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Case report – Comparison of pooled polymerase chain reaction testing to non-pooled antigen capture enzyme-linked immunosorbent assay to detect bovine viral diarrhea virus persistently infected stocker calves

Brendan Kraus,[1] *DVM*; **Robert W. Fulton,**[2] *DVM, PhD*; **Bill J. Johnson,**[2] *DVM*; **David B. Sjeklocha,**[3] *DVM*
[1]*Spur Ridge Veterinary Hospital, PA, Marion, KS 66861*
[2]*Oklahoma State University Center for Veterinary Health Sciences, Stillwater, OK 74078*
[3]*Cattle Empire, LLC, Satanta, KS 67870*
Corresponding author: Dr. David Sjeklocha, Cattle Empire, LLC, Satanta, KS 67870

Abstract

There are several different methods of testing cattle for persistent infection with bovine viral diarrhea virus (PI-BVDV), including immunohistochemistry (IHC), polymerase chain reaction (PCR), virus isolation, and antigen capture enzyme-linked immunosorbent assay (ACE). The purpose of this report is to describe a case in which a pooled PCR test (28 specimens/pool) was compared for sensitivity against an ACE test (tested as single specimens). Ear notch specimens (fresh) were collected from beef calves purchased by a stocker operation. From January through March 2010, this stocker operation received 2,424 calves and took two ear-notch specimens from each calf for PI-BVDV testing. One specimen from each calf was sent to a commercial PI-BVDV testing laboratory that utilizes real-time PCR technology in specimen pools of 28 (Laboratory A), and a second specimen was sent to a commercial PI-BVDV testing laboratory that utilizes ACE (NS23) technology using non-pooled specimens (Laboratory B). Laboratory A detected four positive specimens out of the 2,424 specimens submitted (0.165% PI prevalence), while Laboratory B detected 12 positive specimens out of the 2,424 specimens submitted (0.495% PI prevalence).

The 12 specimens detected by Laboratory B included the same four detected by Laboratory A, as well as eight additional positive specimens. Upon receiving these discordant results, the attending veterinarian requested that the 12 positive specimens detected by Laboratory B be re-tested using the original specimens. These original 12 ACE-positive specimens were then re-tested as single specimens at Laboratory B using a commercially available ACE test (Erns) and real-time PCR technology. The 12 original ACE-positive specimens were again positive by these different tests. Follow-up testing on the 12 calves was conducted more than 30 days after initial testing with a second set of samples from the eight surviving calves (four of the 12 calves died between the original and the second collection). The second set of samples from the eight surviving calves initially ACE-positive by Laboratory B were tested using serum, formalin-fixed ear notches, and fresh ear notches in phosphate buffered saline. All specimens were positive by gel-based reverse transcriptase PCR on serum and ear notches (non-pooled), and all eight formalin-fixed notches were also positive by IHC.

Key words: cattle, BVDV, persistent infection, testing

Résumé

La détection de l'infection persistante des bovins par le virus de la diarrhée virale des bovins (IP-VDVB) s'effectue suivant diverses méthodes, notamment l'immunohistochimie (IHC), la réaction en chaîne de la polymérase (PCR), l'isolement du virus et la capture d'antigènes par la technique ELISA. Dans ce rapport, nous comparons la sensibilité entre un test par PCR effectué sur des spécimens regroupés (28 spécimens par regroupement) et un test par capture d'antigènes effectué sur des spécimens distincts. Nous avons prélevé des biopsies cutanées d'oreille (conservées fraîches) sur des veaux de boucherie achetés par un parc d'élevage. De janvier à mars 2010, ce parc a reçu 2 424 veaux, sur chacun desquels furent prélevées deux biopsies pour la détection de l'IP-VDVB. Nous avons envoyé un échantillon de biopsie de chaque veau dans un laboratoire commercial qui réalise la détection de l'IP-VDVB par PCR en temps réel par regroupements de 28 échantillons (Laboratoire A) et l'autre échantillon de biopsie dans un

second laboratoire commercial qui détecte l'IP-VDVB par capture d'antigènes (NS23) sur des échantillons non regroupés (Laboratoire B). Le Laboratoire A a détecté quatre spécimens positifs sur les 2 424 échantillons analysés (une prévalence d'IP de 0,165 %) et le Laboratoire B a détecté 12 spécimens positifs sur les autres 2 424 échantillons des mêmes veaux (une prévalence d'IP de 0,495 %).

