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NOTES

Neutralizing Antibodies to Type 1 and 2 Bovine Viral Diarrhea Viruses: Detection by Inhibition of Viral Cytopathology and Infectivity by Immunoperoxidase Assay

R. W. FULTON,[1]* J. T. SALIKI,[1,2] L. J. BURGE,[1] J. M. d'OFFAY,[1] S. R. BOLIN,[3] R. K. MAES,[4] J. C. BAKER,[5] AND M. L. FREY[6]

Department of Infectious Diseases and Physiology[1] and Oklahoma Animal Disease Diagnostic Laboratory,[2] Oklahoma State University, Stillwater, Oklahoma 74078; National Animal Disease Center, Agricultural Research Service,[3] and National Veterinary Service Laboratory, Animal and Plant Health Inspection Service,[6] U.S. Department of Agriculture, Ames, Iowa 50010; and Animal Health Diagnostic Laboratory[4] and Large Animal Clinical Sciences,[5] Michigan State University, East Lansing, Michigan 48909

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Neutralizing antibodies to type 1 and 2 bovine viral diarrhea virus (BVDV) strains were measured by a microtiter virus neutralization test (MVNT) in cell culture. Antibodies (neutralizing) were detected by inhibition of viral infectivity, by the absence of viral cytopathology for cytopathic strains, or by immunoperoxidase staining for noncytopathic strains. The immunoperoxidase-stained monolayers could be detected without the aid of light microscopy. Twenty BVDV strains were used as challenge viruses in the in vitro MVNT, including 14 type 1 and 6 type 2 strains. Representative noncytopathic and cytopathic strains of both types were used. Positive control serum samples available for diagnostic testing contained both type 1 and type 2 BVDV antibodies. There did not appear to be major differences in antibody titers among the respective type strains, regardless of biotype (cytopathic or noncytopathic). In a study with sera from calves receiving a modified live virus or inactivated BVDV vaccine, the calves receiving type 1 strains responded with higher antibody titers to type 1 strains than to type 2 strains.

Bovine viral diarrhea virus (BVDV) infects cattle, is associated with several clinical forms, and is present worldwide (2). BVDV is classified as a single-stranded enveloped RNA virus and is a member of the *Pestivirus* genus of the family *Flaviviridae* (13). There are two BVDV biotypes based on the presence or absence of visible cytopathology in infected cell cultures: cytopathic (CP) and noncytopathic (NCP) (2). BVDV strains can be separated into two types, types 1 and 2, on the basis of differences in the viral genome and antigenic differences (22).

Diagnosis of BVDV in cattle has included detection of infectious virus or antigen, or both, in tissues and excretions or BVDV antibodies in the sera of infected animals. Detection of BVDV antibodies has been used to determine an immune response by cattle receiving BVDV vaccines. Examples of tests that are used to detect BVDV antibodies include immunodiffusion test (17), primary binding assays with infected cell cultures (indirect fluorescent-antibody and immunoperoxidase tests) (1), an enzyme-linked immunosorbent assay (19, 20), and a fluorescent-antibody neutralization test (1). Microtiter virus neutralization tests (VNTs) that use inhibition of infectivity (cytopathology) have been used to detect BVDV-neutralizing antibodies (6, 14). In addition, a microtiter VNT has been used to detect neutralizing antibodies to NCP BVDV strains (18). The VNTs used in the United States have generally used the type 1 BVDV strains Singer or NADL as the challenge virus in the VNT.

Antigenic diversity has been detected among the BVDV strains (3, 8, 11, 12, 21, 22, 24). The antigenic diversity issue has been heightened by the separation of BVDV into two types on the basis of genotypic and antigenic differences (18). Recent concerns include whether available diagnostic reagents detect type 2 and type 1 BVDV strains and if vaccines prepared from type 1 strains induce antibodies to the type 2 BVDV strains. Potentially, the results of this study would indicate that selected positive antisera would detect both type 1 and type 2 BVDV antibodies. Also, this study may indicate that type 1 BVDV vaccines induce lower levels of type 2 BVDV antibodies than type 1 BVDV antibodies.

The objective of this study was to detect neutralizing antibodies to several type 1 and type 2 BVDV strains, including both CP and NCP biotypes of each type, in reference positive control sera and sera from calves receiving type 1 BVDV vaccines.

