Multiple diagnostic tests to identify cattle with *Bovine viral diarrhea virus* and duration of positive test results in persistently infected cattle

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Abstract

Several tests for *Bovine viral diarrhea virus* (BVDV) were applied to samples collected monthly from December 20, 2005, through November 27, 2006 (day 0 to day 342) from 12 persistently infected (PI) cattle with BVDV subtypes found in US cattle: BVDV-1a, BVDV-1b, and BVDV-2a. The samples included clotted blood for serum, nasal swabs, and fresh and formalin-fixed ear notches. The tests were as follows: titration of infectious virus in serum and nasal swabs; antigen-capture (AC) enzyme-linked immunosorbent assay (ELISA), or ACE, on serum, nasal swabs, and fresh ear notches; gel-based polymerase chain reaction (PCR) testing of serum, nasal swabs, and fresh ear notches; immunohistochemical (IHC) testing of formalin-fixed ear notches; and serologic testing for BVDV antibodies in serum. Of the 12 animals starting the study, 3 died with mucosal disease. The ACE and IHC tests on ear notches had positive results throughout the study, as did the ACE and PCR tests on serum. There was detectable virus in nasal swabs from all the cattle throughout the study except for a few samples that were toxic to cell cultures. The serum had a virus titer $\geq \log_{10} 1.60$ in all samples from all the cattle except for 3 collections from 1 animal. Although there were several equivocal results, the PCR test most often had positive results. The BVDV antibodies were due to vaccination or exposure to heterologous strains and did not appear to interfere with any BVDV test. These findings illustrate that PI cattle may be identified by several tests, but differentiation of PI cattle from cattle with acute BVDV infection requires additional testing, especially of blood samples and nasal swabs positive on initial testing. Also, calves PI with BVDV are continual shedders of infectious virus, as shown by the infectivity of nasal swabs over the 11-mo study.

Résumé

Plusieurs analyses pour mettre en évidence le virus de la diarrhée virale bovine (BVDV) ont été effectuées sur des échantillons prélevés mensuellement du 20 décembre 2005 jusqu'au 27 novembre 2006 (jour 0 au jour 342) à partir de 12 bovins infectés de manière persistante (PI) avec les sous-types retrouvés chez les bovins aux États-Unis : BVDV-1a, BVDV-1b et BVDV-2a. Les échantillons prélevés incluaient du sang coagulé pour le sérum, des écouvillons nasaux et des encoches d'oreille fraîches et fixées dans la formaline. Les analyses effectuées étaient les suivantes : la titration du virus infectieux dans les échantillons de sérum et les écouvillons nasaux; une épreuve immuno-enzymatique (ELISA) de capture de l'antigène (AC), désignée ACE, sur le sérum, les écouvillons nasaux et les encoches d'oreille fraîches; une réaction d'amplification en chaîne par la polymérase (PCR) dans un support gélifié sur le sérum, les écouvillons nasaux et les encoches d'oreille fraîches; une épreuve immunohistochimique (IHC) sur des encoches d'oreille fixées dans la formaline; et une épreuve sérologique pour détecter des anticorps anti-BVDV dans le sérum. Trois des 12 animaux qui étaient présent au départ de l'expérience sont morts de maladie des muqueuses. Les épreuves ACE et IHC sur les encoches d'oreilles ont montré des résultats positifs tout au long de l'étude, tout comme les tests ACE et PCR sur les échantillons de sérum. Du virus a été détecté dans les écouvillons nasaux de tous les bovins tout au long de l'étude, sauf pour quelques échantillons qui se sont avérés toxiques pour les cultures cellulaire. Tous les échantillons de sérum avaient un titre de $\geq log_{10} 1,60$ pour tous les bovins sauf pour 3 prélèvements chez un animal. Bien qu'il y avait plusieurs résultats équivoques, l'épreuve PCR avait un résultat positif le plus fréquemment. Les anticorps anti-BVDV étaient dus à la vaccination ou à une exposition à des souches hétérologues et n'ont pas semblé interférer avec aucune des épreuves pour détecter le BVDV. Ces trouvailles illustrent bien que les bovins PI peuvent être identifiés par plusieurs analyses, mais que la distinction entre bovins PI et bovins avec une infection aigüe par BVDV nécessite des tests supplémentaires, plus spécialement sur les échantillons de sang et les écouvillons nasaux qui se sont avérés positifs lors des tests initiaux. Également, les veaux avec PI et BVDV sont des excréteurs continuels de virus infectieux, tel que démontré par l'infectivité des écouvillons nasaux durant la période d'étude de 11 mois.

