

## Comparison of ear notch immunohistochemistry, ear notch antigen-capture ELISA, and buffy coat virus isolation for detection of calves persistently infected with bovine viral diarrhea virus

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**Abstract.** Two techniques performed on skin biopsy samples (ear notches), immunohistochemistry (IHC) and antigen-capture ELISA (AgELISA), were compared for detection of bovine viral diarrhea virus (BVDV) persistent infection (PI) in 559 Angus calves between the ages of 1 and 5 months. The calves also were tested for BVDV infection using virus isolation (VI) and reverse transcription (RT)–PCR on buffy coat samples and for antibodies to BVDV types 1a and 2 by serum neutralization (SN). Sixty-seven of 559 (12.0%) calves tested positive at initial screening by IHC, AgELISA, or VI, and all 67 were kept for a minimum of 3 months and retested monthly by IHC, AgELISA, VI, RT-PCR, and SN. Of the calves positive at initial screening, 59/67 (88.1%) were determined PI and 8/67 (11.9%) were determined acutely infected. Both IHC and AgELISA detected 100% of PI calves; however, IHC and AgELISA also detected 6 and 8 acutely infected calves, respectively, at initial screening. Furthermore, IHC and AgELISA continued to detect 3 and 4 acutely infected calves, respectively, 3 months after initial screening. Three acutely infected calves had IHC staining indistinguishable from PI calves at initial screening. Both IHC and AgELISA are accurate at detecting BVDV-infected calves, but veterinarians and producers should be advised that both tests detect some calves acutely infected with BVDV in addition to PI animals. Repeat testing using VI or RT-PCR on buffy coat samples should be performed at 30 days after initial screening to conclusively discriminate between acute and PI.

**Key words:** Acute infection; bovine viral diarrhea virus; ELISA; immunohistochemistry; persistent infection.

### Introduction

Bovine viral diarrhea virus (BVDV) is an important viral pathogen of cattle causing many disease syndromes, including reproductive failure (embryonic loss, abortion, stillbirth, congenital defects, and birth of weak calves that fail to thrive), birth of persistently infected (PI) calves, acute disease in older animals, mucosal disease, and contributing to bovine respiratory disease complex.<sup>1,4</sup> Many strategies have been designed to eliminate BVDV from cattle herds, most relying in part on accurate identification and removal of PI calves from herds. Persistently infected calves are recognized as the most significant source of BVDV within most herds and are capable of shedding infectious virus throughout their lifetimes.<sup>3,9</sup> Currently, a variety of diagnostic tests are used to detect PI calves, including VI and variations on VI (immunoperoxidase

monolayer assay and monolayer ELISA), traditional and real-time reverse transcription (RT)–PCR, antigen-capture ELISA (AgELISA), and immunohistochemistry (IHC).<sup>7,10,13</sup>

Diagnostic tests used to detect BVDV PI calves need to be robust, accurate, economical, and have rapid turnaround to maximize benefits to veterinarians and producers. Because of recognized time, labor, and sensitivity drawbacks associated with some traditional BVDV tests (VI and its derivatives) and the high cost of some newer BVDV tests (PCR), many diagnostic laboratories now rely on IHC assays as a primary means of screening calves for PI status. Immunohistochemistry assays based on examination of sections of haired skin are popular because of convenience of sample collection (ear notching), perceived robustness of the tests, ability to analyze large numbers of samples, and reports of reliability of these tests when compared with traditional detection methods such as VI.<sup>6,10</sup> Immunohistochemistry is not without drawbacks, however, including significant labor expenditures to prepare, process, and examine samples, multistep processes prone to technical error, and reliance on subjective criteria when determining results of the tests. An

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alternative BVDV detection technique recently became available to veterinary diagnostic laboratories in commercial format, the AgELISA. This assay was developed for use with serum samples, but was adapted by the manufacturer for use with ear notch samples in phosphate-buffered saline (PBS). The objective of this study was to compare this new AgELISA technique with IHC for the detection of BVDV PI calves (confirmed by VI and RT-PCR) using ear notch samples.