Les 12 spécimens positifs détectés par le Laboratoire B comprenaient les quatre mêmes spécimens déclarés positifs par le Laboratoire A, plus huit autres spécimens positifs. À la vue de ces résultats discordants, le médecin vétérinaire en poste a demandé que soient retestés les 12 spécimens détectés positifs (par capture d'antigènes) par le Laboratoire B à partir des veaux d'origine. Le second test réalisé par le Laboratoire B sur ces 12 spécimens testés positifs fut effectué sur des échantillons individuels (distincts) au moyen d'un test de capture d'antigènes commercial (Erns) et par analyse de PCR en temps réel. Les 12 spécimens positifs se sont à nouveau révélés positifs selon ces deux tests. Un test fut une nouvelle fois effectué plus de 30 jours plus tard sur les mêmes veaux (qui n'étaient plus que huit, la maladie en ayant tué quatre entre le premier et le deuxième prélèvement). Chez ces huit veaux survivants testés positifs par capture d'antigènes par le Laboratoire B, nous avons analysé le sérum, les biopsies d'oreilles conservées dans le formol et des biopsies fraîches conservées dans une solution saline tampon phosphatée. Tous les spécimens se sont révélés positifs selon la PCR sur gel avec réverse-transcriptase, effectuée sur le sérum et les biopsies fraîches d'oreille (non regroupées) et l'IHC a déclaré positifs tous les huit échantillons de biopsies d'oreille conservées dans le formol.

Introduction

Infection with bovine viral diarrhea virus (BVDV) has been shown to be a source of financial loss for cattle producers.[6] Cattle persistently infected (PI) with BVDV are the primary source for new infections within and among herds. The advent of diagnostic tests that utilize ear notches (skin) has made testing for PI-BVDV cattle more cost and time efficient compared to repeated collections and submissions for virus isolation. However, in order to be effective, the test must detect a positive PI animal among a group with low prevalence. A test should have a relatively high sensitivity—the ability to correctly identify the true positive—otherwise the threat from PI-BVDV may not be removed. Pooling of specimens offers the benefit of decreased testing costs, but extensive pooling may reduce the ability to detect all the PI-BVDV positive animals.[10] This case report questions the diagnostic sensitivity of determining the PI-BVDV status using pooled samples for certain polymerase chain reaction (PCR) tests, which has been reported by Edmondson[1] and Ridpath.[9]

History

In 2009, a stocker operation in eastern Kansas utilized a commercial laboratory (Laboratory A) for testing incoming cattle for PI-BVDV. These crossbred calves had genetics that included English, Continental, and Brahman influence, and their vaccination history was unknown. They were purchased by an order-buyer from livestock auctions in Missouri, Arkansas, and Kansas. In all, Laboratory A tested 1,183 head of 450-500 lb (200-230 kg) calves for this stocker operation in 2009, and reported three calves positive for PI-BVDV (0.25% prevalence). Previous testing experience (at Laboratory B[a]) on this operation resulted in a prevalence of 0.40%. Earlier studies using calves of similar weight and origin suggest a higher prevalence as well when cattle were tested using antigen capture enzyme-linked immunosorbent assay (ACE).[6] Beginning in January of 2010 and running through March, a comparison was conducted to evaluate the difference in outcomes between real time PCR testing utilizing 28 specimens per pool and the ACE[a] test utilizing non-pooled specimens.

Materials and Methods

Cattle were received into a commercial stocker operation utilizing standard animal health protocols as established by the author (BK). At initial processing, two identical-sized ear notch specimens were taken from each calf and placed into separate vials according to a standard protocol established by each respective laboratory. Each vial was individually identified, corresponding to the eartag number assigned to each calf. Specimens were frozen immediately after collection in a frost-free freezer at 0° F (-17.8° C) and shipped via overnight carrier to Laboratory A and Laboratory B in batches of 300-600 every two to three weeks until the study was completed. All samples were collected, handled, and shipped according to protocol developed by each of the respective laboratories. Laboratory A placed the ear notch specimens in pools of 28, and used real-time PCR technology for PI-BVDV detection, while Laboratory B tested specimens as non-pooled specimens using ACE[a] technology. To verify results, Laboratory B re-tested all positive specimens using real-time PCR,[b] but as non-pooled specimens, and a different commercially available Erns-based ACE test,[c] again as non-pooled specimens. Once confirmed, fresh specimens were obtained from all surviving PI-BVDV positive animals (eight of the 12) at least 30 days (range 36-66 days) after initial sampling for further verification of results. The second set of specimens included two ear notches (one in phosphate buffered saline (PBS) and one

in 10% formalin) and one serum specimen per calf. These specimens were sent to Oklahoma State University for testing using reverse transcriptase PCR (gel-based) on non-pooled specimens of serums and fresh notches, and immunohistochemistry (IHC) testing on 10% formalin-fixed ear notches using described procedures.[3]

