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Reverse transcription-polymerase chain reaction on pooled samples to detect bovine viral diarrhea virus by using fresh ear-notch–sample supernatants

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Abstract. Ear-notch samples from 3,599 yearling heifers were collected to detect persistently infected (PI) animals with suspect bovine viral diarrhea virus (BVDV). Individual immunohistochemistry (IHC), individual antigen-capture enzyme-linked immunosorbent assay (AC-ELISA), and reverse transcription-polymerase chain reaction (RT-PCR) tests with pooled ear-notch supernatants were compared with samples from 3,016 heifers, whereas RT-PCR ear-notch pools and individual AC-ELISA tests were compared with samples from all 3,599 heifers. Four heifers were identified positive by both IHC and AC-ELISA, whereas the remaining heifers were identified negative by both tests. When supernatant from ear notches from 100 animals was randomly pooled and RT-PCR was accomplished on each pool, RT-PCR identified 2 pools that contained 1 positive AC-ELISA sample and 1 pool that contained 2 positive AC-ELISA samples. Further evaluation of the pooled RT-PCR ear-notch supernatant detected 100% ($n = 36$) samples spiked with supernatant from a single randomly selected positive AC-ELISA ear notch. Although follow-up confirmatory tests were not completed, all 3 methods correlated 100% in detecting suspect PI animals, with a kappa value of 1. The use of RT-PCR on pooled ear-notch supernatant could provide an initial, rapid, cost-effective method of screening cattle herds for BVDV PI animals. Subsequent serial testing with an AC-ELISA to evaluate individual samples included in the positive pool could minimize the length of time other animals are exposed to the virus.

Key words: Bovine viral diarrhea virus; enzyme-linked immunosorbent assay; immunohistochemistry; persistent infection; reverse transcription-polymerase chain reaction.

Bovine viral diarrhea virus (BVDV) is a major viral disease affecting beef cattle reproduction and performance.^{1,3} The key source of BVDV infection is the BVDV persistently infected (PI) animal.¹⁶ Such animals are the result of fetal exposure to the virus before the development of its immune system approximately between day 18 and day 125 of gestation.¹ Exposure to the virus before day 18 may result in embryonic death and apparent infertility, whereas exposure after day 125 is more commonly associated with abortion, stillbirths, or congenital abnormalities.³ Persistently infected calves are more efficient than transiently in-

fecting ones in spreading BVDV to other animals.¹⁶ Current initiatives by the National Cattlemen Beef Association, American Association of Bovine Practitioners, the Academy of Veterinary Consultants, and state livestock associations to develop effective BVDV-control programs are underway. Control programs hinge on removal of the PI animal to eliminate the most important source of exposure, on effective vaccination programs, and on herd-level biosecurity.

Currently available methods to detect PI cattle include virus isolation (VI), reverse transcription-polymerase chain reaction (RT-PCR), immunohistochemistry (IHC), and antigen-capture enzyme-linked immunosorbent assay (AC-ELISA).^{4,5,9–11} Diagnostic tests such as VI, RT-PCR, IHC on ear notches, AC-ELISA on serum, or ear-notches supernatant are some of the most commonly used tests to detect the presence of a PI animal.¹⁵ Virus isolation and RT-PCR are costly when used on individuals within a population, whereas IHC and AC-ELISA are cost friendly, with charges

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ranging nationally from \$3 to \$5 per animal tested. The cost difference between IHC on ear notches and AC-ELISA is moderate, with the IHC approximately \$0.50 per head less expensive than the AC-ELISA. However, the AC-ELISA test can be accomplished in less than 5 hours of laboratory time as compared with 3–5 days for IHC, thereby allowing animals to be removed sooner and lessening the potential threat of acute infections. This study verified the results of AC-ELISA on ear-notch supernatant with IHC of ear notches to detect the presence of BVDV. All current tests require that follow-up testing be completed at least 3 weeks later to confirm initial positive samples are truly positive and identify PI animals and not acutely infected animals. However, regarding the subject animals in this report, the management decision was made to eliminate the animals after a single initial positive test and to forgo the follow-up second testing, thus decreasing the time to removal of PI animals and minimizing the risk of further infection.

Additionally, the study evaluated the use of a pooled RT-PCR to screen large groups of heifers for the presence of the BVDV. Reverse transcription PCR screening was accomplished on the same phosphate-buffered saline (PBS) supernatant solution that the AC-ELISA test used. Reverse-transcriptase PCR on pooled samples can be accomplished cost effectively and has a rapid laboratory turnaround time of less than 48 hours.