### Materials and methods

**Calves.** Five hundred and fifty-nine red and black Angus calves from 3 herds in Wyoming were screened for BVDV infection using ear notch IHC, ear notch AgELISA, and virus isolation (VI) on buffy coat samples. The calves were between 1 and 5 mo of age at initial testing. On all 3 ranches, heifers and cows were vaccinated annually (usually at branding time, before the breeding season) with several different commercially available products containing modified-live BVDV type 1. Of the 559 calves, 67 (12.0%) tested positive for BVDV by 1 or more of the diagnostic assays. All 67 calves were donated to the Wyoming State Veterinary Laboratory (WSVL), University of Wyoming, at 2–6 mo of age, for follow-up testing (20 from the first ranch, 36 from the second ranch, and 11 from the third ranch). These 67 calves were kept at the WSVL for a minimum of 3 mo after initial screening and retested by 4 BVDV detection techniques (IHC and AgELISA on ear notches, VI and RT-PCR on buffy coats) once monthly. Serum samples from calves were tested for virus-neutralizing antibodies to BVDV types 1a and 2 once monthly. Calves were considered PI animals if they tested positive for BVDV by 1 or more of the tests at initial screening and tested positive by VI and RT-PCR on buffy coats at each subsequent monthly examination for at least 3 mo.

**Sample collection.** For initial screening of calves, skin biopsy samples (ear notches) measuring approximately 2.2 × 2.0 cm were obtained from the dorsal pinna margin of each calf using a large commercial ear notcher<sup>a</sup> or an individual disposable sterile scalpel blade. The ear notcher was rinsed in water and disinfected between calves (flamed in 95% ethanol). Ear notch biopsy samples were divided into 2 pieces using a disposable sterile scalpel blade, with 1 section processed for IHC and 1 section processed for AgELISA. At the same time, whole-blood (for VI and RT-PCR, ethylenediaminetetraacetic acid tubes) and serum samples (for virus neutralization tests, preservative-free tubes) were collected from each calf by means of jugular venipuncture. Identical samples were collected from each calf at subsequent monthly examinations.

**Immunohistochemistry.** Ear notches were fixed in 10% neutral buffered formalin for a maximum of 48 hr and then sectioned, processed, and embedded in paraffin in 6-chambered tissue cassettes.<sup>b</sup> Tissue sections were cut at 5 μm, mounted on silane-coated slides, dried overnight at room temperature, and then incubated at 60° C for 30 min. Mounted tissues sections were deparaffinized, rehydrated, and processed in an automated immunostainer.<sup>c</sup> Briefly, sections were treated with proteinase K for 8 min and then blocked

with commercial blocking solution<sup>d</sup> for 10 min, followed by incubation with a BVDV monoclonal antibody<sup>e</sup> at a dilution of 1:1,000 for 30 min. A commercial alkaline phosphatase detection system<sup>f</sup> was used with fast-red chromogen to demonstrate BVDV antigen in the sections, with light hematoxylin counterstain. Positive and negative control sections were included with each IHC run. Stained sections were examined by a single pathologist without knowledge of other test results. Sections were considered positive if specific red intracytoplasmic staining was observed in keratinocytes of the epidermis or hair follicles (or both).

**Antigen-capture ELISA.** Detection of BVDV antigen in ear notches was performed using a commercially available kit,<sup>g</sup> according to the manufacturer's instructions. Briefly, 1 × 1-cm ear notch biopsy samples were placed in individual sterile tubes containing 2 ml of 0.1 M PBS (pH 7.4) and either frozen at –20° C for later analysis or refrigerated overnight at 4° C for analysis the next day. The samples were vortexed, and 100 μl of supernatant (PBS) from each sample was inoculated into individual wells of 96-well plates pre-coated with anti-BVDV antibody provided with the kit. The plates were held at room temperature for 1 hr and washed 3 times using the provided wash solution. Detector reagent containing secondary anti-BVDV antibody was added to each well, and plates were held for 1 hr at room temperature and washed as above. Horseradish peroxidase-conjugated antibody was added to each well, plates were held for 1 hr at room temperature, and washed 3 times. Chromogen tetramethylbenzidine substrate was added to each well, plates were held at room temperature for 10 min, followed by addition of stop solution with further incubation at room temperature for 5 min. Plates were read on an automated plate reader.<sup>h</sup> The data were analyzed using software supplied with the reader that calculates the normalized optical density (OD) results as follows: normalized OD = raw OD of sample – raw OD of negative control/raw OD of positive control – raw OD of negative control. Samples with normalized OD values below 0.2 were considered negative and those with OD values greater than 0.39 were considered positive. Samples with normalized OD values between 0.2 and 0.39 were considered inconclusive and were subjected to a second test where they were treated with and without antibody in the detector reagent. The normalized OD values for these samples was calculated as follows: normalized OD = raw OD of the sample (with antibody detector) – raw OD of the sample (without antibody detector)/raw OD of positive control – raw OD of negative control. Normalized OD values lower than 0.2 were considered negative, and values higher than 0.2 were considered positive after this second assay.