Results

Upon initial testing of the 2,424 calves, Laboratory A reported four of 2,424 calves positive for PI-BVDV (0.165% prevalence) using the pooled PCR test. These four calves were identified by the stocker operation as numbers 4520, 4614, 4660, and 5211. Laboratory B, using ACE[a] testing on non-pooled specimens, reported these same four calves as positive, but also detected eight additional PI-BVDV positive calves for a total of 12 of 2,424 PI-BVDV positive calves (0.495% prevalence). The eight additional PI-BVDV positive calves were identified as numbers 514, 723, 5237, 5261, 5277, 5279, 5557, and 5653. Laboratory B further tested these 12 positive animals with real-time PCR[b] as non-pooled specimens and a commercially available Erns-based ACE test.[c] All 12 were positive on the second ACE[c] test, but only nine were positive using the PCR[b] test. The three that were negative on the PCR test were numbers 723, 4520, and 4614. These three specimens were removed from their original vial, washed with de-ionized water, re-cut to expose fresh epidermis, and placed in fresh PBS and re-tested 24 hours later, again as non-pooled specimens using PCR.[b] At this point, specimens 723, 4520, and 4614 tested positive (Table 1).

Approximately one to two months (range 36-66 days) after initial tests were complete, a new specimen was obtained from each surviving PI-BVDV positive calf for confirmatory testing. This allowed for eight of the 12 original calves to be re-tested (four of the original 12 positive calves detected by Laboratory B died after the initial sample collection). Serum and two ear notch samples (one ear notch placed in 10% formalin, the other in PBS) were collected from each calf and these specimens were sent to Oklahoma State University for additional confirmatory testing. All specimens were tested as single specimens. IHC was conducted on the 10% formalin-fixed ear notch sample, reverse transcriptase PCR (gel-based) was conducted on the second ear notch sample (placed in PBS) and reverse transcriptase PCR (gel-based) was conducted on the serum from each calf. Calves with ID numbers 723, 5211, 5279, and 5557 died before second samples could be acquired, so Laboratory B sent the initial ear notch specimens from those animals to Oklahoma State University, and these specimens were tested with reverse transcriptase PCR (gel-based). All specimens were positive for PI-BVDV for each respective test (Table 2).

The sensitivity and specificity for Laboratory A's pooled real-time PCR was calculated using the results from the 2,424 calves tested: specificity = number of true negatives/number of true negatives + number of false positives (2412/2412); and sensitivity = number of true positives/number of true positives + number of false negatives (4/4+8). In this case, the pooled reverse transcriptase PCR test utilized by Laboratory A had a specificity of 100% and a sensitivity of 33.3%. These calculations of specificity and sensitivity are based on the results from Laboratory B as the standard.

In this case, it appears that a pooled PCR test did not identify 66.7% (eight of 12) of PI-BVDV calves

Table 1. Results from initial samples/tests.

Calf ID	Lab A PCR pooled	Lab B ACE single	Lab B PCR single	Lab B 2nd ACE single
514	Negative	Positive	Positive	Positive
723	Negative	Positive	Positive (re-cut)	Positive
4520	Positive	Positive	Positive (re-cut)	Positive
4614	Positive	Positive	Positive (re-cut)	Positive
4660	Positive	Positive	Positive	Positive
5211	Positive	Positive	Positive	Positive
5237	Negative	Positive	Positive	Positive
5261	Negative	Positive	Positive	Positive
5277	Negative	Positive	Positive	Positive
5279	Negative	Positive	Positive	Positive
5557	Negative	Positive	Positive	Positive
5653	Negative	Positive	Positive	Positive

Table 2. Results of follow-up testing as compared to initial results from Laboratory A.

