Nested Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) for Typing Ruminant Pestiviruses: Bovine Viral Diarrhea Viruses and Border Disease Virus

Robert W. Fulton, J.M. d'Offay, J.T. Saliki, Lurinda J. Burge, R.G. Helman, A.W. Confer, S.R. Bolin, and J.F. Ridpath

ABSTRACT

A nested reverse transcription (RT) polymerase chain reaction (PCR) assay was evaluated for differentiating reference bovine viral diarrhea virus (BVDV) strains, BVDV from diagnostic accessions, modified-live virus (MLV) BVDV strains in bovine viral vaccines, and a reference border disease virus (BDV). The detection level of this assay was compared to viral infection in cell culture. The PCR assay was used to distinguish 3 ruminant pestiviruses, types 1 and 2 BVDV, and type 3 BDV. The consensus (first) PCR assay detected all 3 ruminant pestiviruses, a result of the shared sequence homology. The consensus PCR product was subjected to a second (nested) PCR which used type-specific primers. The nested PCR was able to differentiate the 3 ruminant pestiviruses. Viral stocks of BVDV were diluted 10-fold and processed for the 2-step PCR assay. The sensitivity of this 2-step PCR assay was compared to viral infectivity in cell culture based on identical volumes of the system tested (cell culture assay and processing for RNA). The RT-PCR type-specific assay differentiated BVDV laboratory reference strains (12), diagnostic laboratory isolates (15), 2 MLV BVDV vaccine strains, and a BDV strain. The 30 ruminant pestiviruses typed included: (1) 27 reference strains and diagnostic laboratory isolates; 18 cytopathic (CP) type 1 strains, 3 CP type 2 strains, 3 noncytopathic (NCP) type 1 strains, and 3 NCP type 2 strains; (2) 2 MLV strains, type 1; and (3) 1 CP BDV type 3. The PCR assay had a detection limit of 10 $TCID_{50}$/0.025 mL of virus when 3 separate BVDV were tested. This 2 step RT-PCR assay would be useful for the typing of ruminant pestiviruses, particularly BVDV isolates from the diagnostic laboratory.

RÉSUMÉ

Une réaction d'amplification en chaîne par la polymérase (ACP) pour détecter la transcriptase réverse (TR) fut évaluée pour sa capacité à différencier des souches de référence du virus de la diarrhée virale bovine (VDVB), des isolats de VDVB provenant d'échantillons soumis pour analyse, de souches vivantes modifiées de VDVB retrouvées dans des vaccins, et d'une souche de référence du virus de la maladie de Border du mouton (VMB). Le niveau de détection de cette épreuve fut comparé à l'infection virale de culture cellulaire. L'épreuve ACP fut utilisée pour distinguer trois pestivirus de ruminant, les types 1 et 2 du VDVB, et le type 3 du VMB. Une première épreuve ACP a permis de détecter les trois pestivirus dû au partage de séquences homologues. Le produit d'ACP fut soumis à une seconde réaction d'ACP utilisant cette fois des amorces spécifiques de type. Cette dernière réaction a permis de distinguer les trois pestivirus de ruminant. Des solutions stocks de VDVB furent diluées 1:10 et préparées pour la réaction d'ACP en deux étapes. La sensibilité de cette réaction d'ACP en deux étapes fut comparée au potentiel infectieux viral en culture cellulaire basé sur des volumes identiques selon le système éprouvé. L'épreuve d'ACP-TR spécifique de type a permis de distinguer les souches de référence de VDVB (12), les isolats provenant de spécimens diagnostics (15), 2 souches contenues dans les vaccins vivants modifiés, et 1 souche de VMB. Les 30 pestivirus de ruminants analysés comprenaient (1) 27 souches de référence et isolats cliniques; 18 souches cytopathogènes (CP) de type 1, 3 souches CP de type 2, 3 souches non-cytopathogènes (NCP) de type 1, et 3 souches NCP de type 2; (2) 2 souches vivantes modifiées; et (3) 1 souche CP de VDVB de type 3. La limite de détection de l'épreuve ACP était de 10 $TCID_{50}$/0,025 mL de virus lorsque 3 souches de VDVB différents furent éprouvés. L'épreuve ACP-TR en deux étapes serait utile pour déterminer le type des pestivirus de ruminant, et plus particulièrement les isolats de VDVB provenant des laboratoires de diagnostic.