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Introduction

Bovine viral diarrhea virus (BVDV) represents a significant cause of disease in cattle worldwide (1). The virus is classified into cytopathic (CP) or noncytopathic (NCP) biotypes according to the presence or absence of observable cytopathologic effects in infected cell cultures (1). Isolates recovered from samples collected from clinically ill cattle are predominantly NCP (2). In addition, the virus is diverse in genomic differences, which are also represented by antigenic differences; 3 major subtypes are found in the United States: BVDV-1a, -1b, and -2a (2–9).

In cattle, disease caused by BVDV ranges from inapparent to severe, with a high mortality rate and potential involvement of 1 or more organ systems (1). Reproductive tract infections resulting in fetal infections with NCP strains in susceptible heifers and cows play a role in reproductive disease, but the more important factor is likely the resulting persistently infected (PI) calf. The outcome of fetal infection with BVDV may be early embryonic death (with recycling), abortion, stillbirth, congenital malformations, or PI in the calf (1). This last outcome results when the susceptible cow or heifer is exposed to NCP strains of BVDV between days 42 to 125 of gestation (10). The PI calves are born alive, are immunotolerant to the infecting strain, and shed virus throughout their lives (1,11). Such cattle are likely the most important reservoir of virus for susceptible cattle, as was illustrated when 70% to 100% of susceptible nonvaccinated penmates became infected after exposure to PI calves (12,13).

Along with *Mannheimia haemolytica, Pasteurella multocida, Histophilus somni,* and *Mycoplasma* spp., BVDV and other viruses, including *Bovine herpesvirus 1* (BoHV-1), *Bovine parainfluenza virus 3* (BPIV-3), *Bovine respiratory syncytial virus* (BRSV), bovine adenoviruses, and bovine coronaviruses, contribute to feedlot and stocker pneumonia (14–17). Under feedlot conditions, BVDV represents a significant contribution to bovine respiratory disease (BRD), including pneumonia. Two studies reported that 0.4% and 0.3% of cattle entering feedlots were PI with BVDV (11,18). In the earlier study, the likelihood of initial treatment for BRD was increased in cattle exposed to a PI calf, and 15.9% of initial cases of BRD were attributed to exposure to PI calves (18). A high level of immunity to BVDV at entry to the feedlot was associated with increased protection against BRD and with economic benefits. Calves with higher concentrations of BVDV antibodies had lower BRD morbidity rates, lower cost of treatments, and fewer treatments for BRD (19). Thus, the absence of PI animals should assist in disease reduction. Control of BVDV requires proper biosecurity to prevent additions of PI animals, optimal BVDV vaccination to maximize immunity, and continual vigilance to identify and remove PI cattle. A major consideration is the use of diagnostic testing to identify PI cattle.

There are numerous methods available for diagnosing both persistent infection and acute or transient infection with BVDV. These include antigen-capture (AC) enzyme-linked immunosorbent assay (ELISA), or ACE, immunohistochemical (IHC) testing, gel-based reverse-transcription (RT) or real-time polymerase chain reaction (PCR), and virus isolation in cell culture (20). There are numerous serologic methods, including ELISA and viral neutralization tests (VNTs) in cell culture, for measuring seroconversion from acute infection. Attention has been focused on tests identifying PI cattle with the use of skin samples, usually ear notches, for ACE or IHC testing (11,21). The utility of ear notches greatly facilitates collection and submission for testing. Other methods, such as testing blood or serum by ACE or virus isolation, may be complicated by the presence of maternal antibodies in young calves (22). In 1 study, calves acutely infected remained ACE- and IHC-positive 3 mo after initial screening (23). Thus, studies of diagnostic testing continue to address several issues complicating the identification of PI cattle.

The purpose of this study was to use multiple diagnostic tests for BVDV on PI cattle over an 11-mo period to determine (a) the duration of positive test results, (b) the level of virus infectivity in the blood and nasal secretions of the PI cattle during the study period, (c) the potential for seroconversion from exposure to heterologous BVDV strains via commingling or vaccination, and (d) the role of antibody, if any, in diagnostic tests.