Two triangular ear notches ~2 cm in height with a base of ~1 cm were collected from 3,016 yearling heifers. Additionally, a single notch was collected from 583 yearling heifers. All the heifers sampled were owned by a single commercial cattle operation and represented 7 distinct herds. Pairs of tubes were sequentially numbered beginning at 1 and ending at 3,016; only single tubes were numbered from 3,016 to 3,599. A record recording the tube number and an animal identification was logged so positive animals could be identified and removed. One ear notch from each replacement heifer was placed in a sterile 10-ml clot tube and shipped via a commercial carrier to a branch veterinary diagnostic laboratory, where 2 ml of PBS was added to each tube. Antigen-capture ELISA testing was accomplished on the fluid contained in the individual tubes by using the protocol as outlined in the test-kit package insert provided by the manufacturer.^a The additional ear notches from the first 3,016 samples were placed into a dry tube and shipped to a second laboratory for individual animal testing by IHC. Upon arrival at the laboratory, ear notches were fixed in 10% buffered formalin for a minimum of 24 hours, embedded in paraffin with 6-chambered cassettes,^b sectioned, mounted, and then stained with a commercial autostainer^c and BVDV monoclonal antibody^d at a working dilution of 1:500. Because of the

remote location of the cattle and inclement weather, the in-transit time for shipment ranged from 3 to 10 days.

Pools of ear-notch PBS supernatant were formed by grouping tubes 1–100 into pool 1, tubes 101–200 into pool 2, and so on until pool 36, which contained tubes 3501–3599. A 250- μ l aliquot of the PBS solution was withdrawn from each tube with a sterile disposable polyethylene transfer pipette.^e To minimize the possibility of contamination among samples, pipettes were changed after each tube and a complete cleanup between pools was accomplished. Laboratory technicians processed samples and assembled pools in approximately 20 minutes per pool of 100. Each sample aliquot was pooled to form 35 pools representing 100 animals and 1 pool representing 99 animals. A 350- μ l aliquot was then withdrawn from each of the 36 pools so that individual RT-PCRs could be accomplished on each pool. Once pools were formed, RNA extraction was accomplished with a commercial kit.^f Kit selection was based on current laboratory availability and not with the intent that one kit might release more viral RNA from cells contained in the supernatant than another kit. Reverse-transcriptase PCR on each of the 100 sample pools was performed by using primer sequences previously described to detect type I and type II BVDV from the 5' untranslated region of the genome; however, individual typing was not performed.¹³ Positive and negative controls were accomplished with each RT-PCR run to ensure test validity. Because of the low number of positive pools identified from the subject cattle and to further evaluate the RT-PCR pooling concept, another 36 pools from 99 AC-ELISA-negative samples were spiked with PBS solution from a single positive AC-ELISA ear notch, accomplishing an additional check of the procedure. Each of the positive AC-ELISA ear notches used in spiking was randomly selected from a cross section of positive notches submitted to the laboratory.

Evaluators of the individual IHC, AC-ELISA, and pooled RT-PCR tests were blinded as to the outcome of the concurrent testing results. Statistical analysis of the results was accomplished by using DAG_Stat.⁸ There was no difference in the individual samples tested by IHC and those tested by AC-ELISA, with a kappa value of 1. The results of RT-PCR on pooled PBS were positive for BVDV when at least 1 positive AC-ELISA ear notch was contained in the pool of 100 samples. No RT-PCR pools were falsely classified when they did not contain a positive AC-ELISA (Table 1). When pooled in groups of 100 samples, the pooled RT-PCR detected the presence of BVDV in the 3 pools that corresponded to the 4 positive AC-ELISA samples. Two of the positive samples were contained in the same pool of 100 samples. The results of the ad-

Table 1. Results of 3 techniques to detect the presence of bovine viral diarrhea virus (BVDV).*