(Traduit par le docteur Serge Messier)

INTRODUCTION

The *Pestivirus* genus of the Flaviviridae contains 3 viruses affecting the livestock industries of cattle, sheep, and swine (1,2). These 3 viruses include bovine viral diarrhea virus (BVDV), border disease virus (BDV) of sheep, and hog cholera virus (HCV). Two genotypes of BVDV

Department of Infectious Diseases and Physiology (Fulton, d'Offay, Saliki, Burge); Department of Anatomy, Pathology and Pharmacology (Helman, Confer); Oklahoma Animal Disease Diagnostic Laboratory, (Saliki, Helman); College of Veterinary Medicine, Oklahoma State University, Stillwater, Oklahoma 74078 USA; and the Enteric and Food Safety Research Unit, National Animal Disease Center, Agricultural Research Service, United States Department of Agriculture, P.O. Box 70, Ames, Iowa 50010 USA (Bolin, Ridpath).

Address correspondence and reprint requests to Dr. Robert W. Fulton, telephone: (405) 744-6737; fax: (405) 744-8263; e-mail: rfulton@okway.okstate.edu.

Received June 3, 1999.

are found in cattle. Both members of the BVDV and BDV are present in the United States, whereas HCV is considered a foreign disease. Bovine viral diarrhea virus is a frequent viral isolate from diagnostic laboratory accessions of bovine samples. There are many clinical forms of BVDV infections of cattle including 1) acute infections: subclinical, digestive tract disease, respiratory disease, immunosuppression, hemorrhagic disease; 2) fetal infections: abortions, stillbirths, congenital defects, and calves born immunotolerant and persistently infected; 3) chronic infections with possible multiple organ systems affected; and 4) classical mucosal disease (3–5). Accurate and relevant diagnosis of BVDV infections is important to the clinician/ diagnostician in evaluating clinically ill cattle or necropsy specimens submitted to the diagnostic laboratory. Persistently infected (PI) animals occur as a result of fetal infection with non-cytopathic BVDV strains in early pregnancy and the calves survive (6,7). The PI animals are infected for life, shed virus continuously, and are important for transmission of BVDV to susceptible animals (6). Identification and removal of PI cattle from herds is important for the prevention and control measures for BVDV in cattle.

Diagnosis of BVDV infection utilizes 1) serologic detection of rising serum antibody titers in paired sera indicating active infection, (2) virus isolation in cell culture, (3) fluorescent antibody staining of tissue sections with BVDV specific antiserum, and (4) immunohistochemistry on formalin-fixed tissues (8). Also various PCR assays have been used to detect BVDV in infected cell cultures and/or infected animal tissues, including blood (9–21). Based on growth characteristics in cell cultures, BVDV strains are divided into 2 biotypes: cytopathic (CP) or non-cytopathic (NCP), as CP strains cause visual cytopathic effects, whereas NCP strains do not (1–3). Both CP and NCP BVDV biotypes can be divided into 2 separate genotypes, types 1 and 2, using polymerase chain reaction (PCR) testing (18,19). These genotype differences coincide with antigenic differences. Recently, the type 1 strains have been further divided into types 1a and 1b (20).

A nested reverse transcription (RT) PCR assay was evaluated for differentiating ruminant pestiviruses in the United States (16). The purpose of this current study was to evaluate that 2-step PCR assay, to classify reference BVDV strains, BVDV viruses from diagnostic accessions, bovine vaccines containing modified-live virus (MLV) strains, and a reference BDV strain. Both CP and NCP BVDV and BDV CP reference strains, BVDV viruses from diagnostic laboratories, and bovine vaccines were included in the typing. The detection level of this PCR assay compared to viral infectivity in cell culture was determined for various BVDV.

