Challenge with *Bovine viral diarrhea virus* by exposure to persistently infected calves: protection by vaccination and negative results of antigen testing in nonvaccinated acutely infected calves

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Abstract

Calves persistently infected (PI) with *Bovine viral diarrhea virus* (BVDV) represent an important source of infection for susceptible cattle. We evaluated vaccine efficacy using calves PI with noncytopathic BVDV2a for the challenge and compared tests to detect BVDV in acutely or transiently infected calves versus PI calves. Vaccination with 2 doses of modified live virus vaccine containing BVDV1a and BVDV2a protected the calves exposed to the PI calves: neither viremia nor nasal shedding occurred. An immunohistochemistry test on formalin-fixed ear notches and an antigen-capture enzyme-linked immunosorbent assay on fresh notches in phosphate-buffered saline did not detect BVDV antigen in any of the acutely or transiently infected calves, whereas both tests had positive results in all the PI calves.

Résumé

Les veaux infectés de manière persistante (PI) avec le virus de la diarrhée virale bovine (BVDV) représentent une source d'infection importante pour les bovins susceptibles. L'efficacité d'un vaccin a été évaluée en utilisant des veaux PI avec une souche non-cytopathogène de BVDV2a pour les infections défis et des épreuves comparées pour détecter le BVDV chez des veaux infectés de manière aigue ou transitoire et les comparés à des veaux PI. Une vaccination avec 2 doses du vaccin vivant modifié contenant BVDV1a et BVDV2a protégeait les veaux exposés aux veaux PI; aucune virémie ou excrétion virale n'ont été détectées. Une épreuve immuno-histochimique sur des tissus d'oreille fixés dans la formaline et une épreuve immuno-enzymatique de capture d'antigène sur des morceaux d'oreille frais dans de la saline tamponnée n'ont pas permis de mettre en évidence du BVDV dans aucun des veaux infectés de manière aigue ou transitoire, alors que chez les veaux PI les deux épreuves ont donné des résultats positifs.

(Traduit par Docteur Serge Messier)

Introduction

Bovine viral diarrhea virus (BVDV) infects domestic and wild ruminants worldwide (1), the infections ranging from clinically inapparent to severe disease involving 1 or more organ systems. Historically, BVDV was associated with digestive tract disease and had a high mortality rate. Currently, BVDV is associated frequently with respiratory disease and, depending on the age of the fetus when exposed, abortion, stillbirth, congenital malformations, and birth of a persistently infected (PI) calf (1). When a susceptible heifer or cow is exposed to noncytopathic (NCP) BVDV between 42 and 125 d of gestation, a PI calf can be born (2). A PI calf is immunotolerant of the virus causing the initial infection, is a lifelong shedder of the virus, and is an important source of virus among susceptible cattle (1).

The BVDV can be classified into biotypes and genotypes (1). Biotypes are based on the presence or absence of a visible cytopathic (CP) effect in infected cell cultures. There are 2 biotypes for BVDV: CP and NCP. Genotype classification is based on divergence in the viral genome sequence revealed by phylogenetic analysis (3,4). The genotypic differences are supported by antigenic differences (4,5). Until recent years, there were 2 genotypes: BVDV1 and BVDV2 (3,4). More recently there has been further division, into subgenotypes BVDV1a, BVDV1b, BVDV2a, and BVDV2b in North America (4,6,7).

Control of BVDV incorporates biosecurity measures to prevent exposure of susceptible cattle to BVDV and enhancement of host immunity. Vaccines in the United States are of 2 types: modified live virus (MLV) and inactivated (killed) virus. In addition to safety, potency, and purity, efficacy must be demonstrated for vaccine licensing. Historically, US efficacy studies have used postvaccination challenge by intranasal or aerosol administration of the challenge virus. However, PI animals may better represent the challenge virus under field conditions. Field conditions, or natural exposure, would include direct or close contact with infectious aerosol or virus-laden

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secretions. A recent study in Europe used PI animals as the challenge to determine host immunity (8). This research group demonstrated that PI animals represent an efficient method of virus transmission (9). An extension of this research would be to evaluate the use of PI animals as the challenge to determine vaccine efficacy.