**Virus isolation.** Virus isolation attempts on buffy coat and tissue samples were performed as previously described,<sup>12,15</sup> with the replacement of 24-well tissue culture plates with 48-well plates.

**Reverse transcription-PCR.** Reverse transcription-PCR, as described previously,<sup>12</sup> was performed on buffy coat samples from all donated calves. Using this RT-PCR assay, viruses were identified by genotype (type 1 or type 2).

**Serology.** Virus-neutralizing antibody titers for BVDV type 1a (NADL) and type 2 (125C) were determined as described previously.<sup>16</sup>

**Table 1.** Results of initial screening of calves for BVDV using 3 assays.\*

Test result	Virus isolation†	Immunohistochemistry‡	Antigen-capture ELISA
Positive	59	65	67
Negative	500	494	492
Total	559	559	559

\* Fifty-nine persistently infected calves and 8 acutely infected calves out of 559 tested.

† Virus isolation performed on buffy coat samples.

‡ Immunohistochemistry and antigen-capture ELISA performed on skin biopsy samples (ear notches).

*Data analysis.* Sensitivity, specificity, positive predictive value, and negative predictive value for the IHC and Ag-ELISA tests in detecting PI BVDV calves at initial screening were calculated using VI and RT-PCR on buffy coats as the gold standard.<sup>17</sup> The kappa statistic was used to evaluate the correlation between the IHC and AgELISA tests in detecting BVDV PI calves.

## Results

Of the 559 calves initially screened for BVDV with ear notch IHC, ear notch AgELISA, and buffy coat VI, 67 (12.0%) tested positive by 1 or more techniques (Table 1). Of the 67 positive calves, 59 (88.1%) were determined to be PI, as demonstrated by repeated and consistently positive VI and RT-PCR on buffy coat samples (Table 2) and by isolation of BVDV from multiple tissues collected at necropsy (data not shown). Noncytopathic BVDV, determined by RT-PCR to be type 2, was isolated from buffy coat samples from 56/59 (95.0%) of the PI calves (representing all 3 ranches), and noncytopathic BVDV, determined by RT-PCR to be type 1, was isolated from buffy coat samples from 3/59 (5.0%) of the PI calves (all 3 from the same ranch). The prevalence of PI calves on the 3 ranches ranged from 1.8% to 38.2%.

The remaining 8 calves positive at initial screening were determined acutely infected with BVDV at subsequent monthly testing intervals. Seven of 8 (87.5%) were negative by VI and RT-PCR on buffy coat samples over a 3-month period (Table 2) and 6/7 (85.7%) were negative by VI on tissues collected at necropsy (data not shown). One calf that had been negative for BVDV by VI and RT-PCR at months 1 and 2 was positive by VI at necropsy (month 3) on 6/13 (46%) tissues or samples, including buffy coat, brain, kidney, nasal swab, rectal swab, and skin (data not shown), and one calf is still alive.

Four of the 8 calves tested positive by ear notch AgELISA for 2 consecutive months after donation to the WSVL. These 4 calves became negative by 3 months and remained negative for 4 more monthly testing intervals, at which time they were killed. Three

of the 8 calves tested positive by ear notch IHC for 3 consecutive months. Two calves became negative by 4 months and remained negative thereafter, whereas 1 calf remained positive for BVDV by IHC for 8 months, although distribution and intensity of staining, especially in follicular epithelium, gradually waned over time (Fig. 1C, 1D). Distribution and intensity of BVDV antigen staining detected by IHC in 3/8 (37.5%) acutely infected calves at the time of donation was similar to that observed in PI calves (Fig. 1A, 1B). This staining was comparable with previously described reports for PI calves.<sup>10</sup> All 3 of these calves had consistently high neutralizing titers to both type 1a and type 2 BVDV (minimum of 1:1,024 and maximum of  $\geq 1:8,192$  by day 90 of the study), and virus was never isolated from or detected by RT-PCR in blood samples from any of these 3 calves at monthly testing intervals from donation through 6 months.