Materials and methods

Cattle and sample collection

Calves arriving at a commercial feedlot in southwestern Kansas were processed upon arrival between July 25, 2005, and October 17, 2005. Processing included administration of vaccine A, which contained modified live virus (MLV) BoHV-1, BVDV-1a (Singer), BVDV-2a (5912), BPIV-3, and BRSV, or vaccine B, which contained MLV BoHV-1, BPIV-3, and BRSV and killed BVDV-1a (Singer) and BVDV-2a (5912). At processing, a fresh skin sample (ear notch) was collected, placed in phosphate-buffered saline (PBS), and tested with ACE by the attending veterinarian. Cattle with positive ACE results were moved to quarantine pens, where additional samples were collected later and sent to Oklahoma State University, Stillwater, Oklahoma, USA, for the tests to be described.

The cattle remained in quarantine pens until 12 PI cattle were selected: 4 each infected with BVDV-1a, BVDV-1b, or BVDV-2a. The 12 cattle were then placed in 1 quarantine pen and commingled from December 20, 2005, until November 27, 2006. Three cattle died during the study with mucosal disease signs and lesions. During the 11-mo study, ear notches, blood, and nasal swabs were collected monthly: formalin-fixed notches for IHC testing and fresh notches in PBS for ACE and PCR testing; blood for ACE, PCR testing, and virus isolation and titration; and nasal swabs for ACE, PCR testing, and virus isolation and titration.

Antigen-capture ELISA

Detection of BVDV antigen [ribonuclease region of envelope (Erns)] in skin (ear notch), serum, and nasal-swab samples was performed with the use of a commercially available kit (IDEXX Laboratories, Westbrook, Maine), according to the manufacturer's recommendations (11). Standardized optical density (OD) values were calculated as follows: standardized OD = (raw OD of sample - raw OD of negative control)/(raw OD of positive control - raw OD of negative control). Values < 0.20 were considered negative and values > 0.39 positive. Samples with OD values from 0.20 to 0.39 were retested with detector reagents with or without antibody, and the standardized OD values were recalculated as follows: standardized OD = (raw OD of sample with antibody - raw OD of sample without

Table I. Details of infection with *Bovine viral diarrhea virus* (BVDV), vaccination against several bovine viruses, and antibody responses in persistently infected (PI) cattle commingled for 11 mo

Dates of vaccination and initial diagnosis (2005)	Animal no.	BVDV subtype	Vaccine type[a]	BVDV antibody; titer[b] on day 0/day 342 of commingling		
				1a	1b	2a
8/26, 8/26	8232[c]	2a	MLV	128	32	512
7/25, 9/14	8233	2a	MLV	4/256	4/128	0/0
5/30, 7/30	8234	1b	MLV	0/0	0/0	0/0
7/25, 9/20	8235	1a	Killed	0/128	0/8	0/64
10/4, 10/3	8236	1a	MLV	2048/2048	512/128	1024/512
5/25, 7/25	8237	1b	Killed	0/16	0/4	0/64
10/17, 10/17	8238	1b	MLV	1024/256	4/0	256/64
8/18, 8/18	8239	1b	MLV	2048/1024	128/128	256/64
10/4, 10/3	8240	1a	MLV	1024/256	32/32	256/32
9/22, 9/22	8241	1a	MLV	1024/512	4/4	64/8
8/26, 8/26	8242[c]	2a	MLV	512	32	64
10/11, 9/19	8243[c]	2a	MLV	0	0	0

MLV — modified live virus.

[a] The vaccine contained MLV or killed BVDV-1a Singer and BVDV-2a 5912, among other components.

[b] Reciprocal of the end-point titer in the viral neutralization test (VNT) in the day 0 and day 342 collections; zero indicates a titer of $< 1:4$.

[c] Died with mucosal signs and lesions on May 6 (study day 137), April 18 (day 119), and January 2 (day 13), 2006, respectively.

antibody)/(raw OD of positive control - raw OD of negative control); values < 0.20 were considered negative and values ≥ 0.20 positive. For quality control, for each run to be acceptable, the raw OD values for the negative and positive controls in the kit must have been < 0.5 and > 0.8, respectively. All samples were tested individually; there was no pooling.

Immunohistochemical testing for BVDV

A skin (ear notch) specimen was collected from each calf with positive ACE results and immediately placed in a tube with neutral-buffered 10% formalin. The IHC testing was performed with the use of primary anti-BVDV monoclonal antibody 3.12 Fl, as previously described (11). Positive controls included fixed skin specimens from calves confirmed to be PI by multiple viral isolations performed at least 3 wk apart and positive IHC results: red, distinct, granular intracytoplasmic staining in the epithelium of the stratum spinosum and stratum basale of the epidermis and follicular infundibulum in more than 1 location. Calves from which skin specimens were considered IHC-positive were classified as PI.