Twenty BVDV strains (14 type 1 and 6 type 2 strains) were used in this study. The strains included 12 CP type 1, 2 NCP type 1, 4 CP type 2, and 2 NCP type 2 strains. The sources of the viruses are listed in Tables 1 and 2. All viruses were propagated in Madin-Darby bovine kidney (MDBK) monolayers in minimum essential medium containing Earle's balanced salts plus 2% fetal bovine serum (FBS; Atlanta Biologicals,

* Corresponding author. Mailing address: Department of Infectious Diseases and Physiology, College of Veterinary Medicine, Oklahoma State University, Stillwater, OK 74078-2006. Phone: (405) 744-6737. Fax: (405) 744-8263. E-mail: RFULTON@OKWAY.OKSTATE.EDU.

Norcross, Ga.). The fetal bovine serum was BVDV antibody free, as described below. The MDBK cells were BVDV free by the immunoperoxidase assay, as described below.

Positive control sera, samples NVSL 440 BDV 7801 and NVSL 445 BDV 8201, were obtained from the National Veterinary Service Laboratory (NVSL), Animal and Plant Health Inspection Service (APHIS), U.S. Department of Agriculture (USDA), Ames, Iowa. Serum sample NVSL 440 BVDV 7801 was prepared in a calf by intranasal administration of BVDV strains NADL, Singer, Draper, and C24V (23). The inoculation records for serum sample NVSL 445 BDV 8201 were not available (23). Another positive control serum sample, sample 844, was supplied by S. Bolin, USDA, National Animal Disease Center (NADC), Ames, Iowa. Serum sample 844 was from a calf exposed sequentially to multiple type 1 and type 2 strains. A negative control serum, FBS, was negative for neutralizing BVDV antibodies throughout this study.

A microtiter viral titration assay in 96-well plates was used to detect and quantitate viral infectivity (14–16). For the viral titration assay, 0.025 ml of medium was placed in each well. Tenfold dilutions of each virus were made in medium, with 0.025 ml of each dilution placed in each well and with eight wells used for each dilution. An MDBK cell suspension, 0.05 ml, was added to each well. The plates were then incubated at 37°C for 5 days. The infectivity for the CP strains was detected by daily observation of cytopathology with an inverted light microscope, with the final reading done on day 5. Any visible cytopathology in a well was considered positive for infectivity. The 50% tissue culture infective dose ($TCID_{50}$) was calculated by the Spearman-Karber method (14–16).

The infectivity for the noncytopathic strains was detected by an immunoperoxidase monolayer assay (IPMA). The dilutions, cells, and incubation were the same as those for the cytopathic assay as described above. On day 5, the medium was removed, and each well of the plates was fixed with 150 μl of 70% acetone in phosphate-buffered saline (PBS) for 10 min at room temperature. The acetone was removed, and after drying at room temperature for at least 3 h, the plates were then stored at 4°C until the staining procedure.

Each well of the fixed plates was rehydrated with 200 μl of PBS with 0.05% Tween 20 (PBST), and each well was then reacted with a monoclonal antibody (MAb) mixture, MAbs 20.10.6 and 15C5 (E. Dubovi, Cornell University), at 100 μl for 1 h at 37°C. The MAbs were selected on the basis of their broad reactivities with various BVDV strains and because they recognize two different BVDV proteins: MAb 20.10.6 recognizes P80 and MAb 15C5 recognizes gp48 (11). The MAbs were diluted 1:1000 in PBST-FBS (PBST with 20% FBS, not heat inactivated). The FBS in the diluent served to block nonspecific binding. The wells were then washed three times in PBST, and 100 μl of biotinylated rabbit anti-mouse antibody (Sigma Chemical Co., St. Louis, Mo.) diluted 1:250 in PBST-FBS was added to each well, and the plates were incubated for 1 h at 37°C. The plates were washed three times with PBST. Horseradish peroxidase-streptavidin (Zymed Laboratories, Inc., South San Francisco, Calif.), diluted 1:1,000 in PBST-FBS, was added at 100 μl/well, and the plates were incubated for 1 h at 37°C. Following another washing step, the plate was reacted with 100 μl of a substrate-chromagen mixture consisting of 280 μg of 3-amino-9-ethyl carbazole per ml in acetate buffer and 0.01% hydrogen peroxide. The plates were incubated for 25 min at room temperature. A developing red color indicated a positive reaction for infectivity. The plates were tapped dry on a paper towel and were allowed to dry at room temperature. The color reaction (red for a positive reaction) could be viewed with or without the inverted microscope. Any positive color reaction in a well was considered positive for infectivity. The titer ($TCID_{50}$ per 0.025 ml) was calculated as described above. The plates were stable, with a positive color reaction found for at least 3 months when the plates were stored at room temperature.

