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Rapid Detection of Bovine Viral Diarrhea Virus by Polymerase Chain Reaction†

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The polymerase chain reaction was used to detect genomic sequences of the positive-stranded RNA of bovine viral diarrhea virus (BVDV), a member of the family *Togaviridae*. Using a set of 20-bp primers located within the conserved 3′ region of the BVDV genome, we were able to consistently amplify a 205-bp target sequence from BVDV cDNA. BVDV RNAs from cell culture-propagated BVDV reference strains, diverse unrelated cytopathic and noncytopathic field isolates, and clinical serum samples were transcribed to cDNA by using avian myeloblastosis virus reverse transcriptase and further specifically amplified by using the polymerase chain reaction assay. The amplification assay was sensitive enough to detect one molecule of cloned BVDV cDNA. Reconstitution experiments conducted by adding decreasing amounts of BVDV (NADL strain) to BVDV-free serum indicated that the threshold of sensitivity of the assay was ≤1 50% tissue culture infective dose. These results show that the polymerase chain reaction may be used for the rapid detection of diverse strains of BVDV in cell cultures, biological products, and clinical specimens from cattle.

Bovine viral diarrhea virus (BVDV) is one of the most important pathogens of the bovine fetus (3). BVDV infection inflicts insidious and sometimes devastating losses on both beef and dairy cattle industries as a result of reproductive and respiratory disease (11). The virus has a genome composed of single-stranded (positive-polarity) nonpolyadenylated RNA of about 12.5 kb in length (6). Field BVDV isolates can be divided into two biotypes according to their ability to produce cytopathic effect in cell cultures (24). Infection with the noncytopathic biotype during the first trimester of gestation leads to abortions, stillbirths, or the birth of persistently infected (PI), immunologically tolerant calves. Generally, the PI animal is apparently healthy, but if superinfection with a cytopathic biotype takes place, the animal usually dies of mucosal disease (1, 2). The percentage of PI cattle in individual herds is usually small; however, it is enough to allow transmission of the infection to other cattle, thus perpetuating BVDV in the herd (1). In order to control the disease, it is important to unequivocally identify and remove PI animals from cattle herds. Furthermore, the presence of BVDV in fetal serum and other bovine products used for cell culture poses a great problem for research laboratories and biological industries. BVDV is one of the leading contaminants of cell cultures and veterinary biologic products (14, 18). The techniques currently in use to detect and/or isolate BVDV in contaminated cell cultures as well as in clinical samples obtained from acutely infected and PI animals are immunofluorescence and/or virus isolation in cell cultures. These techniques are time-consuming and do not provide rapid diagnostic results (8, 19). In this paper, we describe a rapid and sensitive method for detection of BVDV by using the polymerase chain reaction (PCR) assay (21).

MATERIALS AND METHODS

Cells. A mycoplasma- and BVDV-free diploid line of bovine turbinate (BT) cells was obtained from R. Van Deusen (National Veterinary Services Laboratories, U.S. Department of Agriculture, Ames, Iowa).

Reference strains. Singer and NADL strains of BVDV were obtained from the American Type Culture Collection, and Oregon and New York strains were obtained from the National Veterinary Services Laboratories (Diagnostic Virology).

BVDV isolates. BVDV samples were isolated in primary bovine testicle cell cultures from clinical samples obtained from animals with mucosal disease in New York State. These isolates were confirmed to be serologically unrelated by monoclonal antibody typing (unpublished results). The presence of BVDV was confirmed after serial passages ($n \geq 3$) by direct immunofluorescence with a BVDV-specific conjugate supplied by the National Veterinary Services Laboratories. These samples included four isolates of the cytopathic biotype obtained from buffy coat (sample 7) and oral swabs (samples 9, 10, and 11) as well as two isolates of the noncytopathic biotype obtained from buffy coat (sample 6) and oral swabs (sample 8).