Serological results from the 67 animals that tested positive at initial screening, by IHC or AgELISA, are presented in Table 2. The dams on all 3 ranches had been vaccinated with 1 or more doses of a modified-live attenuated vaccine containing BVDV type 1a virus for 1 or more years before this investigation.

Calves 1 through 8 (Table 2) were acutely infected animals that tested positive by 1 or both ear notch tests but were negative by VI or RT-PCR on blood samples collected at initial screening. These 8 animals had high neutralizing titers to both types 1a and 2 BVDV or seroconverted while housed at the WSVL. The titers of several of these 8 animals exceeded 1:32,768 against type 2 BVDV in subsequent tests (data not shown). Type 1 virus was isolated from calves 9 through 11, and type 2 virus was isolated from calves 12 through 67 (Table 2). All 3 (100%) calves infected with type 1 virus (calves 9 through 11) had undetectable or very low anti-type 1a and anti-type 2 titers. All 56 (100%) calves 12–67 had low or undetectable levels of anti-type 2 antibody titers and most had moderate to high anti-type 1a antibody titers. The vast majority of calves 68–559 (that tested negative for BVDV by both IHC and AgELISA at initial screening) had titers to both type 1a and 2 BVDV equal or greater than 1:512 (data not shown).

The sensitivity, specificity, positive predictive value, and negative predictive value of the tests for detecting BVDV PI calves, as compared with the gold standard VI and RT-PCR tests, are shown in Table 3. All calves determined to be PI were consistently positive for both VI and RT-PCR on buffy coat samples (Table 2). For calculations shown in Table 3, acutely infected calves (those repeatedly negative by VI and RT-PCR on buffy coats) were considered false positives and not true PI animals. The sensitivity and specificity of the ear notch tests in detecting all BVDV-infected calves (including

**Table 2.** Bovine viral diarrhea virus isolation, reverse transcription-PCR, immunohistochemistry, antigen-capture ELISA, and serum virus neutralization results over 90 days for 67 calves positive for BVDV at initial screening (day 1).