Diagnosis of BVDV infection relies on demonstration of the virus or of an increase in BVDV immunity (evidence of active infection), usually detected by seroconversion. Tests available to demonstrate BVDV in the host include virus isolation in cell culture, fluorescent antibody testing, nucleic acid hybridization, polymerase chain reaction (PCR) to amplify a specific region of the viral genome, immunohistochemistry (IHC) testing to detect BVDV antigen in tissues, and enzyme-linked immunosorbent assay (ELISA) with antigen capture (ACE) to detect BVDV antigen in blood, tissue supernatant, or both (10). The selection of tests and interpretation of results to differentiate acute or transient infection from persistent infection is a challenge for the clinician. Acute or transient BVDV infection is defined as infection in an animal that is not PI, as demonstrated by clearing of virus isolations or seroconversion with a 4-fold rise in antibody titer. After 1 positive result in testing for BVDV by antigen detection, virus isolation, PCR, or IHC, the diagnostic laboratory may suggest a 2nd test 3 to 4 wk later to confirm the PI status. A negative result of the 2nd test indicates that the animal was acutely infected and not PI. In a previous study, it was demonstrated that susceptible cattle do become actively infected (with virus being isolated or seroconversion being demonstrated) after exposure to PI calves (9). Evaluation of the BVDV tests in actively infected calves would be important to the clinician and diagnostician.

The purpose of the current study was 2-fold: (1) to evaluate vaccine efficacy by challenge of vaccinates and nonvaccinates with exposure to PI calves, and (2) to evaluate multiple tests to detect BVDV in both actively or transiently infected calves and PI calves.

Materials and methods

Cattle

The cattle for this study were from 2 sources. There were 72 calves purchased from a ranch in Arkansas and held on the ranch's premises. After weaning, the calves received their initial treatments and vaccinations. Samples taken at this time, day −30 (September 13, 2003), included nasal swabs for virus isolation, blood collected in tubes treated with ethylene diamine tetraacetic acid (EDTA) for virus isolation from peripheral blood leukocytes (PBLs), and clotted blood for viral serologic study. Half of the calves, identified as ranch vaccinated (RV), received an MLV vaccine containing *Bovine herpesvirus-1*, BVDV1a (Singer CP strain), BVDV2a (296 CP strain), *Parainfluenza-3 virus*, and *Bovine respiratory syncytial virus* (Express 5 MLV; Boehringer Ingelheim, St. Joseph, Missouri, USA). The remaining 36 calves received no viral vaccine and were identified as ranch nonvaccinated (RNV). On day −17, the RV calves received a 2nd dose of the MLV vaccine. On day −3, 12 additional calves were purchased by an order buyer at an auction market and moved to the ranch, where they, identified as auction market (AM) calves, were commingled with the other 72 calves. None of the AM calves had received viral vaccines as far as could be determined. The calves were then shipped to research feedyards at New Mexico State University, Clayton, New Mexico. The animal care followed appropriate guidelines, including those of the Institutional Animal Care and Use Committee, as used in other studies (9,11).

On arrival at the feedyard, day 0, the 84 calves were assigned to 6 adjacent pens (40 × 100 ft with a 34-ft-long bunk space), as detailed in Table I; each pen contained 6 RV calves, 6 RNV calves, and 2 AM calves. In addition, 10 calves PI with NCP BVDV2a were available; 1 to 2 were assigned per pen.

Nasal swabs, EDTA-treated blood samples, and clotted blood samples were collected from the calves on days 0, 6, 13, and 35 for virus isolation and serologic testing. Ear notches were collected on days 6 and 13 for ACE and on days 6, 13, and 35 for IHC testing.