Clinical samples. Sera submitted to the Veterinary Diagnostic Center of the University of Nebraska from BVDV-viremic animals with clinical cases of mucosal and respiratory disease were obtained.

Infectious-serum reconstitution. To assess the sensitivity of the amplification test, BVDV strain NADL was added in serial (10-fold) dilutions to BVDV-free fetal bovine serum.

BVDV cDNA. A 2,160-bp cDNA flanked by *Pst*I restriction sites cloned in pBR322 plasmid (pBV-KPB) was obtained from Marc Collett (Molecular Genetics, Inc., Minnetonka, Minn.). This cDNA corresponds to the sequences between bases 9835 and 12225 of the NADL strain genome.

RNA extraction from cell cultures. Approximately 10^7 BT cells grown in minimal essential medium supplemented with 10% horse serum were infected at a multiplicity of infection of 0.2 to 1, and 48 h later cytoplasmic RNA was extracted (10). Briefly, after two washes with cold phosphate-buffered saline, the cell pellet was lysed with 0.5% Nonidet P-40, digested for 15 min at 37°C with proteinase K (200 μg/ml)–sodium dodecyl sulfate (SDS) (0.2% [wt/vol]), and then centrifuged at 800 × *g* for 5 min, and the RNA was extracted

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from the resulting supernatant with phenol-chloroform. The RNA was precipitated with cold 100% ethanol in the presence of 3 M ammonium acetate, pH 5.2, on dry ice for 1 h. The pellet was dried in a desiccator and resuspended in water.

RNA extracted from serum samples. A 100-μl volume of serum was added to 450 μl of STE buffer (100 mM NaCl, 50 mM Tris, 5 mM EDTA; pH 8), and digestion was carried out for 10 min at room temperature with proteinase K (200 μg/ml) and for 20 min at 56°C in the presence of SDS (0.1% [wt/vol]). After two extractions with phenol-chloroform, the RNA fraction was precipitated for 1 h on dry ice, resuspended in 10 μl of distilled water, and submitted for cDNA production by reverse transcription (RT).

Transcription of RNA to cDNA. After different denaturation procedures addressed to preventing or minimizing secondary structure formation were tried (6, 15), the following procedure was selected. A 10-μl volume of RNA was added to 1.5 μl of primer 2.2 (40 μmol), and the solution was heated at 70°C for 5 min and then chilled on ice. For the RT assay, denatured RNA was incubated at 42°C for 2 h in the presence of Schimke solution (10 mM dithiothreitol, 10 mM $MgCl_2$, 70 mM KCl, 80 mM Tris; pH 8), deoxynucleoside triphosphates (7.5 μM; Pharmacia), 1 μl of RNasin (28 U/μl; Promega), and 1 μl of avian myeloblastosis virus reverse transcriptase (19.6 U/ml; Life Sciences). A 5-μl portion of this solution was used for the PCR assay.

Primers for PCR and target fragment. The 20-bp primers 2.1 and 2.2 used for amplification of BVDV have a GC content of 35% and overall free energy coefficients for formation of secondary structure of 0.7 and −1, respectively (CGC program, University of Wisconsin). The primers were synthesized (Genetic Designs, Inc., Houston, Tex.) and purified by electrophoresis in polyacrylamide. The 205-bp target has an overall free energy for secondary structure formation of −24.9 and is located between bases 9893 and 10098 (6). There is a single recognition site for the restriction enzyme *Bst*EII at base 9964 (see Fig. 1).