Pool	Samples	BVD RT-PCR results	AC-ELISA results by sample no.	IHC results by sample no.
1	1–100	not detected	no positives	no positives
2	101–200	not detected	no positives	no positives
3	201–300	not detected	no positives	no positives
4	301–400	not detected	no positives	no positives
5	401–500	not detected	no positives	no positives
6	501–600	not detected	no positives	no positives
7	601–700	not detected	no positives	no positives
8	701–800	not detected	no positives	no positives
9	801–900	not detected	no positives	no positives
10	901–1,000	not detected	no positives	no positives
11	1,001–1,100	not detected	no positives	no positives
12	1,101–1,200	not detected	no positives	no positives
13	1,201–1,300	not detected	no positives	no positives
14	1,301–1,400	not detected	no positives	no positives
15	1,401–1,500	positive	1,433 and 1,442 positive, all others negative	1,433 and 1,442 positive, all others negative
16	1,501–1,600	not detected	no positives	no positives
17	1,601–1,700	not detected	no positives	no positives
18	1,701–1,800	positive	1,718 positive, all others negative	1,718 positive, all others negative
19	1,801–1,900	positive	1,899 positive, all others negative	1,899 positive, all others negative
20	1,901–2,000	not detected	no positives	no positives
21	2,001–2,100	not detected	no positives	no positives
22	2,101–2,200	not detected	no positives	no positives
23	2,201–2,300	not detected	no positives	no positives
24	2,301–2,400	not detected	no positives	no positives
25	2,401–2,500	not detected	no positives	no positives
26	2,501–2,600	not detected	no positives	no positives
27	2,601–2,700	not detected	no positives	no positives
28	2,701–2,800	not detected	no positives	no positives
29	2,801–2,900	not detected	no positives	no positives
30	2,901–3,000	not detected	no positives	no positives
31	3,001–3,100	not detected	no positives	no positives
32	3,101–3,200	not detected	no positives	no positives
33	3,201–3,300	not detected	no positives	no positives
34	3,301–3,400	not detected	no positives	no positives
35	3,401–3,500	not detected	no positives	no positives
36	3,501–3,599	not detected	no positives	no positives

* RT-PCR = reverse transcription-PCR; AC-ELISA = antigen capture ELISA; IHC = immunohistochemistry.

ditional spiked pools of 100 samples showed that RT-PCR detected the presence of the single positive AC-ELISA sample in all 36 spiked samples. Statistical analysis of the RT-PCR PBS 100 sample pools and the corresponding individual AC-ELISA including the spiked samples showed a kappa value of 1, indicating complete agreement.

In this study, no difference was detected in the ability of IHC or AC-ELISA to detect individual positive samples. Previously reported sensitivities of 100% were attained, whereas previously reported specificities of 98.8% for IHC and 98.4% for AC-ELISA could not be evaluated without follow-up testing.² However, the comparison suggests that the specificity of the 2 tests was identical. Furthermore, pooling of PBS solutions from soaked ear notches and performing RT-

PCR on these PBS pools representative of 100 animals provided a screening method that detected all the individual positive IHC and positive AC-ELISA samples. The advantage of subsequent utilization of the AC-ELISA in detecting individual potentially PI animals lies in the ability of the test to be completed in hours versus days of laboratory time. Antigen-capture ELISA also affords the advantage of being a totally objective test, whereas IHC is subjective and has potential for misinterpretation because of technician fatigue or inexperience. The dairy industry has used RT-PCR to successfully detect the presence of BVDV in bulk-tank milk samples, but that particular source is not available in beef cow operations.^{12,14} Pooled sample testing to screen herds by using PCR on blood and methods of establishing pool size are not novel. A po-

tential pitfall of pooled RT-PCR is that the test detects all BVDV whether the source is a PI animal, an acutely infected animal, or a contaminant. With the exception of contamination, a positive RT-PCR indicates that the virus is present within the tested population and the potential of exposure to a PI animal exists or existed even if no AC-ELISA are identified when follow-up testing is accomplished. Another possible use of the RT-PCR ear-notch pools and follow-up AC-ELISA exists in feedlot cattle, where PI cattle are associated with an increase in respiratory tract disease.⁷ Screening ear notches by pooled RT-PCR could offer a low-cost yet highly sensitive test to detect BVDV. Screening ear notches with pooled RT-PCR followed by AC-ELISA with PBS solution to detect individual positive samples offers the potential for performing serial testing to minimize expense and laboratory time^{6,10} and could allow potential PI animals to be removed from the herd in less than 72 hours of laboratory time at a reasonable cost to the producer.