MATERIALS AND METHODS

VIRUSES

Thirty ruminant pestiviruses, including 29 BVDV strains and a reference CP BDV strain were utilized in the study. These included laboratory reference strains and 1 BDV strain from various sources, diagnostic isolates from the Oklahoma Animal Disease Diagnostic Laboratory, Oklahoma State University, and Michigan State University Animal Health Diagnostic Laboratory, and 2 vaccines containing modified-live virus (MLV) strains of bovine herpesvirus 1 (BHV-1), BVDV (NADL strain), parainfluenza-3 virus (PI-3V), and bovine respiratory syncytial virus (BRSV), BoviShield-4 MLV vaccine (Pfizer Animal Health, Exton, Pennsylvania, USA) and Pyramid-4 MLV vaccine (Fort Dodge Laboratories, Fort Dodge, Iowa, USA). The sources of the respective viruses are included in Table I. The BDV CB-5 strain was a CP strain obtained from the USDA, ARS, NADC, Ames, Iowa, USA. The diagnostic isolates had been confirmed as BVDV (Michigan State University Animal Health Diagnostic Laboratory, East Lansing, Michigan, USA) (5). The laboratory reference strains and diagnostic laboratory strains were grown in either bovine turbinate (BT) or Madin Darby bovine kidney (MDBK) monolayer cultures, as described (22,23). The fetal bovine serum (FBS) and the BT and MDBK used to propagate the viruses were tested and confirmed to be free of BVDV and BVDV antibodies (5). Dessicated vaccines were reconstituted with diluent as provided with original package.

RNA EXTRACTION

Viral RNA was extracted and prepared as described, with modifications (20). Total RNA from freeze-thaw extracts of infected monolayers or diluted vaccines was prepared by acid guanidium thiocyanate/phenol/chloroform extraction. This procedure was modified by using 5:1 saturated phenol/chloroform, pH 4.5. A 375-μL aliquot from the virus containing fluids was added to a 1.5-mL microfuge tube, and 1.5 μL of 10 mg/mL tRNA was added. A stock denaturing solution was prepared. The stock solution was prepared by dissolving 250 g guanidium thiocyanate in a solution of 293 mL DEPC-treated deionized H_2O, sterile and nuclease-free (Midwest Scientific, St. Louis, Missouri, USA), 17.6 mL of 0.75 M sodium citrate, pH 7, and 26.4 mL 10% Sarkosyl (N-laurylsarcosine) at 60–65°C with stirring. The denaturing working solution was prepared by adding 0.35 mL 2ME/50 mL, 0.1 M stock solution. Then 375 μL denaturing working solution and 75 μL 2 M sodium acetate, pH 4.2 (Midwest Scientific), were added, and then mixed gently by inverting the tube. A volume of 750 μL phenol/chloroform, pH 4.5 was added, with mixing by gently inverting the tube, and incubating it on ice for 15 min. The tube was then centrifuged at 10 000 × *g* for 15 min with the aqueous phase (about 700 μL) transferred to a fresh 1.5-mL tube. The RNA was precipitated by mixing the aqueous phase with 1 volume of 100% isopropanol prior to incubating at −20°C for 30 min. The tube was then centrifuged at 10 000 × *g* for 10 min and the supernatant was discarded. The pellet was dissolved in 300 μL denaturing working solution with 1 volume 100% isopropanol added and mixed, followed by incubation at −20°C for 30 min. The mixture was then centrifuged for 10 min at 10 000 × *g* with the supernatant subsequently discarded. The pellet was resuspended in 75% ethanol, vortexed, incubated 10–15 min at room temperature and centrifuged at 10 000 × *g* for 5 min. The supernatant was discarded, and the pellet was then dried 5–10 min in

TABLE I. BVDV and BDV strain identification: source, biotype, and genotype

Identification	Source: Laboratory strain	Source: Vaccine	Source: Diagnostic sample	Biotype (CP or NCP)	Genotype (type 1, 2, or 3)
BVDV					
125-C	a			CP	2
1583 B-1	a			CP	1
TGAC 8HB	a			CP	1
88055	a			CP	1
NADL	a			CP	1
NADL	b			CP	1
Singer	c			CP	1
Singer	b			CP	1
C24V	a			CP	1
5960	a			CP	1
93110915			d	CP	1
94020392			d	CP	1
94110544-382			d	CP	1
94080633			d	CP	1
94121250			d	CP	1
94110474			d	CP	1
1345981			e	CP	1
1423783			e	CP	1
1315303			e	CP	1
1321365			e	CP	2
1431535			e	CP	2
96081190-1			d	NCP	2
96080225			d	NCP	1
94111075			d	NCP	1
Nebraska	a			NCP	1
9401309			d	NCP	2
890	a			NCP	2
MLV(NADL)		f		CP	1
MLV(NADL)		g		CP	1
BDV					
CB-5	a			CP	3

a — USDA ARS NADC, Ames, Iowa; b — Texas Veterinary Medical Diagnostic Laboratory, Amarillo, Texas; c — Reference citation: (23); d — Oklahoma Animal Disease Diagnostic Laboratory, Stillwater, Oklahoma; e — Animal Health Diagnostic Laboratory, Michigan State University, East Lansing, Michigan; f — BoviShield-4 MLV Vaccine, Pfizer Animal Health, Exton, Pennsylvania; g — Pyramid-4 MLV Vaccine, Fort Dodge Laboratories, Fort Dodge, Iowa; CP — cytopathic; NCP — non-cytopathic

speed-vac and resuspended in 15 μL DEPC-treated deionized H_2O. The material was then stored at −70°C until used.