PCR. Optimization was carried out by varying (i) the concentrations of $MgCl_2$ in the reaction buffer, (ii) the annealing temperatures, and (iii) the number of cycles. Briefly, the optimized protocol is as follows. A 10-μl volume of 10× buffer (15 mM $MgCl_2$, 400 mM Tris-HCl, 500 mM KCl, 1% gelatin), 16 μl of an equimolar mixture of deoxynucleoside triphosphates (5 mM), 3 μl of each primer (40 μmol), and 5 μl of cDNA template were added in a total volume of 100 μl. For amplification of cDNA obtained by previous RT of BVDV RNA, 2.5 μl of primer 2.2 was added. The mixture was heated at 95°C for 5 min, after which 3 U of Taq polymerase (Perkin-Elmer Cetus) in a 5-μl volume of 1× buffer was added. A layer of 50 μl of Nujol mineral oil (Perkin-Elmer) was added to prevent evaporation of the reagents. The reaction consisted of 40 equal cycles on an automated thermal cycler (Perkin-Elmer Cetus). Each cycle was composed of 1 min at 95°C (denaturation step), 1 min at 55°C (primer-annealing step), and 2 min at 72°C (primer extension step). Controls used in each amplification round included (i) plasmid pBR322, (ii) RNA from uninfected BT cells, (iii) RNA extracted from BVDV-free bovine fetal serum, and (iv) a replicate sample including a mixture of all reagents with no template added.

Analysis of PCR-amplified products. (i) Electrophoresis. A 10-μl sample of each amplified product was run in 2% NuSieve agarose in Tris-borate-EDTA buffer at 70 V for 2 h.

(ii) Control of amplification specificity. The 205-bp amplification fragment has a restriction site for *Bst*EII that produces two sizeable fragments upon digestion (71 and 134 bp) (Fig. 1). A 50-μl portion of each reaction mixture (half of the total volume) was extracted with phenol-chloroform, precipitated with 100% ethanol–3 M ammonium acetate (pH 5.2), and resuspended in 16 μl of water. *Bst*EII (8 U/μl; Bethesda Research Laboratories) digestion was carried out as recommended by the manufacturer. A sample of 10 μl of the digested product was electrophoresed under the conditions described above.

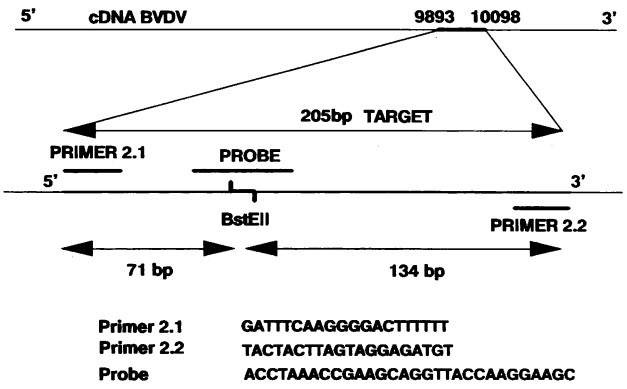

FIG. 1. Illustration of the 205-bp amplification target from BVDV cDNA. Restriction enzyme *Bst*EII cuts the target into two fragments, 71 and 134 bp long.

(iii) Southern blot. After electrophoresis, DNA in the gels was blotted onto a nylon membrane (Hybond; Amersham) and then denaturated and attached to the filter by exposure to UV light (22).

(iv) Hybridization. A specific 30-bp probe overlapping the *Bst*EII site was constructed (Genetic Designs, Inc.). The oligonucleotide was labeled at the 5′ end with ^{32}P. Briefly, 15 ng of probe (10 ng/μl) was mixed with 5 μl of reaction buffer (Tris [pH 7.4], 500 mM; $MgCl_2$, 100 mM; dithiothreitol, 50 mM; spermidine, 10 mM), 1 μl of polynucleotide kinase T4 (10 U/μl; Biolabs), and 10 μl of [γ-^{32}P]ATP (10 mCi/ml; Amersham) in a total volume of 50 μl, and the mixture was incubated for 1 h (7). A total of 50 μl of STE buffer was added to the mixture, and the mixture was passed through a Sephadex G-50 column for 5 min at 2,000 × *g*. Prehybridization was conducted for 30 min at 55°C in 5× SSPE–1× BP (2% bovine serum albumin, 2% polyvinylpyrrolidone)–1% SDS, and hybridization was performed in the same buffer containing at least 4×10^6 cpm of probe per ml for 1 h at 55°C. The filter was washed in 1% SSPE–1% SDS three times for 5 min at room temperature and one time for 3 min at 55°C. After the filter was dried, it was exposed at −70°C to Kodak film (XAR 5) and developed and/or submitted for detection in a radioanalytic imaging system (AMBIS Systems, Inc.). As a negative control for hybridization, a 217-bp fragment from the pseudorabies virus gp50 gene was used.