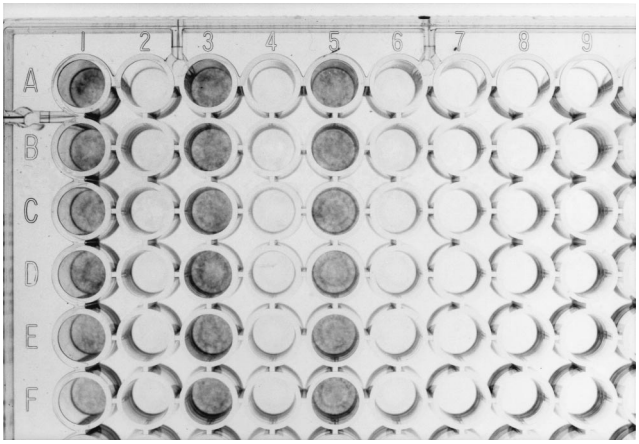

FIG. 1. Representative results obtained by immunoperoxidase staining for BVDV. Wells in column 1 (wells A1 to F1) contained BVDV. Wells in column 2 (wells A2 to F2) contained mock-treated cell cultures. Wells in columns 4 and 6 contained sera with neutralizing antibodies. Wells in column 3 (wells A3 to F3) contained negative control serum and BVDV. Wells in column 4 (wells A4 to F4) contained positive control serum and BVDV. Wells in column 5 (wells A5 to F5) contained calf serum obtained on day 0 (antibody negative) and BVDV. Wells in column 6 (wells A6 to F6) contained calf serum obtained on day 42 (antibody positive) and BVDV.

A microtiter VNT with MDBK cells in 96-well plates was used to quantitate viral neutralizing antibodies to each BVDV strain (14). Sera were heat inactivated at 56°C for 30 min. The plates were incubated at 37°C for 5 days. Each dilution of serum was tested in duplicate rows, with two wells used for each dilution. Infectivity was indicated by (i) visible cytopathic effects (CPEs) for the CP strains or (ii) a red color in the monolayer detected by IPMA for the NCP strains. The VNT antibody titers were expressed as the final dilution of serum (0.025 ml of the serum dilution and 0.025 ml of the virus dilution before adding 0.05 ml of the cell suspension) that completely inhibited infectivity in both wells per dilution. Thus, 1:4 (1:2-diluted serum and virus dilutions) was the lowest dilution tested. For each group of sera tested, positive and negative control sera were included.

Calves seronegative for BVDV were vaccinated with USDA-licensed vaccines according to the instructions on the label. Two calves received an inactivated vaccine containing the Singer strain on days 0 and 28 via the intramuscular route of administration (Triangle 4+PH; Fort Dodge Laboratories, Fort Dodge, Iowa). Two calves received a modified live virus (MLV) vaccine containing the NADL strain (BoviShield-4; SmithKline Beecham Animal Health, Exton, Pa.) intramuscularly on day 0. Sera were collected on days 0 and 42. The sera were heat inactivated and were stored at −20°C until they were assayed for BVDV-neutralizing antibodies as described above.

The assay for antibodies to BVDV type 1 and 2 CP strains used inhibition of virus-induced cytopathology. The procedures described above were used to detect antibodies to the NCP BVDV strains. Figure 1 demonstrates the results of the immunoperoxidase assay for the detection of viral infectivity and neutralizing antibodies to NCP strains.