PRIMER SELECTION

The consensus primers, P1 and P2, were those selected that share maximum homology with all 3 pestiviruses (BVDV types 1 and 2 and BDV) and have no homology to other regions of the pestivirus genomes (16). The P1 and P2 primers (826-bp region) were from the EO region of the pestivirus genome. From that consensus region (826-bp), 3 type specific primers, TS1, TS2, and T3, were prepared (Recombinant DNA/Protein Resource Facility, Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, Oklahoma, USA) (16). The bp count of the amplified DNA for the respective T primers was: TS1, 566; TS2, 488; and TS3, 223. The consensus PCR primers, P1/P2, amplified the 826-bp region. In the second reaction, nested PCR, the consensus PCR product was then reacted with TS1, TS2, TS3, and P2. The TS1/P2 was specific for BDV; TS2/P2 was specific for BVDV type 2; and TS3/P2 was specific for BVDV type 1 (16). The primers were dissolved in DEPC-treated deionized H_2O at 500 μg/mL.

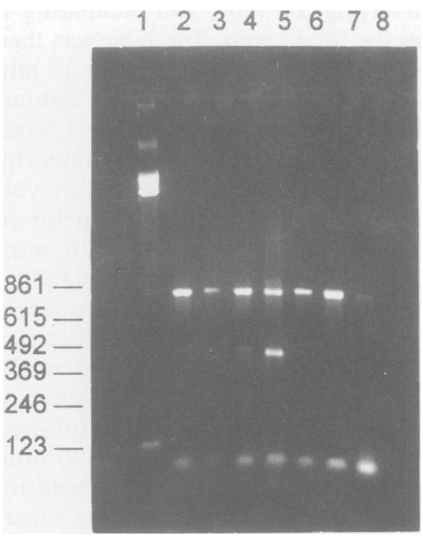

Figure 1. Agarose gel electrophoresis analysis of DNA product of ruminant pestivirus RNA: amplification with consensus primers P1 and P2. Lanes: (1) ladder for bp; (2) Singer; (3) NADL; (4) Oregon C24V, (5) Nebraska; (6) 125-C; (7) 890; and (8) BDV CB-5.

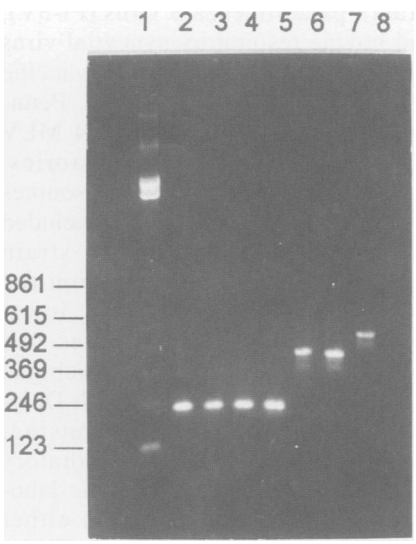

Figure 2. Agarose gel electrophoreses analysis of DNA product with type specific primers, TS1, TS2, and TS3 on DNA product from consensus primers. Lanes: (1) ladder for bp; (2) Singer; (3) NADL; (4) Oregon C24V, (5) Nebraska; (6) 125-C; (7) 890; and (8) BDV CB-5.

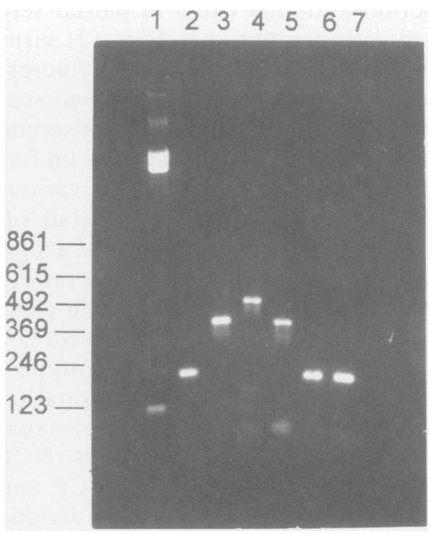

Figure 3. Agarose gel electrophoresis analysis of DNA product with type specific primers. Control reference viruses and selected diagnostic laboratory isolates. Lane: (1) ladder for bp; (2) NADL; (3) 890; (4) BDV; (5) 96081-1190-1; (6) 94110544-382; and (7) 96080225.