RESULTS

Suitability of primers for amplification of cloned BVDV cDNA. The set of primers that consistently amplifies the sequence target within pBV-KPB is shown in Fig. 1. The specificity of the reaction was demonstrated by the size of the amplification product which corresponds to the predicted 205-bp fragment (Fig. 1). Specific cleavage by the restriction enzyme *Bst*EII yielded a pair of segments of predictable sizes (71 and 134 bp), which hybridized specifically with the

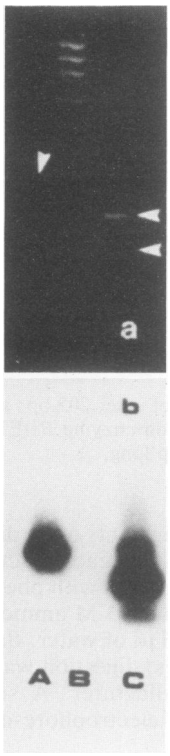

FIG. 2. (a) Agarose gel electrophoresis of the 205-bp amplified fragment before (lane A) and after (lane C) digestion with *Bst*EII. Lane B, *Hae*III-digested fragments from phage ϕX174. Arrows indicate the 205-, 134-, and 71-bp fragments. Amplified products not evident in the figure were visible in the original gel. (b) Southern blot of the gel hybridized with a 30-bp internal probe after a 2-h exposure.

customized internal probe (Fig. 2). By using an optimized protocol for amplification (see Materials and Methods), it was possible to detect 70×10^{-18} g of plasmid template by visualization of the amplification product after electrophoresis in an ethidium bromide agarose gel (Fig. 3a, lane C). When the blotted amplification products were hybridized with the specific internal probe, the detection level was 7×10^{-18} g of cDNA (Fig. 3b, lane D). These quantities correspond to approximately 10 molecules and 1 molecule of insert, respectively. As a negative control, we used plasmid pBR322. Using 1 μg of this plasmid, we consistently failed to produce amplification (data not shown).

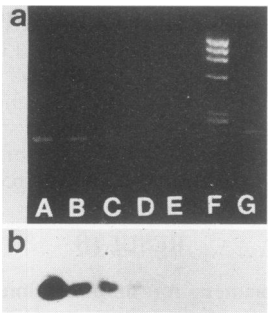

FIG. 3. (a) Agarose gel electrophoresis of PCR products, using decreasing dilutions of template plasmid pBV-KPB. Lanes: A, B, C, D, and E, 7, 0.7, 0.07, 0.007, and 0.0007 fg of pBV-KPB, respectively; F, *Hae*III-digested fragments from phage ϕX174; G, 217-bp amplification fragment from pseudorabies virus gp50 gene. (b) Southern blot of the gel hybridized with a 30-bp internal probe after a 20-h exposure.