Control sera from animals receiving various BVDV strains neutralized all the BVDV strains, both type 1 and type 2

TABLE 1. Antibody titers to type 1 and type 2 BVDV in strains positive control sera

Virus	Titer for serum sample[a]:		
	NVSL 440 BDV 7801	NVSL 445 BDV 8201	Calf 844 NADC
Type 1			
CP			
Singer[b]	512	16,384	2,048
Singer[c]	512	16,384	2,048
NADL[c]	1,024	8,192	4,096
NADL[d]	2,048	8,192	4,096
C24V[d]	2,048	32,768	4,096
88055[d]	512	16,384	2,048
1583-B1[d]	4,096	32,768	4,096
5960[d]	1,024	32,768	2,048
1345981[e]	1,024	16,384	1,024
1315303[e]	1,024	16,384	2,048
94080633[f]	4,096	16,384	8,192
94110544[f]	1,024	32,768	4,096
NCP			
94111075[f]	512	16,384	1,024
Nebraska[d]	1,024	16,384	4,096
Type 2			
CP			
1431535[e]	64	4,096	8,192
1361777[e]	256	8,192	2,048
125-C[d]	256	16,384	8,192
94050290[f]	256	16,384	4,096
NCP			
94101309[f]	64	4,096	8,192
890[d]	128	4,096	16,384

[a] Titers are reciprocal of final dilution inhibiting viral infectivity.
[b] Source cited in references 14 and 16.
[c] Obtained from Texas Veterinary Medical Diagnostic Laboratory, Amarillo.
[d] Obtained from NADC, Agricultural Research Service, USDA, Ames, Iowa.
[e] Obtained from Animal Health Diagnostic Laboratory, Michigan State University, East Lansing.
[f] Obtained from Oklahoma Animal Disease Diagnostic Laboratory, Stillwater.

TABLE 2. Antibody titers to type 1 and type 2 BVDV strains in postvaccination sera

Virus	Titer after vaccination with the following vaccine[a]:			
	Inactivated Singer strain		MLV NADL strain	
	Calf 1-12	Calf 1-16	Calf 1-1	Calf 1-4
Type 1				
CP				
Singer[b]	512	256	256	256
Singer[c]	1,024	1,024	256	128
NADL[c]	512	512	128	64
NADL[d]	1,024	1,024	512	512
C24V[d]	512	512	256	256
88055[d]	1,024	512	512	256
1583-B1[d]	1,024	1,024	256	256
5960[d]	1,024	1,024	256	256
1345981[e]	256	1,024	128	64
1315303[e]	1,024	1,024	128	128
94080633[f]	1,024	1,024	256	256
94110544[f]	1,024	1,024	1,024	512
NCP				
94111075[f]	256	64	16	32
Nebraska[d]	512	256	64	64
Type 2				
CP				
1431535[e]	64	8	4	8
1361777[e]	128	64	16	16
125-C[d]	64	64	8	4
94050290[f]	128	64	16	16
NCP				
94101309[f]	64	8	8	16
890[d]	128	16	4	32

[a] Titers are reciprocal of final dilution inhibiting viral infectivity.
[b] Source cited in references 14 and 16.
[c] Obtained from Texas Veterinary Medical Diagnostic Laboratory, Amarillo.
[d] Obtained from NADC, Agricultural Research Service, USDA, Ames, Iowa.
[e] Obtained from Animal Health Diagnostic Laboratory, Michigan State University, East Lansing.
[f] Obtained from Oklahoma Animal Disease Diagnostic Laboratory, Stillwater.

strains. However, there was a range of neutralizing antibody titers, depending on whether type 1 or type 2 strains were used as the challenge virus. The range of neutralizing antibody titers to the 20 type 1 and type 2 BVDV strains are summarized in Table 1. The range of antibody titers to the type 1 strains by control serum sample NVSL 440 BDV 7801 was 512 to 4,096 for antibodies to the CP strains and 512 to 1,024 for antibodies to the NCP strains. The range of antibody titers to the type 2 strains by serum sample NVSL 440 BDV 7801 was 64 to 256 for antibodies to the CP strains and 64 to 128 for antibodies to the NCP strains.

The range of antibody titers to the type 1 strains by control serum sample NVSL 445 BDV 8201 was 8,192, to 32,768 for antibodies to the CP strains and 16,384 for antibodies to the NCP strains. The range of antibody titers to the type 2 strains was 4,096 to 16,384 for antibodies to the CP strains and 4,096 for antibodies to the NCP strains.