PCR AMPLIFICATION OF PESTIVIRUS RNA

The components in the PCR kit were used including the cycles, reaction times, and temperatures listed in the kit (Gene AMP RNA PCR kit, Perkin Elmer, Foster City, California, USA). Reagents included: MuLV reverse transcriptase (RT) (50 U/μL), RNase inhibitor (20 U/μL), *Taq* DNA polymerase (5 U/μL), 10 mM each of dATP, dCTP, dGTP, and dTTP, and 10 × PCR buffer II (500 mM KCl, 100 mM Tris-HCl, pH 8.3) in 25 mM $MgCl_2$ solution. The RT reaction was performed in a final volume of 20 μL containing: 4 μL 25 mM $MgCl_2$; 2 μL 10 × PCR buffer II; 2 μL DEPC-treated deionized H_2O; 2 μL each of dGTP, dATP, dCTP, and dTTP; 1 μL RNAse inhibitor, 1 μL MuLV RT; 1 μL P2; and 1 μL of sample. The RT reaction was carried out at 42°C for 15 min followed by an initial denaturation at 99°C for 5 min, and cooling at 5°C for 5 min. Then 80 μL of the following were added to the tube: 4 μL 25 mM $MgCl_2$ solution; 8 μL 10 × PCR buffer II; 65.5 μL DEPC-treated deionized H_2O; 0.5 μL *Taq* DNA polymerase; 1 μL DEPC-treated deionized H_2O (instead of P2); 1.0 μL P1 and 20 μL of reverse transcription reaction mixture, for a total volume of 100 μL. This consensus PCR reaction was performed at 95°C for 105 s (initial step). The PCR reaction continued for 35 cycles of 95°C for 15 s (melt) and 60°C for 30 s (anneal-extend). A final step of extension at 72°C for 7 min completed the amplification process. The products were stored at 4°C. The amplified products (consensus PCR product), 8 μL of product per well were electrophoresed on 2.0% agarose gels containing ethidium bromide stain, illuminated, visualized, and photographed in a UV light box.

NESTED PCR WITH TYPE-SPECIFIC PRIMERS

A second reaction was performed on the consensus PCR product using type-specific primers (16). The reaction mixture contained 100 μL with the following components: 8 μL 25 mM $MgCl_2$ solution, 10 μL 10 × PCR buffer II; 68.5 μL DEPC-treated deionized H_2O; 2 μL each of the deoxynucleotides; 0.5 μL *Taq* DNA polymerase; 1 μL each of the primers P2, TS1, TS2, and TS3; and 1 μL of sample (1:500 in DEPC-treated deionized H_2O). The sample was subjected to 25 cycles of 94°C for 1 min (denaturation), 50°C for 45 s (primer annealing), and 72°C for 45 s (primer extension). The reaction products were stored at 4°C. The reaction product was electrophoresed on a 2% agarose gel, as described above. The position of the priming with each pestivirus type-specific primer was designed such that the size of the nested DNA produced was characteristic for each pestivirus type (16).

TABLE II. Comparison of sensitivity of RT-PCR and cell culture viral assay

Amount of virus in 25-μL sample	Type 1 CP (NADL)	Type 1 NCP (Nebraska)	Type 2 NCP (890)
10 000[a]	+[b]	+	+
1000	+	+	+
100	+	+	+
10	+	+	+
1	−	−	−

[a] Numerical values represent $TCID_{50}$ of infectious virus found in 25 μL (0.025 mL), based on microtiter assay in cell culture

[b] RNA extracted from sample of diluted virus, reverse transcribed, amplified in an initial reaction with consensus primers, and reamplified in a second reaction with type-specific primers, and the product electrophoresed

RT-PCR DETECTION LEVEL

Cell cultures infected with each of 3 BVDV reference stocks were frozen at −70°C (22). The cell culture supernatants were assayed for viral infectivity in a cell culture microtiter assay (22,23). The viral infectivity was expressed as $TCID_{50}$/0.025 mL (25 μL). Ten-fold serial dilutions of the virus were made in MEM with 2% BFS, and each dilution was then processed for RNA extraction, as described above. The RNA was assayed by the RT-PCR (both steps) and the amplification products were analyzed by gel electrophoresis.