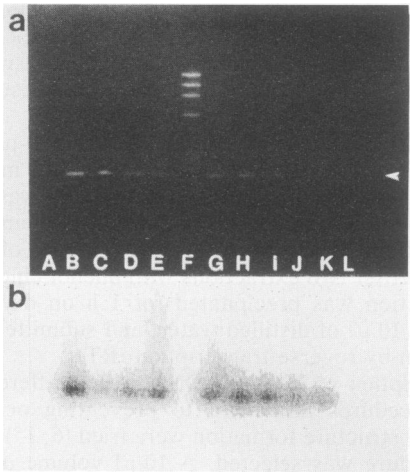

FIG. 4. (a) Agarose gel electrophoresis of PCR products, with cell-propagated reference strains and spiked BVDV strain NADL serum used as targets for amplification. Lanes: A, uninfected BT cell RNA; B through E, BT cells infected with NADL, Oregon, Singer, and New York reference strains, respectively; H, *Hae*III-digested fragments from phage ϕX174; G through L, BVDV-free fetal calf serum reconstituted with 10,000, 1,000, 100, 10, 1, and 0.1 $TCID_{50}$ of BVDV strain NADL per 100 μl. Arrowhead indicates the 205-bp fragment. (b) Southern blot of the gel hybridized with a 30-bp internal probe scanned for 15 min with a radioanalytical image system.

Coupling of BVDV cDNA amplification with BVDV RNA RT. Figure 4, lane B, shows the results of a typical experiment to amplify cDNA prepared by RT of cytoplasmic RNA from BT cells infected with the NADL strain of BVDV. No amplification was detected with RNA extracted from uninfected BT cells (Fig. 4, lane A).

The suitability of the test for direct detection of BVDV in serum was initially assessed by using BVDV-free serum that was artificially infected by the addition of 10^5 50% tissue culture infectious doses ($TCID_{50}$) of BVDV strain NADL per ml. Also in this case, a positive signal was obtained (data not shown). To further assess the sensitivity, we conducted reconstitution experiments by adding known decreasing amounts (10-fold dilutions) of BVDV (NADL strain) to replicate aliquots of BVDV-free bovine serum. These samples were used for the RT plus PCR reaction. The results of this assay are shown in Fig. 4. Upon Southern blotting of the amplified products and hybridization with the internal probe, the threshold of sensitivity of our test in BVDV-infected serum was ≤1 $TCID_{50}$/100 μl of serum (Fig. 4). The BVDV titer of each dilution of reconstituted infectious serum was confirmed by endpoint titration. The amplification products obtained after RT plus PCR of RNAs fulfilled the requisites for specificity as described above.

Ability of the coupled BVDV RT and PCR tests to detect reference strains and isolates of BVDV. The optimized protocol was used successfully to amplify cDNAs transcribed from RNA fractions obtained from BT cells infected with reference strains Singer, Oregon, and NADL (all cytopathic biotypes) and New York (a noncytopathic biotype) (Fig. 4,

TABLE 1. Classification of samples used for BVDV PCR

Isolate	Origin[a]	RNA source	Biotype	PCR result	Viral assay result
NADL	ATCC	CCP[b]	Cytopathic	+	ND[c]
Oregon	NVSL	CCP	Cytopathic	+	ND
Singer	NVSL	CCP	Cytopathic	+	ND
New York	ATCC	CCP	Noncytopathic	+	ND
7	B.c.[d]	CCP	Cytopathic	+	ND
9	O.s.[d]	CCP	Cytopathic	+	ND
10	O.s.[d]	CCP	Cytopathic	+	ND
11	O.s.[d]	CCP	Cytopathic	+	ND
6	B.c.[d]	CCP	Noncytopathic	+	ND
8	O.s.[d]	CCP	Noncytopathic	+	ND
57	Serum	Serum	Noncytopathic	−	ND
57[e]	Serum	CCP	Noncytopathic	+	+
77	Serum	Serum	Noncytopathic	+	+
94	Serum	Serum	Noncytopathic	+	+
87	Serum	Serum	Cytopathic	+	+
14	Serum	Serum	Noncytopathic	−	−

[a] ATCC, American Type Culture Collection; NVSL, National Veterinary Services Laboratories; B.c., buffy coat; O.s., oral swab. Serum, Clinical samples from herds with history of persistent BVDV infection. The samples represent different Nebraska counties encompassing a wide area.
[b] CCP, Cell culture passage.
[c] ND, Not done.
[d] Isolates from New York.
[e] First passage in cell culture.

lanes A through F, and Table 1). In addition, this test was effective in detecting four cytopathic and two noncytopathic unrelated isolates propagated in cell cultures (Table 1).