Antigen-capture ELISA

Commercially available ear notchers (Stone Manufacturing, Kansas City, Missouri, USA) were used to obtain biopsy samples measuring 1 × 1.5 cm. The samples were placed in individual tubes with 2 mL of sterile phosphate-buffered saline. The tubes were stored at 4°C until shipped, within 48 h of collection, to the laboratory, where they were frozen upon arrival and stored at −20°C until analyzed, within 3 wk of collection. A commercially available kit (Bovine Viral Diarrhea Virus [BVDV] Antigen Test Kit; Syracuse Bioanalytical, Ithaca, New York, USA) was used, according to the manufacturer's guidelines, to detect BVDV antigen in the ear notches. Positive-control samples were obtained from other PI calves and negative-control samples from calves previously found to be negative by the ACE test as well as by virus-isolation and IHC testing.

Immunohistochemistry testing

An additional ear-notch sample was immediately placed in a tube with 10% neutral buffered formalin and stored at room temperature until shipped to the laboratory, where it was stored at room temperature until submitted for IHC testing, as previously described (12), within 3 wk of collection. The primary antibody was a monoclonal antibody against BVDV1 (BVDV Mab 3.12 F1), an affinity-purified, murine-origin immunoglobulin (Ig) G_1. An affinity-purified rabbit IgG against mouse antigen served as the secondary antibody. The tertiary antibody was an affinity-purified, biotinylated goat Ig (H + L)-specific antibody against rabbit antigen. The conjugate was streptavidin–horseradish peroxidase, and the chromagen was Nova RED substrate kit for peroxidase (SK-4800; Vector Laboratories, Burlingame, California, USA). The tissue sections were counterstained with Mayer hematoxylin, dehydrated in alcohol, cleared in toluene, and mounted in resinous medium. Positive-control samples were obtained from other PI calves (PI status confirmed by multiple virus isolations at intervals of 3 wk) and negative-control samples from calves previously found to be repeatedly negative by virus-isolation and IHC testing. Positive IHC results were characterized by red intracytoplasmic staining in the epithelial cells of the hair follicle, hair matrix cells of the bulb, and dermal papilla.

Virus isolation and subtyping

Virus was isolated from nasal swabs and PBLs as previously described (9). The BVDV-positive culture supernatants were grown

Table I. Results of tests for *Bovine viral diarrhea virus* (BVDV), antigen, and antibody on the initial day (day 0) of exposure to persistently infected (PI) calves and on subsequent days