Calf No.	VI*†		RT-PCR		IHC‡		AgELISA		VN			
	Day 1	Day 90	Day 1	Day 90	Day 1	Day 90	Day 1	Day 90	BVDV1a		BVDV2	
									Day 1	Day 90	Day 1	Day 90
1	-	-	-	-	+	+	+	-	1:256	1:2,048	≥1:8,192	≥1:8,192
2	-	-	-	-	+	+	+	-	1:4,096	1:2,048	≥1:8,192	≥1:8,192
3	-	-	-	-	+	+	+	-	1:2,048	1:1,024	≥1:8,192	≥1:8,192
4	-	-	-	-	-	-	+	-	1:32	1:256	≥1:8,192	≥1:8,192
5	-	-	-	-	-	-	+	-	1:128	1:256	1:1,024	1:4,096
6	-	-	-	-	+	-	+	-	1:128	1:128	1:16	1:512
7	-	-	-	-	+	-	+	-	1:128	1:128	≥1:8,192	≥1:8,192
8	-	-	-	-	+	-	+	-	1:1,024	1:2,048	1:8,192	≥1:8,192
9	+	+	+	+	+	+	+	+	<1:4	<1:4	<1:4	<1:4
10	+	+	+	+	+	+	+	+	1:16	1:16	<1:4	<1:4
11	+	+	+	+	+	+	+	+	1:64	1:64	<1:4	1:4
12	+	+	+	+	+	+	+	+	1:128	1:64	<1:4	1:4
13	+	+	+	+	+	+	+	+	1:32	1:64	<1:4	1:4
14	+	+	+	+	+	+	+	+	1:128	1:32	1:16	1:8
15	+	+	+	+	+	+	+	+	1:8	ND	≤1:4	ND
16	+	+	+	+	+	+	+	+	1:512	1:256	<1:4	<1:4
17	+	+	+	+	+	+	+	+	1:32	1:16	≤1:4	<1:4
18	+	+	+	+	+	+	+	+	1:128	1:8	1:32	<1:4
19	+	+	+	+	+	+	+	+	1:16	1:512	<1:4	<1:4
20	+	+	+	+	+	+	+	+	1:256	1:64	1:4	<1:4
21	+	+	+	+	+	+	+	+	1:256	1:256	<1:4	<1:4
22	+	+	+	+	+	+	+	+	1:64	1:16	1:8	1:8
23	+	+	+	+	+	+	+	+	<1:4	ND	≤1:4	ND
24	+	+	+	+	+	+	+	+	1:32	1:64	1:8	1:8
25	+	+	+	+	+	+	+	+	1:512	1:64	<1:4	<1:4
26	+	+	+	+	+	+	+	+	1:32	1:8	1:64	1:16
27	+	+	+	+	+	+	+	+	1:64	1:64	1:64	1:32
28	+	+	+	+	+	+	+	+	1:128	1:64	≤1:4	<1:4
29	+	+	+	+	+	+	+	+	1:256	1:512	1:4	<1:4
30	+	+	+	+	+	+	+	+	<1:4	<1:4	<1:4	<1:4
31	+	+	+	+	+	+	+	+	1:2,048	1:1,024	<1:4	<1:4
32	+	+	+	+	+	+	+	+	1:2,048	1:512	<1:4	<1:4
33	+	+	+	+	+	+	+	+	1:4,096	1:1,024	1:4	<1:4
34	+	+	+	+	+	+	+	+	1:1,024	1:64	1:128	1:64
35	+	+	+	+	+	+	+	+	1:128	1:16	<1:4	<1:4
36	+	+	+	+	+	+	+	+	1:4	<1:4	<1:4	<1:4
37	+	+	+	+	+	+	+	+	1:2,048	1:512	1:128	1:8
38	+	+	+	+	+	+	+	+	1:512	1:512	<1:4	<1:4
39	+	+	+	+	+	+	+	+	<1:4	<1:4	<1:4	<1:4
40	+	+	+	+	+	+	+	+	1:8,192	1:2,048	<1:4	<1:4
41	+	+	+	+	+	+	+	+	<1:4	<1:4	<1:4	<1:4
42	+	+	+	+	+	+	+	+	1:1,024	1:256	≤1:4	1:8
43	+	+	+	+	+	+	+	+	1:2,048	1:1,024	1:8	≤1:4
44	+	+	+	+	+	+	+	+	1:1,024	1:256	<1:4	<1:4
45	+	+	+	+	+	+	+	+	1:4	<1:4	1:32	<1:4
46	+	+	+	+	+	+	+	+	1:4,096	1:1,024	1:32	1:16
47	+	+	+	+	+	+	+	+	1:128	1:64	<1:4	<1:4
48	+	+	+	+	+	+	+	+	1:4	<1:4	1:8	1:4
49	+	+	+	+	+	+	+	+	1:512	1:1,024	≤1:4	1:4
50	+	+	+	+	+	+	+	+	1:8	<1:4	1:16	1:16
51	+	+	+	+	+	+	+	+	1:1,024	1:512	<1:4	<1:4
52	+	+	+	+	+	+	+	+	1:32	1:16	1:16	1:128
53	+	+	+	+	+	+	+	+	1:512	1:128	<1:4	<1:4
54	+	+	+	+	+	+	+	+	1:512	1:1,024	<1:4	<1:4
55	+	+	+	+	+	+	+	+	1:512	1:256	<1:4	≤1:4
56	+	+	+	+	+	+	+	+	1:2,048	1:256	1:16	1:4
57	+	+	+	+	+	+	+	+	1:32	1:16	<1:4	<1:4
58	+	+	+	+	+	+	+	+	1:1,024	1:1,024	≤1:4	≤1:4
59	+	+	+	+	+	+	+	+	1:256	1:128	<1:4	≤1:4