Multiplex RT-PCR assay

A gel-based nested multiplex RT-PCR assay was used to detect BVDV in serum, nasal-swab samples, and fresh ear notches collected into PBS, as previously described (11,24). The primers were designed for the NS5B gene. The typing detected types 1 and 2 of BVDV but did not distinguish subgenotypes. Reference strains included in each RNA extraction were BVDV-1a (Singer) and BVDV-2a (125c). All samples were tested individually; there was no pooling.

Virus subtyping

Serum samples (100 μL each) were inoculated into freshly seeded Madin–Darby bovine kidney (MDBK) cells in a flask containing 6 mL of medium. Cultures were incubated for 6 d, subjected to a freeze–thaw cycle, and clarified by centrifugation. Supernatants were stored at -70°C until subtyping was performed. The BVDV isolates were subtyped as BVDV-1a, -1b, -2a, or -2b on the basis of phylogenetic comparison of sequences of the 5′-untranslated region (5′-UTR) of the virus genome, as previously described (7,11). Reference BVDV-1a, -1b, and -2b strains were included in the analysis.

Virus titration

A microtiter virus titration assay in 96-well plates was used to quantitate BVDV infectivity of serum and nasal-swab specimens (5,11). The median tissue culture infective dose ($TCID_{50}$) per 0.025 mL of sample was calculated by the Spearman–Kärber method. Ten-fold dilutions and 4 wells per dilution were used for each sample. To calculate the virus quantity per 1.0 mL of serum, a dilution factor of 40 was used ($\log_{10}$ 1.60). For nasal-swab specimens, it was determined that each swab would absorb 100 μL (0.1 mL). After arrival at the laboratory, each nasal-swab specimen was placed in 2 mL of cell culture medium. A 0.025-mL volume of a 1:10 dilution of the diluted specimen was used as the inoculum. Thus, to determine the virus

Table II. Virus infectivity of serum samples from the PI cattle on study days 0 to 342

Animal	BVDV	Quantity of BVDV (log_{10}) per 1.0 mL of serum; study day											
no.	subtype	0	31	63	98	121	154	189	213	241	273	304	342
8232	2a	1.60	1.60	3.10	2.60	2.40							
8233	2a	2.60	2.90	3.35	2.90	2.40	1.60	< 1.60	1.60	< 1.60	< 1.60	2.35	1.60
8234	1b	4.40	5.10	4.85	3.90	4.60	3.60	3.35	3.90	4.10	4.35	4.35	3.35
8235	1a	4.40	4.90	5.35	4.90	4.90	5.60	4.35	4.10	4.10	5.10	3.85	4.10
8236	1a	4.10	3.60	4.60	4.60	4.10	4.10	4.10	4.40	4.10	5.10	5.35	4.10
8237	1b	2.90	1.60	3.35	2.60	3.60	3.10	2.35	3.90	4.10	4.10	2.85	3.10
8238	1b	4.40	2.60	2.60	3.90	2.90	3.85	2.35	3.40	3.85	4.35	4.35	3.60
8239	1b	4.90	4.90	2.85	4.40	4.40	5.35	4.10	4.90	5.10	5.60	4.10	4.85
8240	1a	4.60	5.10	5.10	5.60	4.60	3.35	4.85	4.60	3.85	4.85	4.10	4.10
8241	1a	1.60	4.10	3.35	4.40	3.10	3.60	3.10	3.10	1.60	3.35	3.60	2.85
8242	2a	3.10	3.10	3.60	3.90								
8243	2a	3.10											

Table III. Virus infectivity of nasal secretions collected from the PI cattle on study days 0 to 342