		BVDV1a titer[a]		BVDV1b titer[a]		BVDV2a titer[a]		Virus isolation[b]				IHC testing			ACE	
Pen	Calf	Day 0	Day 35	Day 0	Day 35	Day 0	Day 35	Day 0	Day 6	Day 13	Day 35	Day 6	Day 13	Day 35	Day 6	Day 13
A	RV1	1 024	512	1 024	512	256	64	−	−	−	−	−	−	−	−	−
	RV2	256	2 048	512	2 048	32	512	−	−	−	−	−	−	−	−	−
	RV3	512	4 096	1 024	4 096	512	4 096	−	−	−	−	−	−	−	−	−
	RV4	256	2 048	512	2 048	64	256	−	−	−	−	−	−	−	−	−
	RV5	512	4 096	128	1 024	64	256	−	−	−	−	−	−	−	−	−
	RV6	1 024	4 096	128	512	32	128	−	−	−	−	−	−	−	−	−
	RNV1	0	8	0	8	0	256	−	−	−	−	−	−	−	−	−
	RNV2	256	2 048	512	4 096	32	2 048	−	−	−	−	−	−	−	−	−
	RNV3	0	0	0	0	0	0	−	−	−	−	−	−	−	−	−
	RNV4	0	0	0	0	0	64	−	−	−	−	−	−	−	−	−
	RNV5	32	512	64	512	0	32	−	−	−	−	−	−	−	−	−
	RNV6	0	16	0	16	0	512	−	−	−	−	−	−	−	−	−
	AM1	0	8	0	8	0	1 024	−	−	− (+)	−	−	−	−	−	−
	AM2	32	32	64	64	0	32	−	−	−	−	−	−	−	−	−
	PI97[c]	0	NS	0	NS	0	NS	+(+)	NS	NS	NS	NS	NS	NS	NS	NS
	PI103	0	0	0	0	0	0	+(+)	+(+)	+(+)	+(+)	+	+	+	+	+
B	RV7	2 048	2 048	128	512	64	1 024	−	−	−	−	−	−	−	−	−
	RV8	512	512	32	128	8	64	−	−	−	−	−	−	−	−	−
	RV9	2 048	2 048	4 096	8 192	512	1 024	−	−	−	−	−	−	−	−	−
	RV10	256	4 096	256	4 096	32	1 024	−	−	−	−	−	−	−	−	−
	RV11	1 024	4 096	64	1 024	32	1 024	−	−	−	−	−	−	−	−	−
	RV12	512	2 048	1 024	4 096	128	2 048	−	−	−	−	−	−	−	−	−
	RNV7	512	4 096	512	4 096	16	4 096	−	−	−	−	−	−	−	−	−
	RNV8	0	64	0	64	0	1 024	−	−	+	−	−	−	−	−	−
	RNV9	0	16	0	16	0	1 024	−	−	−	−	−	−	−	−	−
	RNV10	0	64	0	64	0	2 048	−	−	−	−	−	−	−	−	−
	RNV11	32	2 048	32	4 096	0	1 024	−	−	−	−	−	−	−	−	−
	RNV12	0	64	0	64	0	1 024	−	−	−	−	−	−	−	−	−
	AM3	0	2 048	0	2 048	0	1 024	−	+(+)	−	−	−	−	−	−	−
	AM4	0	8	0	16	0	1 024	−	−	+(+)	−	−	−	−	−	−
	PI98[d]	0	NS	0	NS	0	NS	+	+	+(+)	NS	+	+	NS	+	+
	PI104	0	0	0	0	0	0	+(+)	+(+)	+(+)	+(+)	+	+	+	+	+
C	RV13	1 024	2 048	128	512	32	1 024	−	−	−	−	−	−	−	−	−
	RV14	1 024	4 096	256	1 024	32	1 024	−	−	−	−	−	−	−	−	−
	RV15	512	1 024	256	1 024	128	512	−	−	−	−	−	−	−	−	−
	RV16	1 024	2 048	1 024	1 024	256	1 024	−	−	−	−	−	−	−	−	−
	RV17	512	1 024	1 024	2 048	256	1 024	−	−	−	−	−	−	−	−	−
	RV18	256	2 048	256	1 024	32	1 024	−	−	−	−	−	−	−	−	−
	RNV13	0	64	0	32	0	2 048	−	+	−	−	−	−	−	−	−
	RNV14	0	16	0	16	0	4 096	−	+	−	−	−	−	−	−	−
	RNV15	256	16 384	256	16 384	64	1 024	−	−	−	−	−	−	−	−	−