**Table 2.** Continued.

Calf No.	VI*†		RT-PCR		IHC‡		AgELISA		VN			
									BVDV1a		BVDV2	
	Day 1	Day 90	Day 1	Day 90	Day 1	Day 90	Day 1	Day 90	Day 1	Day 90	Day 1	Day 90
60	+	+	+	+	+	+	+	+	1:4	1:32	<1:4	<1:4
61	+	+	+	+	+	+	+	+	1:1,024	1:512	<1:4	<1:4
62	+	+	+	+	+	+	+	+	1:64	1:32	1:128	1:128
63	+	+	+	+	+	+	+	+	1:32	1:64	<1:4	<1:4
64	+	+	+	+	+	+	+	+	1:512	1:128	<1:4	<1:4
65	+	+	+	+	+	+	+	+	1:128	1:64	1:4	≤1:4
66	+	+	+	+	+	+	+	+	1:4,096	1:1,024	<1:4	<1:4
67	+	+	+	+	+	+	+	+	1:64	1:128	<1:4	<1:4

\* VI = virus isolation; RT-PCR = reverse transcription-PCR; IHC = immunohistochemistry; AgELISA = antigen-capture ELISA; VN = virus neutralization; ND = not determined.

† VI and RT-PCR performed on buffy coat samples.

‡ IHC and AgELISA performed on skin biopsy samples (ear notches).

acutely infected calves) is unknown because reference test results are not available for comparison. The kappa statistic of correlation between the 2 ear notch tests (IHC and AgELISA) was 1.0—there was complete agreement between the tests in detecting PI calves.

### Discussion

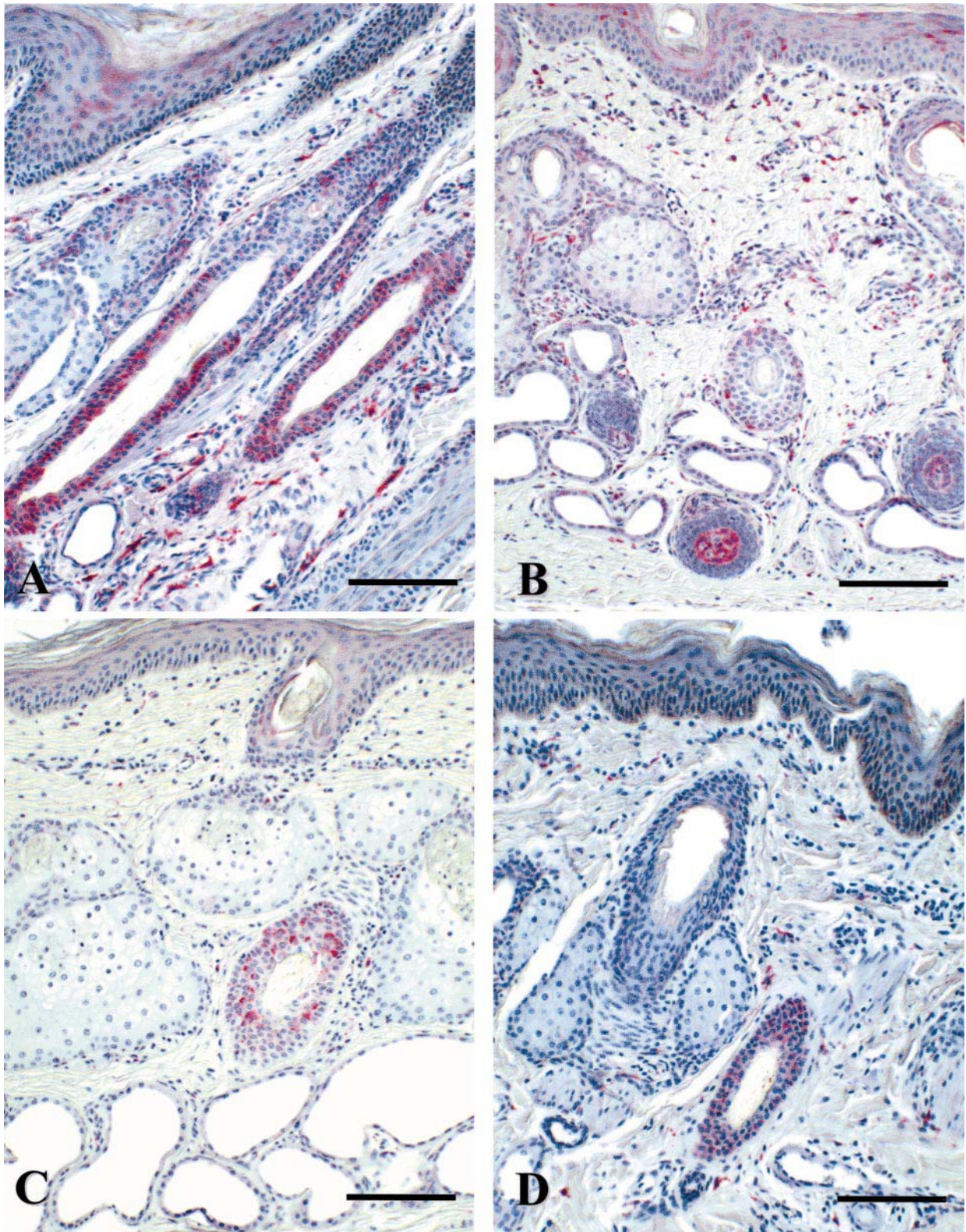
Immunohistochemistry performed on skin biopsy sample (including ear notch) sections is an accurate and effective means of screening calves for BVDV infection.<sup>6,10,14</sup> The accuracy of IHC in detecting BVDV PI calves was confirmed again in this study, and AgELISA using ear notch samples also was an efficient technique for diagnosis of PI calves. Both tests detected 100% of PI calves; however, both tests also detected several acutely infected calves at initial screening and for several months thereafter. Similar findings have been reported in other studies, using IHC on skin and other tissues.<sup>5,6,8</sup> Only by testing calves in this study over a 3-month period, using a battery of techniques (including VI and RT-PCR on buffy coat samples), were acutely infected calves accurately discriminated from PI calves. After concluding the study, it was determined that testing all ear notch (IHC or AgELISA) BVDV-positive calves by VI or RT-PCR on buffy coat samples 30 days after initial screening could consistently discriminate between acutely and persistently infected animals.