Animal	BVDV	Quantity of BVDV (log_{10}) per 1.0 mL of nasal secretions; study day											
no.	subtype	0	31	63	98	121	154	189	213	241	273	304	342
8232	2a	4.15	3.65	3.65	5.85	5.10							
8233	2a	3.90	T	T	5.10	5.60	4.85	4.10	3.85	4.90	4.10	4.85	6.10
8234	1b	5.15	5.65	4.15	6.35	5.85	5.85	4.60	T	6.10	6.10	6.35	6.85
8235	1a	4.15	5.40	4.40	5.10	5.60	5.60	5.85	3.85	5.90	6.35	5.85	4.10
8236	1a	3.65	3.40	3.90	4.85	5.35	5.35	5.10	T	5.40	5.85	5.60	6.35
8237	1b	4.15	4.65	3.65	3.60	4.85	6.10	5.10	T	4.60	5.35	5.85	5.85
8238	1b	4.90	5.40	4.65	6.35	7.85	6.10	5.85	5.85	6.60	6.85	6.60	6.35
8239	1b	4.90	5.40	T	6.85	6.10	7.60	6.10	6.35	6.40	6.85	6.10	6.85
8240	1a	5.40	4.90	4.40	6.60	5.60	6.60	5.60	3.85	6.10	6.60	6.35	5.60
8241	1a	5.40	5.15	3.90	5.85	6.10	7.10	4.35	T	5.60	6.60	6.10	5.85
8242	2a	4.15	3.90	2.90	3.85								
8243	2a	4.65											

T — Toxicity: the inoculum was toxic to the cell cultures and caused complete loss of the monolayer.

quantity per 1.0 mL of the nasal-swab specimen, a dilution factor of $40 \times 2 \times 10$ (800) was used (log_{10} 2.90). During the study a second type of swab was used that came with 3 mL of sterile transport medium. After use, that type of swab was immersed in the transport medium and then frozen until shipped to the lab. The dilution factor for this system was log_{10} 3.30.

Serologic tests for BVDV antibodies

A VNT in MDBK cells in 96-well plates was used to quantitate virus neutralizing antibodies to BVDV-1a, -1b, and -2a (5,12). The viruses used were CP BVDV-1a (Singer), CP BVDV-1b (TGAC 8HB), and CP BVDV-2a (125C). The 1:4 final dilution was the lowest dilution tested.

Results

Although all 12 PI cattle included in the study had NCP strains of BVDV detected by ACE in ear notches collected before day 0 (December 20, 2005), animal 8243 had a CP strain in the serum on day 0; this animal died 13 d later (on January 2, 2006) with mucosal disease. Two other animals also died with mucosal disease during the study: no. 8242 on April 18, 2006 (day 119), and no. 8232 on May 6, 2006 (day 137). Table I presents for each animal the BVDV subtype and date of initial positive results, along with vaccination details and antibody findings.

Ten of the PI cattle received MLV BVDV vaccine approximately 7 to 2 mo before day 0 and had no evidence of mucosal disease after vaccination prior to the initiation of the experiment. However the 3 animals that died during the study had received MLV vaccine prior to day 0: animals 8243, 8242, and 8232. On day 0, 8 of the 12 cattle had low (4) to high (2048) titers of antibody to BVDV, whereas the other 4 cattle had no antibodies to any BVDV subtype, despite being in quarantine pens before the study. Of the 9 survivors, 3 seroconverted during the 11-mo study: animal 8233, PI with BVDV-2a, produced antibodies to both BVDV-1a and BVDV-1b, but not BVDV-2a; animal 8235, PI with BVDV-1a, produced antibodies to all 3 BVDV subtypes; and animal 8237, PI with BVDV-1b, produced antibodies to BVDV-1a and BVDV-2a but not BVDV-1b. The other 6 survivors

Table IV. Results of gel-based polymerase chain reaction (PCR) assays for BVDV in nasal secretions collected from the PI cattle on study days 0 to 342

Animal	BVDV	Study day						
no.	subtype	0	31	63	98	121	189	342
8232	2a	+	+	+	+	+		
8233	2a	+	+	+	+	+	+	+
8234	1b	+	+	+	+	+[a]	+	+
8235	1a	+	+	+	+	+	+	+
8236	1a	+	+	+	+	+[a]	+	+
8237	1b	+	+	+	+	+	+	+
8238	1b	+	+	+	+	+	+	+
8239	1b	+	+	+[a]	+	+[a]	+	+
8240	1a	+	+	+	+	+	+	+
8241	1a	+	+	+[a]	+	+	+	+
8242	2a	+	+	+	+			
8243	2a	+						

[a] The initial result was negative; 1 to 4 retests were required to obtain a positive result.

