PCR assay of skin samples**—All skin samples were submitted to the Diagnostic Center for Population and Animal Health at Michigan State University. One half of each skin sample was processed for routine diagnostic testing, which was performed via RNA extraction of homogenated pooled samples (10 skin samples/pool). The RNA was then tested by use of a hydrolysis probe–based real-time RT-PCR assay that targeted the 5′ untranslated region of the BVDV genome.[b] An internal RNA control sample was used to monitor inhibition of the RT-PCR assay. The detection limit of the assay

was 0.8 $TCID_{50}$.[16] Laboratory protocol required all individual samples that comprised a pool that had positive results be retested by use of fluorescent antibody staining of fresh-frozen sections of tissue.

All individual skin samples were retested by use of a gel-based RT-PCR assay that amplified a region of the 5′ untranslated region spanning bases 139 to 394 of the genome of the BVDV National Animal Disease Laboratory reference virus. A positive result for an individual skin sample would be confirmed by use of virus isolation or viral genotype–specific RT-PCR assays.

**Virus isolation**—Virus isolation was performed on serum samples, nasal swab specimens, and WBCs harvested from whole blood. Approximately 200 µL of each sample was inoculated onto BT cells in 24-well flat-bottom cell culture plates. Transport medium for the nasal swab specimens was passed through a 0.45-µm syringe filter prior to inoculation. The BT cells were free of adventitious BVDV, as determined by use of the RT-PCR assay. The growth medium for the BT cells was free of adventitious BVDV and antibody against BVDV as determined via virus isolation, RT-PCR assay, and VN assay against type 1 and 2 BVDV. Cell monolayers were observed for cytopathic effect, and potential BVDV-infected cells were subpassaged after 5 to 8 days to fresh monolayers of BT cells. After culture for 2 to 5 days (duration of culture was dependent on the detection of cytopathic effect), RNA was extracted from infected cells by use of reagent[c] as recommended by the manufacturer and stored frozen until used for genetic analysis.

To obtain the BVDV vaccine viruses, the viruses in the reconstituted vaccine were biologically cloned. The reconstituted BVDV vaccine was diluted 100-fold in cell culture medium, and then serial 4-fold dilutions were made from the diluted vaccine. The serial dilutions were inoculated onto BT cells that had been seeded into 96-well microtitration plates. Culture fluid was harvested from wells with cytopathic effect typical of BVDV and inoculated onto fresh BT cells grown in 24-well plates. After culture for 2 to 3 days, RNA was extracted from infected cells of the 24-well plates.

**VN**—Serum samples were tested for VN antibodies against genotypes 1a, 1b, and 2a BVDV by use of standard microtitration assay procedures.[16] The cytopathic Singer strain was used as the genotype 1a BVDV reference strain, cytopathic strain TGAC as the genotype 1b reference strain, and cytopathic 125C strain as the genotype 2a BVDV reference strain. The VN test was conducted by use of BT cells that were free of adventitious BVDV.[17] Fetal bovine serum that was used to supplement cell culture medium had negative results when tested for live adventitious BVDV, RNA from BVDV, and antibody against BVDV. Serial 2-fold dilutions (range, 1:4 to 1:4,096) were made for each serum sample. The antibody titer was considered to be the highest serum dilution at which the cytopathic effect of the BVDV reference strain was completely inhibited.

**Genetic analysis**—The genotypes of isolated viruses were predicted by use of genotype-specific RT-PCR assays that targeted the 3′ end of the genomic region encoding viral protein NS5B and most of the 3′ untranslated region of the viral genome.[18,19] To derive nucleic acid sequences for genetic analysis, RNA from select viral isolates was amplified to obtain a segment of the viral genome that included approximately 100 bp of the 3′ end of the 5′ untranslated region and all of the genomic region encoding the $N^{pro}$ and capsid viral proteins. Samples of amplified nucleic acid were submitted to the Michigan State University Research Technology Support Facility for DNA sequencing. Sequences were obtained by use of a capillary sequencer.[d] Sequences were aligned and trimmed by use of software as described elsewhere.[20] Phylogenetic analyses were conducted by use of integrated software as described elsewhere.[21]

## Results

**RT-PCR assay of skin samples**—All skin samples collected from the vaccinated cattle at each time point had negative results for BVDV. Results were negative for RT-PCR assay of both pooled and individual skin samples.

**Virus isolation**—For seropositive steer calves, BVDV was not isolated from serum samples or nasal swab specimens. For seronegative heifers, BVDV was not isolated from nasal swab specimens, but 20 BVDV isolates were identified in serum and buffy coat samples. Four of 7 heifers had positive results for virus isolation from serum on at least 1 day (11 total isolates), and 5 of 7 had positive results for virus isolation from buffy coats on at least 1 day (9 total isolates; **Table 1**).