For this study, pool size was determined based upon data management and the ease of handling the volume of the generated pools. Further attention to pool size might be given when considering 3 key components in selecting a pool size. The first is the size of the herd being tested, for some herds of interest might not contain 100 animals. If pools of fewer than 100 samples are encountered, the testing protocol may need to be adjusted. A second consideration in determining pool size is the ability of the test to detect the presence of the virus at various pool sizes. Initially, an RT-PCR was conducted on a shammed pool of 399 negative samples and 1 positive AC-ELISA. The test successfully detected the presence of viral RNA within the pool. For this study, 72 pools of 100 correlated 100% to the results of AC-ELISA. A statistical analysis of these results would indicate an average sensitivity of 100% with a lower confidence limit (CL) at the 95% level of 90.97%, coupled with an average specificity of 100% with a lower CL at the 95% level of 89.42%. Increasing the pool size above 100 might result in a failure to detect the virus if the dilution were to become great enough. Conversely, decreasing the pool size could allow for more frequent detection of transient infections and a subsequent decrease in the number of times the pool was confirmed to contain a positive AC-ELISA. The other component of the pool size is the economics of serial testing. The size of pools could be decreased, but the advantage of doing so must be carefully considered. In this group of animals, the 3,599 samples were pooled in groups of 100 for a producer cost of \$1,800 (\$35 for each RT-PCR and \$0.15 per sample). The detection of individual positive samples in the 3 positive pools cost the producer an additional \$1,200 for a total of \$3,000 to test this pop-

ulation of cattle. Pool sizes of fewer than 100 animals could be used, thus decreasing the number of tests within a pool needed to identify positive individuals, but there would be an increase in the total charges for the pools. Hypothetically, pools of 50 samples would have cost the producer \$42.50 (\$35 for each RT-PCR and \$0.15 for each sample pooled). Seventy-two pools of 50 samples would be required to test this population of cattle at a cost of \$3,060, with additional charges to identify the individual. The determination of pool size is in part a function of previous knowledge of the potential of BVDV infection and the likelihood of PI animals being present. When considering BVD control and eradication programs, the utilization of RT-PCR pools followed by AC-ELISA or IHC on individual animals in the pool are viable methods to economically and rapidly screen herds and identify suspect animals. The use of either AC-ELISA or IHC to identify individual potential PI cattle in positive pools is a more economical approach than using RT-PCR alone.¹⁰

Sources and manufacturers

- a. Bovine Virus Diarrhea Antigen Test Kit®, IDEXX laboratories, Westbrook, ME.
- b. Fisher Tissue Path Microsette Biopsy Cassettes®, Fisher Scientific, Denver, CO.
- c. BenchMark®, Ventana Medical Systems Inc., Tuscon, AZ.
- d. Anti BVD Monoclonal antibody®, IDEXX laboratories, Westbrook, ME.
- e. Disposable Polyethylene Transfer Pipets®, Fisher Scientific, Pittsburgh, PA.
- f. Qiagen QIAamp®, RNA blood, Qiagen Sciences, Germantown, MD.

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Detection of foot-and-mouth disease virus: comparative diagnostic sensitivity of two independent real-time reverse transcription-polymerase chain reaction assays

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Abstract. Rapid and accurate diagnosis is central to the effective control of foot-and-mouth disease (FMD). It is now recognized that reverse-transcription polymerase chain reaction (RT-PCR) assays can play an important role in the routine detection of FMD virus (FMDV) in clinical samples. The aim of this study was to compare the ability of 2 independent real-time RT-PCR (rRT-PCR) assays targeting the 5' untranslated region (5'UTR) and RNA polymerase (3D) to detect FMDV in clinical samples. There was concordance between the results generated by the 2 assays for 88.1% (347 of 394) of RNA samples extracted from suspensions of epithelial tissue obtained from suspect FMD cases. The comparison between the 2 tests highlighted 19 FMDV isolates (13 for the 5'UTR and 6 for the 3D assay), which failed to produce a signal in 1 assay but gave a positive signal in the other. The sequence of the genomic targets of selected isolates highlighted nucleotide substitutions in the primer or probe regions, thereby providing an explanation for negative results generated in the rRT-PCR assays. These data illustrate the importance of the continuous monitoring of circulating FMDV field strains to ensure the design of the rRT-PCR assay remains fit for purpose and suggest that the use of multiple diagnostic targets could further enhance the sensitivity of molecular methods for the detection of FMDV.

Key words: Foot-and-mouth disease virus; real-time reverse transcription-polymerase chain reaction; 5' untranslated region.

Foot-and-mouth disease (FMD) is a contagious viral disease affecting cloven-hoofed livestock, principally cattle, pigs, sheep, and goats. The 2001 outbreak in

the United Kingdom highlighted the devastating impact of FMD in a country previously free from the disease. This outbreak lasted for approximately 7 months, requiring the slaughter of 6.5 million animals for its control, and is estimated to have cost £8 billion to the UK economy. Rapid and accurate diagnosis plays an important role for the implementation of effective measures to control the spread of disease. The causative agent, FMD virus (FMDV), is a highly variable RNA virus that exists as 7 immunologically distinct serotypes, which in most cases can be further subdivided into a number of topotypes.⁵ Recently, real-time reverse transcription-polymerase chain reaction

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