We also tested six unrelated serum samples with a previous record of positive virus isolation which had been stored at −70°C for an extended period of time. As a positive control, we used reconstituted infectious serum (by addition of NADL strain, 10^3 $TCID_{50}$ per ml). In four of six samples, positive amplification products were obtained directly from the serum material (Table 1). In one of the negative cases, although no virus was detected in the original serum material, positive amplification was achieved when the test was performed on supernatant of the first blind passage of this sample in cell culture, which also had been preserved at −70°C (Table 1). The other sample remained negative by PCR and also by viral isolation throughout a total of three blind passages. The amplification products fulfilled the criteria for specificity described above. No amplification was observed with cDNA (reverse-transcribed RNA) from uninfected BT cells, from BVDV-free bovine fetal serum, or from serum from a BVDV-free calf.

DISCUSSION

From our results, it is evident that it is feasible to specifically amplify RNA sequences of BVDV by coupling the BVDV reverse transcriptase reaction and BVDV cDNA amplification. In addition, the set of primers selected was suitable for amplification of RNA from a wide range of BVDV isolates obtained either after serial propagation of the isolates in cell cultures or directly from clinical samples.

Since the primers are used both in the production of specific cDNA and in the amplification of that cDNA by PCR, the design of such sequences is of fundamental importance for the preparation of a protocol to amplify RNA targets. We originally selected several sets of 20-bp primers mapping to the conserved region of the BVDV genome which is close to the 3′ end. We based this selection on genomic areas of high homology between two published sequences of the BVDV genome (6, 16). At the same time, we selected the primers because of their relatively low GC content and their acceptable free energy coefficient (i.e., a free energy coefficient that is positive or near zero, which indicates a low probability of secondary structure formation). Of the two sets of primers that we tested under different conditions of amplification, only one (herein reported) specifically amplified BVDV RNA. The 205-bp target for amplification is included in the region that codes for the putative precursor of the RNA polymerase (5). This also suggests that our target sequence lies within a highly conserved area of the BVDV genome. This high level of conservation is a desirable feature for a primer-target sequence selected for diagnostic purposes, because it contributes directly to detection of a wide range of diverse virus strains. Using the set of primers described above, we were able to detect 15 diverse isolates of both BVDV biotypes. This is particularly important when considering the reported difficulties in finding a universal synthetic oligonucleotide sequence which would be able to recognize RNAs from different BVDV isolates by means of filter hybridization (17). Our results may reflect the fair number of mismatches that PCR tolerates (23). However, the specificity is still high, as evidenced by the absence of nonspecific amplification.

We failed to detect BVDV-specific amplification in two clinical serum samples. However, we could detect the virus by amplification in the supernatant of the first passage of one of these samples in cultured cells. Although the failure to directly detect BVDV in one serum sample apparently challenges the sensitivity of our test, other factors should be taken into account, such as the fact that hemoglobin was observed in both negative serum samples. Compounds derived from heme (porphyrins) have been reported to be inhibitors of PCR (13).

Although intensive, the coupled protocol for RT plus PCR provides a rapid diagnosis of BVDV. In all cases, results were obtained within 24 to 36 h. This is significantly less time than the minimal time required to obtain results by using standard viral isolation techniques.

Since its development, the PCR has been used for the detection of several RNA viruses, such as human immunodeficiency virus (12), rubella virus (4), enterovirus (20), rhinovirus (9), and hepatitis delta virus (25). Our results show that this technique should be useful for the diagnosis of BVDV in cell lines, bovine serum, and tissues because of its sensitivity and rapid performance.

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