Table V. Results of gel-based PCR assays for BVDV in ear notches collected from the PI cattle on study days 0 to 342 and placed in phosphate-buffered saline

Animal	BVDV	Study day				
no.	subtype	0	31	154	189	342
8232	2a	+	+	+		
8233	2a	+	+[a]	+	+	+
8234	1b	+	+[a]	–	+	+
8235	1a	+	+	+	+[a]	+[a]
8236	1a	+	+	+	+	+
8237	1b	+	+	+	+[a]	+
8238	1b	+	+	+	+	+
8239	1b	+[a]	+	+	+	+[a]
8240	1a	+	+	+	+	+[a]
8241	1a	+	+	+	+	+
8242	2a	+	+			
8243	2a	+				

[a] The initial result was negative; 1 to 4 retests were required to obtain a positive result.

did not seroconvert; 5 had high initial VNT titers of antibody to BVDV-1a (512 to 2048), and 1 was among those that initially had no antibodies to any BVDV subtype.

Ear notches collected on day 0 and monthly to day 342 were positive by both ACE and IHC in all the PI cattle.

The serum from the monthly collections was uniformly positive for virus infectivity (Table II) except for that from animal 8233: on days 189, 241, and 273 the level of infectivity was below the detection limit of $\log_{10}$ 1:60, and on days 154, 213, and 342 the level was just at the detection limit. Some calves, such as nos. 8235 and 8240, had high levels throughout the study, at $\log_{10}$ 3.85 to 5.60 and $\log_{10}$ 3.35 to 5.60, respectively.

The monthly collection of nasal swabs from the PI cattle indicated high levels of virus infectivity (Table III), at $\log_{10}$ 2.90 to 7.85, and therefore virus shedding throughout the 11-mo study. Except for 7 instances in which toxicity of the samples destroyed the monolayer cultures in the assay system, all nasal-swab samples contained infectious virus. For example, the titers ranged from $\log_{10}$ 3.65 to 5.40 on day 0 and from $\log_{10}$ 4.10 to 6.85 on day 342.

Serum from the monthly collections, except for day 98 (which were not tested), was consistently positive in ACE testing for BVDV antigen. However, the OD readings were much lower for the serum than for the ear notches, many being in the suspect range initially. Retesting of the suspect samples according to the kit yielded positive results. One example was animal 8232: the sample from day 31 was initially suspect, but gave positive results with retesting; the level of infectivity in the serum of this animal on day 31 was only $\log_{10}$ 1.60.

The nasal-swab samples collected on days 0, 213, 241, 273, 304, and 342 were also tested by ACE for BVDV antigen and were positive except for 1 collection from animal 8237, on day 213; this sample was toxic for the monolayer cultures in the virus-titration assay. However, the other 6 samples that were toxic in the virus-titration assay were ACE-positive.

The gel-based RT-PCR assay was used on serum and nasal-swab samples from days 0 and 342 as well as numerous monthly collections. The assays were performed on single samples and not pooled samples. All of the day 0 and day 342 serum samples were PCR-positive, as were all those tested from the collections on days 31, 63, 121, and 154. However, the serum from animal 8234 on day 189 was initially PCR-negative; retesting yielded positive results. The level of infectivity did not appear to be an issue, for the virus titer was $\log_{10}$ 3.35 in the animal's serum that day. The PCR results for the nasal-swab samples (Table IV) were more problematic: those obtained for samples collected on days 0, 31, 98, 189, and 342 were consistently positive, but several of the samples collected on days 63 and 121 were initially negative, and up to 4 retests were required to obtain a positive result. These discrepant findings were not always related to toxicity in the virus-titration assay or the level of virus infectivity; for example, the day-63 samples of animals 8239 and 8241 were initially negative, but only the sample from animal 8239 was toxic, and the sample from animal 8241 had a virus titer of $\log_{10}$ 3.90. Also, there were PCR-positive results for 2 samples, from animal 8233 on days 31 and 63 that were toxic to cell cultures.

The gel-based RT-PCR assay was also used to test individual fresh ear notches placed in PBS on days 0, 31, 154, 189, and 342. Although several specimens had to be retested up to 4 times after initially negative results (Table V), all but 1 specimen yielded positive results. The exception was animal 8234, on day 154.

In all the PCR assays the bands were consistent for type 1 or 2 of the persistently infecting BVDV strain: a band of 604 base pairs (bp) represented the BVDV-2 strains, and a 360-bp band represented the BVDV-1 strains (24).