RESULTS

CONSENSUS PCR PRODUCT OF PESTIVIRUS GENOME

RNA was isolated from 30 ruminant pestiviruses and reacted in the RT PCR. The consensus PCR products of the 826-bp region were visualized with P1 and P2 primers (Fig. 1). Of the 30 ruminant pestiviruses, 25 yielded PCR products of the expected bp size. Of the 5 viruses without visible consensus PCR bands, 1 was a reference virus, 2 were diagnostic laboratory isolates, and 2 were MLV BVDV strains.

TYPE-SPECIFIC PCR PRODUCT

The DNA products of the amplification of the consensus pestivirus genomes were subjected to a second round of amplification in the nested PCR reaction using TS1, TS2, TS3, and P2 primers and were visualized in the agarose gel (Fig. 2). Each of the 30 ruminant viruses had bands at the expected regions for the respective type. The 5 viruses that lacked a visible band after the first round PCR (consensus) had a visible band after the second round of type-specific PCR. The RT-PCR type specific assay differentiated both BVDV laboratory reference strains (12), viruses from 2 diagnostic laboratories (15), 2 MLV BVDV vaccine strains, and a CP BDV reference strain (Table I). An example of 3 diagnostic laboratory isolates compared to the 3 reference types is demonstrated in Figure 3. The 27 BVDV reference and diagnostic laboratory isolates included 18 CP BVDV type 1 strains; 3 CP BVDV type 2 strains; 3 NCP BVDV type 1 strains; and 3 NCP BVDV type 2 strains. The CB-5 BDV strain is cytopathic. The 2 MLV vaccines each contained a type 1 strain, which is consistent for the NADL strain according to package label inserts.

RT-PCR DETECTION LEVEL

The nested PCR assay was performed with RNA extracted from a 375-μL fraction of ten-fold dilutions of virus with titers expressed as $TCID_{50}$/0.025 mL (25 μL). Then 375 μL of the initial sample resulted in a 15-μL sample at completion of RNA extraction. Then 1 μL of the RNA sample was then amplified in the 2-step PCR assay. Thus, the 1 μL

used in the RT-PCR represented $TCID_{50}$/0.025 mL, or 25 μL, based on the RNA extraction.

The sensitivity of the nested PCR assay using the 2-step RT-PCR compared to viral infectivity is shown in Table II. For each of the 3 BVDV, the RT-PCR detected 10 $TCID_{50}$/0.025 mL of the initial sample in the amplification product at the end of amplification with type specific primers.

DISCUSSION

The results of this current study support those findings of the prior report whereby these same primers P1, P2, TS1, TS2, and TS3, detected type-specific sequence differences unique to each of the 3 types (16). All 3 type specific primers were in the second amplification and only a single amplified product was detected for the respective virus. Our findings were somewhat different in that 5 BVDV strains did not yield a visualized product on the first amplification of the consensus PCR assay. However, all 5 products of the first amplification produced a type-specific product in the second amplification. The viruses were not titrated for infectivity prior to RNA extraction. It is possible that a relatively low level of virus genome was present for the first amplification. There were, on occasion, extra bands for the consensus PCR amplification as observed in Figure 1 (lane 5). These were unique to the respective virus and observed on repeated PCR amplification.

Our study confirms the use of consensus primers and type-specific primers to differentiate ruminant pestivirus from a prior study (16). However, there were differences between our studies. This current study utilized modifications in the consensus PCR and type-specific PCR. This study utilized different volumes of reactants, sources of reactants, and reaction conditions of RT and consensus PCR reaction from the prior study (16). However, the conditions of the second round amplification, with type-specific primers for denaturation, primer annealing, and primer extension, were the same. In a prior study (16) with these same primers, 6 reference strains were used: 2 bovine strains, NADL and 890; and 4 ovine strains, BD78, BD31, BDSC, and OV97. Of those 6 strains, our study used the NADL and 890 strains. Our study, with these same primers, confirmed the same genotypes for NADL strain (type 1) and 890 strain (type 2). This current study examined additional strains previously typed with a total of 10 reference strains (19) and 5 diagnostic isolates examined (Michigan State University, Animal Health Diagnostic Laboratory, East Lansing, Michigan, USA). Another area unique to this study is the addition of the biotype for each BVDV typed, CP or NCP (Table I).