Table I ***(continued)***

Pen	Calf	BVDV1a titer[a]		BVDV1b titer[a]		BVDV2a titer[a]		Virus isolation[b]				IHC testing			ACE	
		Day 0	Day 35	Day 0	Day 35	Day 0	Day 35	Day 0	Day 6	Day 13	Day 35	Day 6	Day 13	Day 35	Day 6	Day 13
	RNV16	64	2 048	128	4 096	8	1 024	−	−	−	−	−	−	−	−	−
	RNV17	0	8	0	16	0	1 024	−	−	+	−	−	−	−	−	−
	RNV18	0	16	0	8	0	1 024	−	−	−	−	−	−	−	−	−
	AM5	0	16	0	32	0	512	−	−	+	−	−	−	−	−	−
	AM6	0	256	0	256	0	512	−	+	−	−	−	−	−	−	−
	PI99[e]	0	NS	0	NS	0	NS	+(+)	+(+)	NS	NS	+	+	NS	+	+
	PI105	0	0	0	0	0	0	+(+)	+(+)	+(+)	+	+	+	+	+	+
D	RV19	256	4 096	4 096	8 192	1 024	2 048	−	−	−	−	−	−	−	−	−
	RV20	2 048	4 096	4 096	8 192	1 024	2 048	−	−	−	−	−	−	−	−	−
	RV21	512	8 192	128	4 096	32	1 024	−	−	−	−	−	−	−	−	−
	RV22	2 048	8 192	128	1 024	64	512	−	−	−	−	−	−	−	−	−
	RV23	2 048	1 024	4 096	2 048	512	1 024	−	−	−	−	−	−	−	−	−
	RV24	1 024	4 096	256	1 024	64	1 024	−	−	−	−	−	−	−	−	−
	RNV19	0	16	0	32	0	1 024	−	−	−	−	−	−	−	−	−
	RNV20	0	32	0	8	0	1 024	−	−	−	−	−	−	−	−	−
	RNV21	0	16	0	32	0	1 024	−	−	+	−	−	−	−	−	−
	RNV22	0	16	0	32	0	1 024	−	−	−	−	−	−	−	−	−
	RNV23	4	8	0	8	8	512	−	−	−	−	−	−	−	−	−
	RNV24	0	16	0	16	0	1 024	−	−	−	−	−	−	−	−	−
	AM7	0	128	0	32	0	512	−	−	−	−	−	−	−	−	−
	AM8	0	16	0	8	0	512	−	−	−	−	−	−	−	−	−
	PI100	0	0	0	0	0	0	+(+)	+(+)	+	+(+)	+	+	+	+	+
	PI106	256	128	16	32	0	0	+(+)	+(+)	+	+(+)	+	+	+	+	+
E	RV25	1 024	4 096	256	512	64	1 024	−	−	−	−	−	−	−	−	−
	RV26	1 024	1 024	128	256	32	128	−	−	−	−	−	−	−	−	−
	RV27	1 024	4 096	64	256	64	512	−	−	−	−	−	−	−	−	−
	RV28	8 192	4 096	1 024	1 024	128	128	−	−	−	−	−	−	−	−	−
	RV29	1 024	4 096	256	8 192	64	1 024	−	−	−	−	−	−	−	−	−
	RV30	1 024	4 096	128	1 024	64	1 024	−	−	−	−	−	−	−	−	−
	RNV25	0	16	0	16	0	512	−	−	−	−	−	−	−	−	−
	RNV26	64	16 384	128	8 192	16	4 096	−	−	−	−	−	−	−	−	−
	RNV27	0	4	0	8	0	256	−	−	−	−	−	−	−	−	−
	RNV28	0	8	0	32	0	128	−	−	−	−	−	−	−	−	−
	RNV29	512	16 384	512	16 384	128	4 096	−	−	−	−	−	−	−	−	−
	RNV30	128	2 048	512	8 192	32	1 024	−	−	−	−	−	−	−	−	−
	AM9	0	8	0	64	0	1 024	−	+	+(+)	−	−	−	−	−	−
	AM10	0	64	0	64	0	1 024	−	+	−	−	−	−	−	−	−
	PI107	128	128	32	16	0	0	+(+)	+(+)	+(+)	+(+)	+	+	+	+	+
F	RV31	1 024	16 384	1 024	16 384	512	8 192	−	−	−	−	−	−	−	−	−
	RV32	1 024	16 384	128	4 096	16	1 024	−	−	−	−	−	−	−	−	−
	RV33	4 096	8 192	512	2 048	256	4 096	−	−	−	−	−	−	−	−	−
	RV34	2 048	4 096	512	1 024	32	256	−	−	−	−	−	−	−	−	−

Table I (*concluded*)