The level of BVDV serum antibodies present at day 0 or acquired during the study did not have an adverse effect on the various tests used to detect BVDV antigen or infectious virus. The antibody levels ranged from 0 (< 4) to 2048 on both day 0 and day 342, yet the test results were positive.

Discussion

This study investigated the use of several tests to detect BVDV in various samples, as well as the duration of positivity, over an 11-mo interval. With ear notches, ACE and IHC testing had positive results throughout the study for all the PI cattle. With serum, ACE and PCR testing also had positive results throughout the study. Virus-infectivity testing by means of titration assays of nasal secretions indicated that the PI cattle were continual shedders of BVDV over the 11 mo. Although PCR assays of nasal secretions and ear notches initially gave negative results on a few occasions in a few cattle, retesting eventually yielded positive results in all but 1 calf, for which a single ear notch collected into PBS was persistently negative.

In PI cattle, infectious virus has been demonstrated in several tissues, including serum, feces, urine, vaginal mucus, and uterine flush medium (25,26). Thus, identifying PI cattle is critically for biosecurity and control of BVDV by preventing exposure to cattle that shed BVDV. Being able to use 1 or more of the numerous tests that detect BVDV to screen for PI is the goal; however, the ability of these tests to distinguish transiently infected cattle from PI cattle with 1 positive result is not always definitive. Early on in PI testing, virus isolation from peripheral blood leukocytes (PBLs) or serum samples was used; animals whose results were initially positive were retested in 3 to 4 wk, and a 2nd positive result was considered evidence of PI status, since virus would have cleared by then from an acutely infected animal (12,13).

With development of ACE and IHC testing came a move toward using skin samples (formalin-fixed for IHC and fresh in PBS for ACE) to detect BVDV in PI cattle. Usually ear notches were collected (11,20,21). These tests have been used extensively in screening for PI cattle. Whether 1 positive ACE or IHC result is sufficient for a diagnosis of PI status is not totally clear. In 1 study, IHC and ACE detected PI in 100% of the cases; however, 6 and 8 acutely infected calves were initially IHC- and ACE-positive, respectively (23). Of the 8 ACE-positive calves, 4 remained ACE-positive for 2 consecutive months and were negative by the 3rd mo; 3 of the 8 were IHC-positive for 3 mo. These 8 calves had negative results of virus isolation attempts and RT-PCR of PBLs at days 1 and 9. In another study, 78 susceptible cattle (negative at day 0 in both serologic tests and attempts at virus isolation) became acutely infected, as defined by seroconversion, virus isolation, or both, after exposure to PI cattle in the same pen (13). The ear notches from days 6, 13, and 35 were IHC-negative, and those from days 6 and 13 were ACE-negative. Days 6 and 13 were those expected for virus isolation from PBLs for acute infection. The PI cattle in that study remained IHC- and ACE-positive in all collections.

Results were all positive for ACE testing of serum and skin samples and for real-time PCR of serum from PI calves with negative results of ELISA testing for BVDV antibodies (27).

Pooling of fresh ear-notch supernatant from several animals has been used for gel-based RT-PCR screening for persistent infection with BVDV (28). The goal of pooling is to minimize the economic costs of testing, with the assumption that pools will detect the single PI animal's sample. The positive samples in a pool are then tested individually by ACE to identify the respective BVDV positive animal. In the current study, which did not pool samples, numerous samples from PI cattle initially had negative results with gel-based RT-PCR. These animals would have been missed in pooled PCR assays.

Use of PCR tests of serum for diagnosis of BVDV infection appears attractive with the potential for pooling. In the current study, the results of RT-PCR testing were positive for all serum collections from the PI cattle, but the serum of acutely infected animals may also be PCR-positive. In another study, all acutely infected calves studied 4 to 13 d after infection had IHC-negative ear-notch specimens, but only a few had any PCR-positive serum samples (29). With 1 exception, all of the acutely infected calves that were PCR-positive on days 4 and 9 were PCR-negative on day 13. In another study, seronegative bulls were infected with BVDV to evaluate testicular BVDV infection (30). An RT-PCR assay detected BVDV in the serum of 3 bulls between days 4 and 17 after exposure, but subsequent serum collections were PCR-negative. These 2 studies illustrate that after positive results have been obtained in suspect animals, PCR tests should be repeated in 3 to 4 wk to differentiate acute infection from persistent infection.