Our results for genotyping were consistent with other laboratories working with these same viruses. All of the viruses obtained from the USDA-ARS, NADC laboratory had the same genotyping as in our study (Table I) (19). Also, the 5 BVDV strains from Michigan State University had the same genotype as originally reported. The 2 MLV vaccines containing the NADL strain had the type 1 genotype as expected for the NADL strain. Interestingly, 3 individual viruses (laboratory strains often used for serology) had not been previously genotyped. These included 2 Singer strains from separate laboratories (Oklahoma State University and Texas Veterinary Medical Diagnostic Laboratory, Amarillo, Texas), and a NADL strain (Texas Veterinary Medical Diagnostic Laboratory) (22,23). The typing in this study was the expected type as priorly published reports on this strain by another laboratory have stated (19). This indicates that these reference strains had not become contaminated with type 2 BVDV. The FBS may be contaminated with BVDV (24). In a recent study, 1000 FBS lots collected by a commercial supplier were tested for BVDV by cell culture procedures (24). Analysis indicated 203 lots with BVDV contamination (198 NCP and 5 CP isolates) with 115 containing BVDV type 1, 65 with BVDV type 2, and 23 with ≥ 2 BVD isolates. Although FBS is usually screened for BVDV by cell culture inoculation and confirmation by BVDV-positive antisera, concern exists for possible contamination of these reference strains. The nested PCR assay used in this study may be an excellent supplemental test for surveillance of BVDV adventitious virus contamination.

The published detection levels of various PCR assays are varied (9,10,21). The different detection limits were reached using various methods, including different dilutions and samples. Several studies may have used serums from infected animals processed for both PCR and cell culture infectivity or, in the case of our study, viral stocks with predetermined infectivity in cell culture. Examples of studies of detection limits of PCR to BVDV viral infectivity where volumes were described are: 6 $TCID_{50}$/300 μL of fetal fluid; (21); 10 μL of serum (10^4 $CCID_{50}$/mL) (9) and ≤ 1 $TCID_{50}$/100 μL of serum (10). Obviously comparisons of assays are difficult with the different protocols.

Also, our study used a limited number of isolates from 2 diagnostic laboratories. While this was not a prevalence/incidence survey for BVDV, we were able to type all the BVDV strains which had been isolated after cell culture inoculation and in which the presence of BVDV was confirmed, based on reaction with BVDV-positive antiserum. This nested RT PCR assay is easily adapted to laboratories interested in improving how they identify and characterize field BVDV isolates from cattle and/or bovine necropsy specimens. For example, a recent report described a nested PCR assay capable of detecting BVDV in aborted bovine fetuses (21). This information is extremely useful for the epidemiology of ruminant pestiviruses, and also for vaccine-related diagnostic challenges.

ACKNOWLEDGMENTS

The authors express appreciation to Ms. Sherl Holesko for photography and figure preparation. Supported by OKLO 1990 project, Oklahoma Agricultural Experiment Station.

REFERENCES

1. **COLLETT MS.** Genomic structure of BVDV. In: Program and Abstracts of the International Symposium Bovine Viral Diarrhea Virus: A 50-Year Review. Ithaca: Cornell University Press 1996: 18–23.
2. **MEYERS G, TAUATZ N, DUBOVI EJ, et al.** Origin and diversity of cytopatho-