The range of antibody titers to the type 1 strains by calf serum sample 844 was 1,024 to 8,192 for antibodies to the CP strains and 1,024 to 4,096 for antibodies to the NCP strains. The range of antibody titers to the type 2 strains was 2,048 to 8,192 for antibodies to the CP strains and 8,192 to 16,384 for antibodies to the NCP strains.

The range of neutralizing antibody titers to the 20 type 1 and type 2 BVDV strains in the sera of the calves receiving either inactivated vaccine (Singer strain) or the MLV vaccine (NADL strain) are summarized in Table 2. The responses of the calves to BVDV types 1 and 2 ranged from seronegative (titer, <4) to seropositive (titer, ≥ 4) after vaccination with the inactivated or MLV vaccines. All four calves had higher antibody levels to the CP type 1 strains than to the CP type 2 strains. Calves 1-12 and 1-16 received the inactivated Singer vaccine and responded with the following titers: for calf 1-12, 256 to 1,024 to type 1 CP strains, 256 to 512 to type 1 NCP strains, and 64 to 128 to type 2 CP and NCP strains; for calf 1-16, 256 to 1,024 to type 1 CP strains, 64 to 256 to type 1 NCP strains, 8 to 64 to type 2 CP strains, and 8 to 16 to type 2 NCP strains.

Calves 1-1 and 1-4 received the MLV NADL vaccine and responded with the following titers: for calf 1-1, 128 to 1,024 to type 1 CP strains, 16 to 64 to type 1 NCP strains, 4 to 16 to type 2 CP strains, and 4 to 8 to type 2 NCP strains; for calf 1-4, 64 to 512 to type 1 CP strains, 32 to 64 to type 1 NCP strains, 4 to 16 to type 2 CP strains, and 16 to 32 to type 2 NCP strains.

Prior studies have detected a range of antibody titers to diverse BVDV strains in BVDV-vaccinated calf sera by neutralization tests in cell culture (5, 7–9). Use of various MAbs have placed various BVDV strains into groups. Recently, differences in PCR results along with antigenic differences have

resulted in two types, types 1 and 2. These respective types include both CP and NCP strains (22).

This study reports on the application and modification of the immunoperoxidase assay for the detection of BVDV infectivity by a microtiter VNT (18). The MAbs used in this study detected both type 1 and type 2 strains. Thus, this immunoperoxidase detection of BVDV infection permitted the detection of neutralizing antibodies to several BVDV strains, including both type 1 and type 2 strains. In general, the antibody titers to both the CP and NCP strains of the respective types were similar. However, the titers were not always identical, indicating some antigenic diversity among strains of each type.

The detection of BVDV neutralizing antibodies has application (i) in assessing the defense mechanisms of cattle challenged with virulent virus and (ii) for measuring the immune responses of cattle receiving BVDV vaccines. Calves with low to intermediate passively acquired antibody titers of 2 to 256 were not protected from viral challenge with the strain 890, a type 2 strain (10). However, as the antibody titers increased, the disease severity and disease duration decreased. The antibody response in calves is a measure of the immunogenicity of MLV BVDV vaccines (4). Bolin (4) cited the *Code of Federal Regulations*, 1990, Title 9, Part 1, Sections 113.215 and 113.311. Nineteen of 20 calves receiving the MLV vaccine must have a neutralizing titer of $\geq$8 within 28 days. Also, neutralizing antibodies are used to evaluate killed (inactivated) vaccines. A neutralizing titer of $\geq$8 must be present in four of five calves 14 days or more after the final dose was administered.

These results suggest that diagnosticians and/or investigators studying the immune response to BVDV may have an alternate way of measuring antibodies, particularly to those NCP strains. However, because the differences between type 1 and 2 strains can be detected by either inhibition of the CPE or the inhibition of infectivity detected by immunoperoxidase staining, one might choose the inhibition of the CPE because the immunoperoxidase test is more labor intensive, consuming both time and reagents. Interestingly, in preliminary studies (data not shown), infectivity to a type 1 strain was identical when both procedures, CPE detected by light microscopy and infectivity detected by immunoperoxidase staining, were used. In order to detect antibodies to either type 1 or type 2 BVDV strains, these two procedures are options. Perhaps only one strain of each type should be used.

Further studies are ongoing in this laboratory to determine the immune response to these BVDV strains in archived sera from calves receiving several different vaccines containing NADL, C24V, Singer, and nonidentified strains.

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