In 3/8 (37.5%) acutely infected animals, the pattern of IHC ear notch staining was indistinguishable from PI calves at initial screening. In contrast to a previous report describing characteristic staining differences between acutely infected and PI calves,<sup>10</sup> multifocal to diffuse staining was observed in skin from 3 acutely infected calves in this study. Staining was both epidermal and follicular, including cells of hair bulbs or dermal papillae (Fig. 1A, 1B). Not only was such staining present at initial screening, but 2 calves also

remained IHC positive for 4 months, and 1 calf for 8 months, in the absence of any other evidence of PI (Fig. 1C, 1D; Table 2). Distribution and intensity of staining did wane over time in these 3 calves, but reliance on IHC or AgELISA alone, without complementary tests (VI and RT-PCR), would have led to these calves being diagnosed as PI based on published criteria. No explanation for the persistence of antigen over 8 months was identified in the 1 calf; this calf was negative consistently for BVDV by VI and RT-PCR on buffy coat samples and consistently had high antibody titers for both BVDV1a and BVDV2 by virus neutralization. Persistently and acutely infected animals were housed together for the first month after arrival at the WSVL, and repeated exposure to BVDV likely occurred in non-PI calves. Similarly, another calf that tested positive by 1 ear notch test (AgELISA) at initial screening, and subsequently tested negative for BVDV by both ear notch tests and VI and RT-PCR on blood at months 1 and 2, was positive by VI on a variety of tissues at necropsy just after month 3 (including buffy coat, brain, kidney, nasal swab, rectal swab, and skin). The virus isolated from this calf was noncytopathic and determined to be type 2 by RT-PCR, and this calf had been housed with up to 10 BVDV type 2 PI calves during its stay at the WSVL. These findings were interpreted as consistent with infection while at the laboratory, and a 4-fold rise in anti-type 2 BVDV titer between donation and necropsy further supported this hypothesis (calf No. 5, Table 2).