**VN**—Eleven of 12 seropositive steer calves had neutralizing antibodies against BVDV before vaccination, and the remaining calf had neutralizing antibodies against BVDV by day 18 after vaccination. Conversely, all 7 seronegative heifers lacked detectable concentrations of neutralizing antibodies against BVDV before vaccination but had neutralizing antibodies against BVDV genotype type 1a and 1b by day 18 after vaccination; 6 of 7 seroconverted to BVDV genotype type 2a by day 18 (**Table 2**).

**Biological cloning and sequence analysis**—Biological cloning of the reconstituted vaccine yielded 34 viral isolates that were tested by use of RT-PCR assay for identification of viral genotype. Of the 34 isolates, only

Table 1—Genotype of BVDV isolated from serum and buffy coat samples obtained from 7 seronegative heifers after the cattle were vaccinated with a commercially available modified-live BVDV vaccine.

| Day* | Serum† | Buffy coat† |
|---|---|---|
| 7 | 1a (1) | 1a (2) |
| 8 | 1a (1) | 1a (1); 2a (1) |
| 9 | 1a (1) | ND |
| 10 | 1a (1) | ND |
| 11 | 1a (2) | 1a (1) |
| 12 | 1a (3) | 1a (2); 2a (1) |
| 13 | 1a (2) | 1a (1) |
| 14 | ND | ND |

Numbers in parentheses are the number of cattle from which the genotype was isolated.
*Day of vaccination was designated as day 0. †No BVDV was isolated from 2 cattle.
ND = Not detected.

Table 2—Median (range) of VN results for serum samples obtained from 12 seropositive steer calves and 7 seronegative heifers after cattle were vaccinated* with a commercially available modified-live BVDV vaccine.

| | Singer 1a | | TGAC 1b | | 125C 2a | |
|---|---|---|---|---|---|---|
| **Cattle** | **Day 0** | **Day 18** | **Day 0** | **Day 18** | **Day 0** | **Day 18** |
| Seropositive steer calves | 64 (4–4,096) | 128 (16–2,048) | 16 (4–1,024) | 24 (4–512) | 48 (4–1,024) | 64 (4–2,048) |
| Seronegative heifers† | < 4 | 64 (16–512) | < 4 | 61 (< 4–128) | < 4 | 4 (4–128) |

*Day of vaccination was designated as day 0. †Cattle were considered seronegative at an antibody titer < 4.

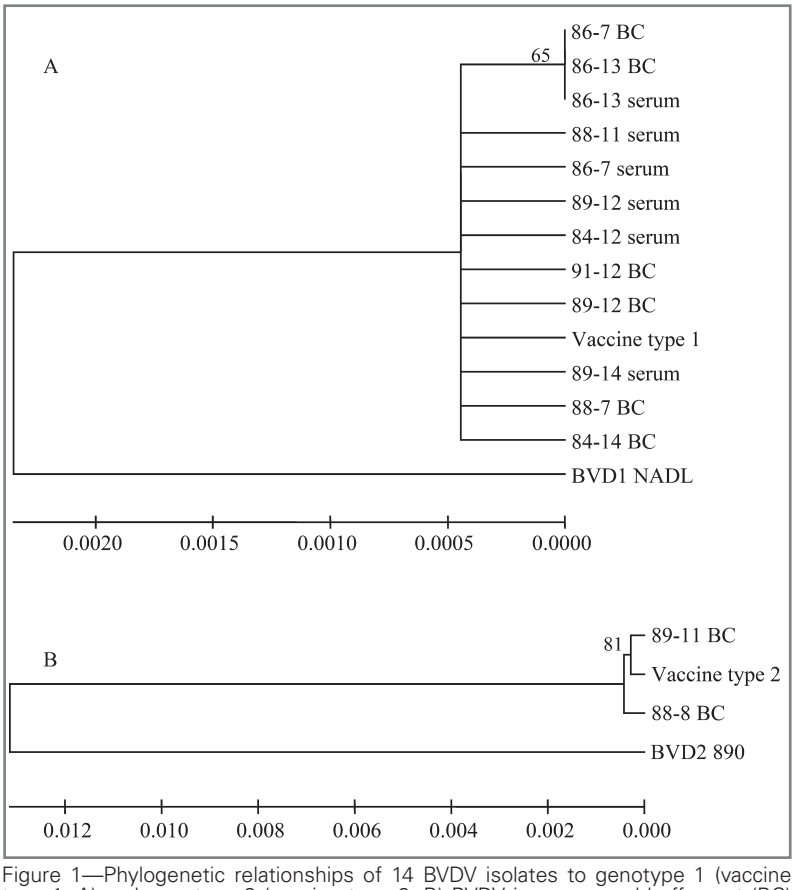


Figure 1—Phylogenetic relationships of 14 BVDV isolates to genotype 1 (vaccine type 1; A) and genotype 2 (vaccine type 2; B) BVDV in serum and buffy coat (BC) samples obtained from 7 seronegative heifers after the cattle were vaccinated with a modified-live BVDV vaccine. National Animal Disease Laboratory (NADL) BVD1 and BVD2 890 were used as reference viruses in panel A and B, respectively. The neighbor-joining method was used to generate the phylogenetic tree. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed by use of the Jukes-Cantor method. The scale at the bottom of each panel represents the number of base substitutions per site; notice that the scales differ between the panels.

