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Efficiency of various cloned DNA probes for detection of bovine viral diarrhea viruses

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ABSTRACT

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We have evaluated 24 cytopathic (CP) and 37 noncytopathic (NCP) strains of bovine viral diarrhea virus (BVDV) with a dot blot assay using four different genome segments of the NADL strain as hybridization probes (p80, p54, gp53, and gp62). The p80 and p54 probes hybridized to 23/24 (96%) and 22/24 (92%), respectively, of CP strains examined. In contrast, these same two probes only detected 16/37 (43%) and 5/37 (13%), respectively, of the NCP strains examined. The gp53 probe detected 18/24 (75%) and the gp62 probe detected 19/24 (79%) of the CP strains. In contrast, these latter two probes only detected 9/37 (24%) and 7/37 (20%), respectively, of NCP strains. This low detection rate of NCP strains suggests a need for developing a probe based on NCP sequences for identification of NCP strains.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is an enveloped, positive-strand RNA virus and the prototype member of the Pestivirus genus (Westaway et al., 1985; Sumiyoshi et al., 1987). Pestiviruses have recently been suggested as a likely new genus with the family Flaviviridae (Collett et al., 1988b; Collett et al., 1989). The various BVDV strains differ in their ability to cause cytopathic (CP) changes in cell culture. Thus, CP and noncytopathic (NCP) biotypes of BVDV are identified. The CP strains induce cytoplasmic vacuolation in infected cells, where NCP strains do not. In the United States, serological surveys have demonstrated that 70 to 80% of bovine samples examined

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showed neutralizing antibody to BVDV (Ames, 1986). The ubiquitous nature of BVDV has a marked economic impact on the cattle industry (Brownlie, 1985; Baker, 1987).

Current methods to detect BVDV infection of cattle include virus isolation and viral neutralization tests. These methods are expensive, time consuming, and difficult to use in large scale screening of animals or animal products for BVDV (Baker, 1987; Radostits and Littlejohns, 1988). Nucleic acid hybridization techniques using cloned probes are finding application in assays of clinical specimens in research and diagnostic laboratories. Such probes, highly specific in their nature, have widespread applications in the detection of routine microbial diagnostic assays (Highfield and Dougan, 1985; Lin et al., 1985; Pettersson and Hyypia, 1985; Shockley et al., 1987). Application of dot blot assays, particularly with *in situ* hybridization, to avoid the difficulties of RNA extraction has not been used for BVDV. This technique would solve many of the problems associated with current assays. The dot blot assay could be used in 96-well plate for screening of animals or biologic products. The purpose of this study is to test the cloned cDNA regions of the BVDV genome to determine sequences most useful for detection and differentiation of a large number of field strains of BVDV.

MATERIALS AND METHODS

Viruses

Noncytopathic ($n=37$) and cytopathic ($n=24$) isolates of BVD virus were propagated in bovine turbinate (BT) cells. Classification of the cytopathic viral biotype was based on the induction of cytoplasmic vacuolation and cell death within 48 h of infection of culture cells. The viruses were isolated over a 30-year period from cattle, fetal calf serum, swine, deer, and cell cultures infected with adventitious BVD virus. Confluent monolayers of BT cells were inoculated with virus at a multiplicity of infection of five. After 24 to 32 h for cytopathic viruses or 48 h for noncytopathic viruses, the infected BT cells were harvested by treatment with trypsin-versene solution. The cells were pelleted by centrifugation and suspended in Eagle's minimum essential medium containing 10% fetal calf serum and 10% dimethyl sulfoxide, and frozen at -70°C until further use. The BT cells used for growth of virus were tested by direct fluorescent antibody staining procedures and were found free of adventitious BVD virus. The fetal calf serum used in the cell culture medium was free of adventitious BVD virus by virus isolation and antibodies to BVD virus by viral neutralization and indirect immunoperoxidase staining procedures.