As in IHC, AgELISA also detected 4/8 (50%) acutely infected calves for 2 months after the study began, using ear notch samples. These calves became negative on AgELISA, 1 month before turning negative by IHC. The 1 calf that remained IHC positive over 8 months was AgELISA negative at 4 months. Possibly the amount of BVDV antigen, waning by IHC at this





**Figure 1.** **A**, haired skin/pinna from a calf persistently infected with bovine viral diarrhea virus (BVDV). Immunohistochemical staining of BVDV antigen within epidermal, follicular, and hair bulb cells. Alkaline phosphatase technique, fast-red chromogen, and hematoxylin counterstain. Bar = 100  $\mu$ m. **B**, haired skin/pinna from a calf acutely infected with BVDV. Immunohistochemical staining similar to persistently infected calf in **A**. Alkaline phosphatase technique, fast-red chromogen, and hematoxylin counterstain. Bar = 100  $\mu$ m. **C**, haired skin/pinna from a calf acutely infected with BVDV, day 60 after initial screening. Fairly intense, but focal immunohistochemical staining of BVDV antigen in epidermal and hair follicle cells. Alkaline phosphatase technique, fast-red chromogen, and hematoxylin counterstain. Bar = 100  $\mu$ m. **D**, haired skin/pinna from the same calf as **C**, day 120 after initial screening. Focal BVDV antigen staining in a hair follicle, with loss of epidermal staining. Alkaline phosphatase technique, fast-red chromogen, and hematoxylin counterstain. Bar = 100  $\mu$ m.



**Table 3.** Comparison of immunohistochemistry and antigen-capture ELISA, using ear notches to detect BVDV persistently infected calves, with buffy coat virus isolation and RT-PCR as the gold standards.\*

	Immunohistochemistry	Antigen-capture ELISA
Sensitivity (%)	100.0	100.0
Specificity (%)	98.8	98.4
Positive predictive value (%)	90.8	88.1
Negative predictive value (%)	100.0	100.0

\* Acutely infected calves that tested positive were considered false positives (i.e., not true persistently infected calves).

point, was insufficient to give a positive AgELISA result past 4 months in this calf.

The serological test results from this investigation are consistent with the VI, IHC, and PCR findings and were useful in discriminating between acutely and persistently infected animals. Initial antibody titers against type 1a and type 2 in the non-PI calves were appreciably higher than those of the PI animals. The type 1a antibodies of calves from the 2 ranches where only type 2 virus was isolated decreased over time, suggesting that titers were acquired by passive transfer in colostrum. Conversely, the type 2 antibody titers increased over time, suggesting that preexisting type 1a antibodies were unable to prevent repeated type 2 reinfections. Interestingly, serum samples containing relatively high type 1a titers (Table 2) did not neutralize either type 2 strain 125C or the type 2 virus isolated from the PI calves. Moreover, the lack of cross-reactivity with the heterologous virus suggests that a different set of antibodies is produced against the heterologous virus. This observation should not be interpreted as a lack of cross-protection because seroneutralization does not account for the potential role of cell-mediated immunity.

The results of this study demonstrate that both IHC and AgELISA are accurate and effective at detecting BVDV-infected calves, including PI calves. Both lend themselves to use when screening large numbers of calves attributable to ease of sample collection (ear notching) at branding or other times when calves are handled. Both tests performed well in calves older than 4 months and also in calves younger than 4 months, unlike other BVDV diagnostic tests including VI and microtiter techniques using serum samples.<sup>2,11</sup> Producers and veterinarians should be advised that both techniques may detect some acutely infected animals and that follow-up testing, using VI or RT-PCR on buffy coat or serum samples may be required to discriminate between acutely and persistently infected calves. Based on the results of this study, repeat testing should be performed at 30 days after the initial screening test. Both IHC and AgELISA are cost-effective; however,

IHC required an average of 5 days for sample preparation and assay completion in this study versus 1 day for AgELISA. Furthermore, the AgELISA required 1 technician to run, compared with a multitude of technicians and 1 diagnostic pathologist to complete IHC assays and sample examinations.

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### Sources and manufacturers

- Stone Manufacturing and Supply Company, Kansas City, MO.
- Sakura/Tissue-Tek®, Torrance, CA.
- Autostainer Plus Universal Staining System, DakoCytomation, Carpinteria, CA.
- Protein Block Serum-Free, DakoCytomation, Carpinteria, CA.
- Monoclonal antibody 15.C.5, Syracuse Bioanalytical Inc., Ithaca, NY.
- LSAB®2 System, DakoCytomation, Carpinteria, CA.
- Bovine Virus Diarrhea Antigen Test Kit, Syracuse Bioanalytical Inc., Ithaca, NY.
- MRX® Revelation™ TC, Dynex Technologies Inc., Chantilly, VA.

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