Pen	Calf	BVDV1a titer[a]		BVDV1b titer[a]		BVDV2a titer[a]		Virus isolation[b]			IHC testing			ACE	
		Day 0	Day 35	Day 0	Day 35	Day 0	Day 35	Day 0	Day 6	Day 13	Day 35	Day 6	Day 13	Day 35	Day 6	Day 13
	RV35	2 048	4 096	1 024	2 048	32	2 048	–	–	–	–	–	–	–	–	–
	RV36	512	2 048	32	256	64	1 024	–	–	–	–	–	–	–	–	–
	RNV31	0	32	0	16	0	2 048	–	–	+	–	–	–	–	–	–
	RNV32	0	8	0	16	0	512	–	–	–	–	–	–	–	–	–
	RNV33	64	4 096	64	2 048	16	1 024	–	–	–	–	–	–	–	–	–
	RNV34	64	16 384	128	16 384	64	4 096	–	–	–	–	–	–	–	–	–
	RNV35	4	4	4	16	64	32	–	–	–	–	–	–	–	–	–
	RNV36	0	16	0	8	0	1 024	–	–	–	–	–	–	–	–	–
	AM11	0	0	0	128	0	64	–	– (+)	–	–	–	–	–	–	–
	AM12	0	4	0	8	0	256	–	–	–	–	–	–	–	–	–
	PI108	64	128	0	4	0	0	+(+)	+(+)	+(+)	+(+)	+	+	+	+	+

IHC — immunohistochemistry (staining of formalin-fixed ear notches); ACE — antigen-capture enzyme-linked immunosorbent assay (of fresh ear notches in phosphate-buffered saline); RV — ranch vaccinated; RNV — ranch nonvaccinated; AM — auction market; NS — no sample

[a] Reciprocal of highest dilution with complete neutralization of virus in both wells; 0 — no neutralization at 1:4, the lowest dilution tested

[b] + — from peripheral blood leukocytes; (+) — from nasal swabs

[c] Died on day 5

[d] Died on day 22

[e] Died on day 22

in cell culture and subsequently subtyped by sequencing of the 5′ untranslated region (UTR) (13).

Serologic testing

A virus neutralization test in Madin–Darby bovine kidney cell monolayers in 96-well microtiter plates was used to quantitate virus-neutralizing antibodies to BVDV (9,11). The viruses used were CP BVDV1a (Singer strain), CP BVDV1b (TGAC 8HB), and CP BVDV2a (125-C). A 1:4 dilution was the lowest tested, and titers of less than 1:4 were considered negative.

Statistical analysis

The differences in virus isolation between the RV and RNV cattle groups were compared with use of the 2-tailed Fisher's exact test and SAS software (PROC FREQ), version 8.2 (SAS Institute, Cary, North Carolina, USA).

Results

Viral and serologic status on days −30 and 0

For all 72 ranch calves, no virus was isolated from the nasal swabs or the PBLs collected on day −30. Most of these calves were seronegative for BVDV1a, 1b, and 2a at this point. Some had antibody to BVDV1a (21 [29%]), BVDV1b (21 [29%]), or BVDV2a (23 [32%]), which was considered to be due to maternal transfer, as the titers in those that were RNV declined from day −30 to day 0. In almost all the calves, the BVDV2a antibody titers were lower than the BVDV1a or 1b antibody titers.

On day 0 all 72 ranch calves were negative for virus in nasal swabs and PBLs (Table I). All 36 RV ranch calves were seropositive for BVDV1a, 1b, and 2a. Among the 36 RNV ranch calves, 23 were seronegative for BVDV1a, 24 for BVDV1b, and 25 for BVDV2a. Of the 12 AM calves, 11 were seronegative for all 3 BVDV genotypes.

Seroconversion after exposure to PI calves

Of the 36 RV calves with pre-existing BVDV2a antibodies on day 0 (the day they were initially exposed to calves PI with BVDV2a), 30 had seroconverted, showing a 4-fold or greater rise in BVDV2a antibody titer, by day 35 after exposure, and all 30 showed consistent BVDV1a and 1b seroconversion. For the 6 calves not showing BVDV2a seroconversion, the day 0 titers were 128 to 1024. Blockage of seroconversion by these titers was not complete, however, as seroconversion did occur in some RV calves with these titers (examples: RV3, 512 → 4096; RV15, 128 → 512; and RV16, 256 → 1024).