The variable results of repeated PCR tests of the PBS supernatant of fresh ear notches from the PI cattle in our study were difficult to interpret. In numerous animals the results were negative initially and then positive in repeated testing. One explanation for the initially negative results would be the presence of inhibitors to the test in the PBS. This suggests the need to monitor for such inhibitors with the use of controls. Positive BVDV control samples were used in each run's RNA extraction and PCR.

Multiple reports have indicated the potential that maternal antibodies in calves will interfere with BVDV detection. In calves that were PI and had passively transferred antibodies, the ability to detect viremia was reduced until the circulating levels of virus neutralizing antibodies dropped (26). In that study, mature PI cattle were followed for approximately 2 y. In 1 PI animal no virus was detected in the serum, but there were low levels of virus in the PBLs. Coincident with reduced virus infectivity of the serum was the development of neutralizing antibody. Thus, the potential for interference of maternal antibody with virus isolation from the blood of PI calves after colostrum ingestion was evident, as was the potential for active antibody production by the PI calves. In the current study the presence of antibodies to BVDV at day 0 or their acquisition during the study did not affect the titers of infectious virus.

The impact of maternal antibodies on ACE or IHC testing of skin samples from calves is relevant, as attempts are made to identify PI calves as soon as possible to minimize exposure of other calves and pregnant cattle. One report (31) indicated positive results of ACE and RT-PCR testing of PBLs from PI calves before the ingestion of colostrum, whereas on day 7 after colostrum ingestion, virus was isolated in cell culture for only 4 of 25 PI calves, and PBLs from only 10 of the 25 calves were ACE-positive. However, the RT-PCR test detected BVDV in all 18 PI calves tested at day 7. This indicates that maternal antibody will potentially interfere with virus isolation and ACE testing of PBLs from PI cattle but not with PCR testing of PBLs. In a subsequent study, PI calves without or with maternal antibodies as detected by an indirect ELISA were tested for BVDV with an indirect immunoperoxidase (IPX) assay in cell culture and an ACE test on serum (32). All 14 PI calves with an ELISA antibody

titer < 1:10 were ACE-positive, and 11 were IPX-positive. Of the 9 samples with an ELISA antibody titer between 1:10 and 1:1250, 5 were ACE-positive and none IPX-positive. Thus, maternal antibody interfered with both tests, but less with the ACE test. Another study investigated the role of maternal antibody in ACE testing of fresh ear notches and serum from PI calves (33). After ingestion of colostrum (1 to 2 d post partum), all PI calves had negative ACE results in serum. All ear notches, whether collected before or after colostrum ingestion, were ACE-positive. Thus, maternal antibody to BVDV did not negatively affect the ACE test of ear notches. Five diagnostic tests were performed on skin samples, serum, or both, from cattle in another study (22). Only PI animals were identified by positive ACE and IHC results for the skin samples. The real-time PCR results were also positive in the serum of PI calves; there were instances of false- positive results, perhaps from contamination. Negative ACE results with serum from 2 PI calves were believed to be due to maternal antibodies. The results of the current study indicate that low or high levels of BVDV antibodies do not interfere with virus isolation or titration of BVDV from serum or nasal secretions, ACE testing of serum, nasal secretions, or ear notches, PCR testing of serum, or IHC testing of formalin-fixed ear notches.

The virus infectivity of the nasal secretions of the PI cattle was greater than that of the serum from the respective collections in this study. For example, on day 0 animal 8232 had 2.55 $\log_{10}$ greater nasal-swab infectivity than serum infectivity, and on day 342 animal 8234 had 3.50 $\log_{10}$ more infectious virus in the nasal swab than in the serum. A potential explanation is local production of BVDV by the cells of the upper respiratory tract. Confer et al (34) reported on BVDV antigen reactivity in the respiratory epithelium, including the mixed tubuloalveolar glands of the nasal cavity, the serous secretory cells and ductular epithelium, and the squamous and ciliated columnar epithelium of the respiratory tract. These results indicate that the serous secretions of the nasal cavity would contain locally produced virus from active infections of the upper respiratory tract of PI cattle.

The ACE test on the nasal secretions of PI cattle represents a rapid test for infectivity. In this study, with 1 exception, all the nasal-swab samples from the PI cattle were ACE-positive. Whether this finding will be considered diagnostic of persistent infection will require further studies to determine whether acutely and transiently infected cattle also have positive results in ACE testing of nasal swabs. It is likely that the ACE test results mirror the high levels of virus demonstrated in the nasal secretions of PI cattle.

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