Calf ID	Lab A PCR pooled (initial test)	OSU IHC follow-up test	OSU PCR serum (single) follow-up	OSU PCR notch (single) follow-up
514	Negative	Positive	Positive	Positive
723	Negative	N/A Dead	N/A Dead	Positive (initial sample)
4520	Positive	Positive	Positive	Positive
4614	Positive	Positive	Positive	Positive
4660	Positive	Positive	Positive	Positive
5211	Positive	N/A Dead	N/A Dead	Positive (initial sample)
5237	Negative	Positive	Positive	Positive
5261	Negative	Positive	Positive	Positive
5277	Negative	Positive	Positive	Positive
5279	Negative	N/A Dead	N/A Dead	Positive (initial sample)
5557	Negative	N/A Dead	N/A Dead	Positive (initial sample)
5653	Negative	Positive	Positive	Positive

when compared to two ACE tests using non-pooled specimens, and under special processing conditions, a PCR test on non-pooled specimens. Also, a third laboratory verified these results using PCR on the four original samples from the calves that died and, on a second set of specimens from the surviving eight head, IHC (on 10% formalin-fixed ear notches) and PCR on serum and fresh ear notches were utilized.

Discussion

Although the ACE test detected three times as many PI-BVDV cattle (12 vs four) as the pooled PCR test, the possibility exists that not all PI-BVDV cattle were detected in this case by either testing method. Indeed, only eight of the 12 were verified as persistently infected as four died prior to confirmatory testing. It can be said that the four calves that died were infected with BVDV, but it cannot be accurately stated that these four were in fact persistently infected because follow-up testing was not possible, therefore leaving some room for error. Results of this comparison indicate that the pooled real-time PCR methods utilized by Laboratory A did not detect all of the PI-BVDV cattle in this case report (four of 12 or 33.3% detected).

Use of specificity alone may be misleading in validating diagnostic tests, especially when dealing with a disease with a low prevalence. In this case, commercial Laboratory A had a specificity of 100% as it recognized all of the negatives when compared to the ACE test; however, the calculated sensitivity was 33.3% as the pooled PCR test failed to detect all positive specimens. When using PCR on non-pooled specimens, Laboratory B was able to detect all 12 PI-BVDV positive animals, but it was still necessary to freshen the cut surface of the epidermis on three of these non-pooled specimens.

Extensive pooling reduces test costs for the laboratory, and the laboratory then determines how this cost savings is passed on to clients. Publicly accessible information from a variety of websites indicates that the cost for non-pooled ACE testing ranges from $2.50 to $5.00 per sample. In comparison, the cost of pooled PCR testing ranges from $1.95 to $3.95 per sample, and non-pooled PCR testing is listed at $25.00 to $60.00 per sample. This shows a definite cost-saving advantage to pooling PCR tests. The disadvantage demonstrated in this report is that PCR testing of 28-specimen pools has a much lower diagnostic sensitivity relative to the non-pooled ACE.

In this case, it appears that the pooled PCR test did not detect all PI cattle. Ridpath[9] demonstrated that pooling real-time PCR in 10-specimen pools may decrease sensitivity to the point of failure to detect 10% of positive specimens. Further, she showed that 100-specimen pools may result in failure to detect over 50% of PI-BVDV positive specimens. The Wyoming State Veterinary Diagnostic Laboratory has also investigated pooling of ear notch samples with reverse transcriptase PCR, and determined that test performance was not sufficiently acceptable to continue pooling (J. Cavender, personal communication, 2010). In contrast, Kennedy[8] reported that pooling with reverse transcriptase PCR detected 100% of cattle positive for PI-BVDV, and in another study[7] he showed that reverse transcriptase PCR

detected more PI-BVDV positive cattle than did ACE. The discrepancies in these references indicate that more controlled sensitivity studies are needed.

Finding and removing PI-BVDV cattle can be an excellent tool for biosecurity; however, a test that detects all positive specimens should be used. In the case presented here, the real-time PCR test pooled at the described level did not detect all the positive specimens. Testing non-pooled specimens using real-time PCR technology detected nine of the 12 specimens that were positive at Laboratory B. The eight fresh follow-up ear notch specimens tested by gel-based reverse-transcriptase PCR at Oklahoma State University were all detected as positive for PI-BVDV as non-pooled specimens. In this case report, PCR diagnostic sensitivity was suspect with pooled and non-pooled specimens. It could be a function of Laboratory A's pool size, laboratory technique, technician error, or a combination of any of these factors. In addition, the low sensitivity may not reflect the sensitivity of pooled PCR testing in other laboratories or for other antigens and, therefore, should not be extrapolated to all pooled PCR testing.