Of the 25 BVDV2a-seronegative RNV calves, 24 seroconverted after exposure, showing a 4-fold or greater rise in BVDV2a antibody titer, as did 10 BVDV2a-seropositive RNV calves; however, in 1 calf, RNV35, the titer fell from 64 to 32. The seronegative calf that did not seroconvert (RNV3) to BVDV2a-positive remained seronegative for BVDV1a and 1b as well. All 11 of the consistently seronegative AM calves (seronegative for all 3 subtypes) seroconverted to BVDV2a-seropositive after exposure, as did the 1 calf that had been seronegative for BVDV2a but seropositive for the other 2 genotypes.

Thus, 36 calves (24 RNV and 12 AM) that had been BVDV2a-seronegative on day 0 seroconverted to BVDV2a-positive after exposure to PI calves. Regardless of BVDV2a serologic status on day 0 for the 36 RNV and 12 AM calves, 47 of 48 seroconverted to BVDV2a-positive after exposure. There were no seroconversions among the PI calves, most remaining seronegative for all BVDV strains or having a change of only 1 dilution.

Virus isolation

Virus was isolated from the nasal swabs or the PBLs (indicating viremia), or both (Table I), that were collected on day 6 or 13, or both, from 14 (6 RNV and 8 AM) calves among the 84 exposed to the PI calves. All 14 isolates in the exposed calves and the isolates from the PI calves were NCP and, except in 3 AM calves positive on day 6, were BVDV2a. Two of the AM calves (AM3 and AM11) were infected with BVDV1a on day 6; these isolates were genetically identical for the 5′-UTR sequenced, which indicates a common source of the virus, likely through exposure to a PI animal at or before shipment to the ranch. Another AM calf (AM6) was infected with BVDV1b, again likely from exposure before shipment. Virus was not isolated from the samples collected on day 0 or day 35 from any of the 84 RV, RNV, and AM calves, but it was isolated from all the nasal and PBL samples collected on days 0, 6, 13, and 35 from all the PI calves, and those isolates were all BVDV2a.

All the RNV and AM calves from whose samples virus was isolated had been BVDV2a-seronegative on day 0. No RV, RNV, or AM calf with BVDV antibodies on day 0 became virus-positive. Thus, of the 37 calves (25 RNV and 12 AM) that were BVDV2a-seronegative on day 0, 38% were virus-positive on day 6 or 13, or both. On the basis of the virus-isolation findings, all 36 RV calves but only 30 of the RNV calves were protected against acute viral infection ($P = 0.0249$).

Ear-notch findings

None of the formalin-fixed ear notches collected from the RV, RNV, and AM calves on days 6, 13, and 35 after exposure to the PI calves were IHC-positive for BVDV antigen, whereas all the samples collected from the PI calves were IHC-positive. None of the fresh ear notches collected from the RV, RNV, and AM calves on days 6 and 13 were ACE-positive for BVDV antigen. The collections on days 6 and 13 were done to coincide with expected potential virus isolation, as indicated from other studies (9,11). However, no calves with acute infection according to virus isolation from nasal swabs or PBLs demonstrated IHC or ACE evidence of BVDV.

Discussion

In this study 2 doses of an MLV vaccine containing BVDV1a and BVDV2a strains given 30 and 17 d before exposure to calves PI with BVDV2a conferred protection against viremia in the challenged calves. Nonvaccinates responded as expected to exposure, with seroconversion and with PBLs collected 6 and 13 d after exposure yielding virus. Interestingly, some of the vaccinated calves with BVDV antibodies at day 0 (the initial day of exposure) had anamnestic responses, with an increase in the titers of BVDV antibodies. Potentially these animals were primed for increased BVDV antibody production through interaction of memory T cells or B cells, or both, with antigens.

The results of this study confirm those of prior studies demonstrating that PI calves can serve as a natural method of challenge (8,9) and show that PI calves can be used to challenge vaccinates for efficacy studies. In a previous study (9), vaccinates and nonvaccinates were exposed to calves PI with BVDV1b, and BVDV1b was isolated from calves 7 to 21 d after exposure; seroconversion occurred in 70% to 100% of the nonvaccinated penmates. Calves receiving a killed BVDV before PI exposure were not completely protected, as viremia was detected in some after exposure. Calves receiving MLV BVDV2a and 1a vaccine only 3 d before exposure were also not completely protected, some becoming viremic. In another previous study, PI cattle were used as the challenge to measure fetal protection afforded by vaccination to heifers before breeding. Exposure at 87 d of gestation resulted in transplacental infection in all 7 unvaccinated dams.