1 was classified as BVDV genotype 1, whereas 25 isolates were classified as genotype 2. The remaining 8 viral isolates were a mixture of genotypes 1 and 2. Genetic sequencing of the 26 type 1 and type 2 isolates revealed 99.9% alignment with the type 1 and type 2 viruses that were reportedly contained within the vaccine.[e]

A representative sample of BVDV isolates (12 type 1 and 2 type 2) from the cattle were selected and sequenced for use in comparison with the vaccine strains as well as to identify genetic variation among viruses within each animal. Genetic sequencing revealed that the viruses isolated had 99.9% homogeneity with each other (**Figure 1**). In addition, the nucleic acid sequences of the viral isolates aligned with the sequences of the type 1 and 2 BVDV contained within the vaccine.

## Discussion

In the study reported here, all cattle, regardless of serologic status, had negative results for BVDV when tested by use of RT-PCR assay on individual and pooled skin samples obtained after vaccination with a modified-live BVDV vaccine. These findings indicated that it is unlikely to detect BVDV vaccine virus in skin samples via RT-PCR assay of individual or pooled skin samples obtained after cattle have been vaccinated with the commercially available BVDV vaccine used in the study. These findings are similar to results of other studies[9,10,12] in which investigators evaluated skin samples (obtained after cattle were vaccinated) by use of immunohistochemical analysis for detection of viral antigen. In those studies, investigators were unable to detect BVDV in skin samples from cattle that were acutely infected[9,10] or inoculated with BVDV vaccine.[12]

Bovine viral diarrhea virus was not detected in any of the samples from seropositive steer calves. This result was expected because of the preexisting VN antibody titers in these calves. In seronegative heifers, use of RT-PCR assays (pooled and individual skin samples) did not reveal virus in skin samples of vaccinated cattle. However, virus isolation from serum and buffy coat and seroconversion of calves indicated that the vaccine viruses were replicating in the animals during the testing period. Results of virus isolation from nasal swab specimens, serum samples, and buffy coat samples of seronegative heifers were similar to findings for seronegative cattle reported in other studies.[9,10,22] Although the present study was not designed to determine the reason that virus could not be detected in skin samples, explanations for this finding could include the reduced virulence of the virus used in the vaccine and modification of the location of replication of the vaccine virus. By their nature, modified-live virus vaccines are of reduced

virulence and do not replicate to high numbers, compared with results for virulent field strains of BVDV. It should be mentioned that when virus isolation was performed on serum and buffy coat samples, cytopathic effect often was not observed until ≥ 5 days after inoculation of cell cultures (data not shown). This suggested that there were low numbers of viable virus in these samples. If low numbers of virus exist in the bloodstream, the likelihood of detecting virus from a skin sample by use of any assay, including RT-PCR assay, is minimal. Furthermore, attenuation of the vaccine may change the sites at which the virus replicates. Cytopathic BVDV can be found in the skin of cattle with mucosal disease[23]; however, to our knowledge, no studies have been conducted to evaluate the distribution of modified-live cytopathic vaccine virus in the skin.

The majority (18/20) of the BVDV isolates in the seronegative heifers were genotype 1a. It could be speculated that genotype 1a grew better and replicated better in the vaccinated cattle than did other genotypes. Genotype 1a could be better adapted in vivo and could replicate more efficiently to higher numbers.

A limitation of the present study was that only 1 commercially available modified-live BVDV vaccine was used. The findings from this study should not be extended to other commercially available modified-live or killed virus vaccines. In addition, nonvaccinated control cattle were not included. Nonvaccinated control cattle would have enabled us to detect extraneous virus exposure during the course of the study. However, this limitation was accounted for by sequencing virus isolates from the cattle and comparing those sequences with the sequences of the vaccine virus used in the study. The fact that all virus isolates detected were genetically identical to the viruses in the vaccine provides substantial evidence that the virus we detected originated from the vaccine virus administered.

Although virus was not detected in skin samples collected from the cattle of the present study, it is presumptuous to assume that this could never happen. Host factors, such as immunosuppression, could allow for vaccine virus to replicate to higher numbers or to disseminate more widely into various tissues. Also, perfect specificity of diagnostic assays is almost never obtainable. Inherent imperfection of an assay or laboratory error could lead to false-positive results. These inherent imperfections are the reason that it is recommended to retest cattle to confirm persistent infection with BVDV.

Results of the present study provided evidence that it is unlikely to detect BVDV via RT-PCR assay performed on skin samples obtained from cattle after they have been vaccinated with a commercially available modified-live BVDV vaccine. When this fact is combined with evidence from other studies that have also failed to detect vaccine virus in skin samples by use of various diagnostic assays, veterinarians and producers should be confident that positive test results for BVDV in skin samples are unlikely to be caused by the vaccine virus after administration of a modified-live virus vaccine to cattle.

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