The pooled PCR test sensitivity in this case report raises several concerns: 1) cattle operations that have utilized pooled PCR testing methods may be operating under the belief that they have removed PI-BVDV animals when there could be undetected cattle positive for PI-BVDV still in the herd; 2) these cattle operations may have marketed their cattle as "PI-Free", and the buyer may have paid a premium for this status when there were still PI cattle present; 3) producers may become disappointed and cease testing for PI-BVDV because they do not perceive the benefits that testing and removing PI cattle may offer, thereby passing up an opportunity to improve herd biosecurity; and 4) the effect of a single PI-BVDV calf not detected by diagnostic testing can be substantial.[2,5]

Conclusions

In this case, PCR in 28-specimen pools did not identify 66.7% (eight of 12) of the cattle positive for PI-BVDV. Four of the 12 calves positive on the ACE test died before follow-up samples could be collected for confirmation testing; however, the available specimens from these four calves were positive to an array of other testing methods, which strongly suggests they were PI-BVDV. Testing of follow-up samples from the surviving eight calves confirmed their PI status. Even though ACE did detect more PI cattle, it is possible that it did not identify all PI-BVDV cattle in this case. However, the ACE test did detect three times more positive specimens than the pooled PCR test. Because of this, more PI cattle were removed from the population using ACE testing than would have been removed using the results of the pooled PCR test. Veterinarians and their clients need to be aware of the testing methods being utilized by PI-BVDV laboratories they use, and consider strengths and weaknesses of those testing methods when choosing a laboratory.

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Dr. Sjeklocha (corresponding author) was an owner of Laboratory B when this manuscript was submitted, but no longer has any financial or operational interest in the laboratory.

Endnotes

[a]Central States Testing® NS23, Central States Testing®, Sublette, KS
[b]Life Technologies®, Austin, TX (formerly Applied Biosystems®, Carlsbad, CA)
[c]IDEXX HerdChek®, Westbrook, ME

References

1. Edmondson MA, Givens MD, Walz PH, Gard JA, Stringfellow DA, Carson RL. Comparison of tests for detection of bovine viral diarrhea virus in diagnostic specimens. *J Vet Diag Invest* 2007;19:376-381.
2. Fulton RW, Briggs RE, Ridpath JF, Saliki JT, Confer AW, Payton ME, Duff GC, Step DL, Walker D. Transmission of bovine viral diarrhea virus 1b to susceptible and vaccinated calves by exposure to persistently infected calves. *Can J Vet Res* 2005;69:161-169.
3. Fulton RW, Hessman B, Johnson BJ, Ridpath JF, Saliki J, Burge LJ, Sjeklocha D, Confer AW, Funk R, Payton ME. Evaluation of diagnostic tests used for detection of bovine viral diarrhea virus and prevalence of BVDV subtypes 1a, 1b, and 2a in persistently infected cattle entering a feedlot. *J Am Vet Med Assoc* 2006;228:578-584.
4. Fulton RW, Hessman BE, Ridpath JF, Johnson BJ, Burge LJ, Kapil S, Braziel B, Kautz K, Reck A. Multiple diagnostic tests to identify cattle with bovine viral diarrhea virus and duration of positive test results in persistently infected cattle. *Can J Vet Res* 2009;73:117-124.
5. Fulton RW, Johnson BJ, Briggs RE, Ridpath JF, Saliki JT, Confer AW, Burge LJ, Step DL, Walker DA, Payton ME. Challenge with bovine viral diarrhea virus by exposure to persistently infected calves: protection by vaccination and negative results of antigen testing in acutely infected calves. *Can J Vet Res* 2006;70:121-127.
6. Hessman BE, Fulton RW, Sjeklocha DB, Murphy TA, Ridpath JF, Payton ME. Evaluation of economic effects and the health and performance of the general cattle population after exposure to cattle persistently infected with bovine viral diarrhea virus in a starter feedlot. *Am J Vet Res* 2009;70:73-85.
7. Kennedy JA. Diagnostic efficacy of a reverse transcriptase-polymerase chain reaction assay to screen cattle for persistent bovine viral diarrhea virus infection. *J Am Vet Med Assoc* 2006;229:1472-1474.
8. Kennedy JA, Mortimer RG, Powers B. Reverse transcription-polymerase chain reaction on pooled specimens to detect bovine viral diarrhea virus by using fresh ear-notch-specimen supernatants. *J Vet Diagn Invest* 2006;18:89-93.
9. Ridpath JF, Hessman BE, Neill JD, Fulton RW, Step DL. Parameters of ear notch specimens for BVDV testing: stability, size and viral load. *Proc Am Assoc Bov Pract Conf* 2006;39:269-270.
10. Ridpath JF, Hessman BE, Neill JD, Fulton RW, Step DL, Zimmerman A, Chase CL. Evaluating stability, size requirements, viral load and pooling of ear notch specimens in BVDV testing [abstract]. *Am Assoc Vet Lab Diagn* 2006;213.