In the current study, the IHC and ACE tests on formalin-fixed and fresh ear notches did not detect any evidence of acute or transient BVDV infection after PI calf exposure. As expected, all the ear notches collected from the PI calves were positive by IHC and ACE testing. Using seroconversion as a broad indicator of acute infection, 66 of the 84 challenged calves (30 RV, 24 RNV, and 12 AM) became acutely infected with BVDV2a after exposure. Many of these calves already had antibodies at the time of exposure. Using an even more strict criterion for acute infection, seronegativity before seroconversion, 36 calves (24 RNV and 12 AM) became acutely infected with BVDV2a after exposure; their IHC and ACE test results remained negative. Of these 36 calves, 14 (40%) were viremic or had virus isolated from nasal swabs. Thus, in this study the IHC and ACE tests on ear notches did not identify acutely infected calves.

The results of this study are consistent with those of some other studies. With histopathologic criteria for immunoperoxidase staining of ear notches, the IHC test has been used to identify PI calves (14–16). In a study of acute BVDV infection in calves exposed to 1 of 3 subtypes (BVDV1a, BVDV1b, and BVDV2), the IHC test on fixed notches was used with other tests, including virus isolation from PBLs, samples being collected 0, 4, 9, and 13 d after nasal challenge; antibody testing for seroconversion was performed on serum samples (17). Of 16 exposed calves, 14 seroconverted, and all 16 became viremic but had negative IHC results.

The results of the current study differ somewhat from those of another recent study (18) of 67 calves found to be positive in screening for BVDV by IHC (65) and ACE (67) testing of ear notches and by virus isolation from PBLs (59). The 67 calves were followed for 90 d with additional IHC and ACE testing, virus isolation and reverse-transcription PCR (RT-PCR) on buffy-coat samples, and serum neutralization tests for antibodies to BVDV types la and 2. The results indicated that cattle acutely infected with BVDV could have positive results by the IHC test on formalin-fixed notches and by the ACE test on fresh notches. Of the 8 calves determined to be acutely infected, 7 had negative virus-isolation and RT-PCR results over the 90 d, and 6 of the 7 had negative virus-isolation results at necropsy. All 8 acutely infected calves became ACE-negative during the study, but 4 remained ACE-positive for 2 mo; 6 of the 8 were also IHC-positive on day 1, and 3 were still IHC-positive at day 90. Thus,

in contrast to the results of other studies, IHC or ACE positivity was not restricted to PI calves. In the current study, none of the acutely infected calves were IHC- or ACE-positive even when viremic or seroconverting. The differences between the 2 studies may be due to a difference in viral load and virulence or in the criteria used to evaluate the IHC test results, or both. One could speculate that the IHC or ACE results might have been different earlier among the ranch or the AM calves. However, after challenge with the PI calves, there were no IHC- or ACE-positive results.

The results of this study reaffirm the requirement to properly identify isolated viruses by subtyping or gene sequencing to determine if the isolates are identical to the challenge strains. In this study 3 isolates (2 BVDV1a, 1 BVDV1b) were different from the BVDV2a challenge strain. It is especially important to distinguish isolates when the calves are from the regular marketing system (auction markets), where exposure to BVDV is likely.

In summary, although the current study did not detect acute BVDV infection by IHC and ACE testing of ear notches after exposure to PI calves, there will likely be some caution about using only 1 test to differentiate acutely infected and PI calves. A negative IHC or ACE ear-notch result appears to offer a substantial basis for eliminating a calf as PI, but a positive result poses a dilemma, as a confirmatory test may be in order for such situations as breeding and purchases. For some facets of the cattle industry, such as purchased stockers and feedlot entry, 1 test is economical in terms of labor, time, and finances. However, such calves should perhaps be segregated until a confirmatory test is performed.

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