genic pestiviruses. In: Program and Abstracts of the International Symposium Bovine Viral Diarrhea Virus: A 50-Year Review. Ithaca: Cornell University Press 1996: 24–34.
3. **BAKER JC.** The clinical manifestations of bovine viral diarrhea infection. Vet Clin North Am Food Anim 1995; 11: 425–445.
4. **SALIKI JT.** Bovine viral diarrhea: clinical picture in South Central United States. In: Program and Abstracts of the International Symposium Bovine Viral Diarrhea Virus: A 50-Year Review. Ithaca: Cornell University Press 1996: 159–164.
5. **SALIKI JT, FULTON RW, HULL SR, et al.** Microtiter virus isolation and enzyme immunoassays for detection of bovine viral diarrhea virus in cattle serum. J Clin Microbiol 1997; 35: 803–807.
6. **KIRKLAND PD, MCGOWAN MR, KAFI M, et al.** Early reproductive loss due to bovine viral diarrhea virus infection. In: Program and Abstracts of the International Symposium Bovine Viral Diarrhea Virus: A 50-Year Review. Ithaca: Cornell University Press 1996: 98–107.
7. **MCCLURKIN AW, LITTLEDIKE ET, CUTLIP RC, et al.** Production of cattle immunotolerant to bovine viral diarrhea virus. Can J Comp Med 1984; 48: 156–161.
8. **BROCK KV.** Diagnosis of bovine viral diarrhea virus infections. Vet Clin North Am Food Anim 1987; 11: 549–561.
9. **BROCK KV.** Detection of persistent bovine viral diarrhea virus infections by DNA hybridization and polymerase chain reaction assay. Arch Virol 1991; Suppl 3: 199–208.
10. **LOPEZ OJ, OSORIO FA, DONIS RO.** Rapid detection of bovine viral diarrhea virus by polymerase chain reaction. J Clin Microbiol 1991; 29: 578–582.
11. **HOOFT VAN IDDEKINGE BJL, VAN WAMEL JLB, VAN GENNIP HG, et al.** Application of the polymerase chain reaction to the detection of bovine viral diarrhea virus infections in cattle. Vet Microbiol 1992; 30: 21–34.
12. **RIDPATH JF, BOLIN SR, KATZ J.** Comparison of nucleic acid hybridization and nucleic acid amplification using conserved sequences from the 5′ noncoding region for the detection of bovine viral diarrhea virus. J Clin Microbiol 1993; 31: 986–989.
13. **ALANSARI H, BROCK KV, POTGEITER LND.** Single and double polymerase chain reaction for detection of bovine viral diarrhea virus in tissue culture and sera. J Vet Diagn Invest 1993; 5: 148–153.
14. **RADWAN GS, BROCK KV, HOGAN JS, et al.** Development of a PCR amplification assay as a screening test using bulk milk samples for identifying dairy herds infected with bovine viral diarrhea virus. Vet Microbiol 1995; 44: 77–92.
15. **HAMEL AL, WASYLYSHEN MD, NAYAR GPS.** Rapid detection of bovine viral diarrhea virus by using RNA extracted directly from assorted specimens and a one-tube reverse transcription PCR assay. J Clin Microbiol 1995; 33: 287–291.
16. **SULLIVAN DG, AKKINA RK.** A nested polymerase chain reaction assay to differentiate pestiviruses. Virus Res 1995; 38: 231–239.
17. **LAAMANEN UI, NEUVONEM EP, YLIVIUHKOLA EM, et al.** Comparison of RT-PCR assay and virus isolation in cell cultures for the detection of bovine viral diarrhea virus (BVDV) in field samples. Res Vet Sci 1997; 63: 199–203.
18. **PELLERIN CJ, VAN DEN HURK J, LECOMTE J, et al.** Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities. Virology 1984; 203: 260–268.
19. **RIDPATH JF, BOLIN SR, DUBOVI EJ.** Segregation of bovine viral diarrhea virus into genotypes. Virology 1994; 205: 66–74.
20. **RIDPATH JF, BOLIN SR.** Differentiation of types 1a, 1b and 2 bovine viral diarrhoea virus (BVDV) by PCR. Mol Cell Probes 1998; 12: 101–106.
21. **HYNDMAN L, VILCEK S, CONNER J, et al.** A novel nested reverse transcription PCR detects bovine viral diarrhea virus in fluids from aborted fetuses. J Virol Methods 1998; 71: 69–76.
22. **FULTON RW, CONFER AW, BURGE LJ, et al.** Antibody responses by cattle after vaccination with commercial viral vaccines containing bovine herpesvirus-1, bovine viral diarrhea virus, parainfluenza-3 virus, and bovine respiratory syncytial virus immunogens and subsequent revaccination at day 140. Vaccine 1995; 13: 725–733.
23. **FULTON RW, SALIKI JT, BURGE LJ, et al.** Neutralizing antibodies to type 1 and 2 bovine viral diarrhea viruses: detection by inhibition of viral cytopathology and infectivity by immunoperoxidase assay. Clin Diagn Immunol 1997; 4: 380–383.
24. **BOLIN SR, RIDPATH JF.** Prevalence of bovine viral diarrhea virus genotypes and antibody against those viral genotypes in fetal bovine serum. J Vet Diagn Invest 1998; 10: 135–139.