Plasmids

The plasmids containing various portions of BVDV (NADL) genome are listed in Table 1. The parent vector for pBV4-p80, pBV4-gp53, and pBV4-gp62 was pGEM-4; for pBV-F2 was pUC-9 (Collett et al., 1988a). Selection and amplification of the plasmids were performed in *Escherichia coli* strain DH5 α by standard procedures (Maniatis et al., 1982).

Preparation of probes

Four cloned cDNA probes were prepared. The p80 probe represented the 917 bp and 1241 bp *Bam*HI non-overlapping fragment prepared from pBV4-p80 plasmid. The gp53 probe was a 950 bp *Eco*RI/*Pst*I fragment prepared from pBV4-gp53. The gp62 probe was a 1370 bp *Eco*RI/*Pst*I fragment prepared from pBV4-gp62. Finally, the p54 probe was a 1130 pb *Bam*HI/HD3 fragment prepared from pBV-F2.

The restriction fragments were purified by preparative agarose gel electrophoresis and electroelution (Maniatis et al., 1982). The probes were radioactively labeled by the oligonucleotide random-priming method using a commercial kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) and [α - 32 P] dGTP (New England Nuclear, Boston, MA). Each 32 P-labeled probe had a specific activity of approximately 5×10^8 cpm/ μ g. Before use, each probe was denatured by boiling.

Preparation of filter for dot blot hybridization

To prepare samples for dot blot, each tube containing the BT cells infected with an individual BVD virus strain was quickly thawed and the cells pelleted by microfuge centrifugation for 30 s. Non-infected BT cells were used as a negative control. The cell pellet was then reconstituted with $1 \times$ PBS and the cell concentration adjusted to 10^6 /ml. A nitrocellulose membrane was wetted in $1 \times$ PBS before application of the sample. This membrane was supported by two sheets of blotting paper in a 96-well dot blot apparatus (Minifold, Schleicher and Schuell, Inc., Keene, NH). A 100 μ l aliquot of diluted cells

TABLE 1

Sources of plasmids and probes used for dot blot assay

Plasmid	Encompasses BVD cDNA nucleotides	Insert site at the vector	Probe
pBV4-p80	5644-7949	<i>Sma</i> I	<i>Bam</i> HI fragment
pBV4-gp53	2530-3575	<i>Sma</i> I	<i>Eco</i> RI/ <i>ps</i> tI fragment
pBV4-gp62	1113-2480	<i>Sma</i> I	<i>Eco</i> RI/ <i>ps</i> tI fragment
pBV-F2	4070-5200	<i>Pst</i> I	<i>Bam</i> HI/ <i>Hind</i> III fragment

prepared as described above were spotted onto the nitrocellulose membrane and vacuum suction was applied for 5 min. The filter was immersed in fixative solution (0.5 M NaCl, 12 mM NaHPO₄, 48 mM Na₂HPO₄ and 1% glutaraldehyde) at 4°C for 1.5 h and rinsed for 5 min with a solution made to contain 10 mM EDTA and 0.1 M Tris-HCl (pH 8.0). The rinsing was repeated twice more, and the filter was then digested in the same solution with proteinase K (20 µg/ml) at 37°C for 0.5 h. The filter was air-dried and exposed in a UV crosslinker (Stratagene, San Diego, CA).

Hybridization

Hybridization conditions were adapted from a previously described method (Paeratakul et al., 1988). Prehybridization solution [5×SSPE (0.18 M NaCl, 10 mM NaH₂PO₄ pH 7.4, 1 mM EDTA pH 7.4), 40% formamide, 1× Denhardt's solution, and 10 µg/ml denatured salmon sperm DNA] was added to a heat sealable bag along with the filter and incubated at 60°C for 4 h. The prehybridization solution in the bag was then replaced with fresh prehybridization solution together with the denatured cDNA probe (10⁶ cpm/cm²).

Fig. 1. Dot blot detection of BVDV RNA from BT cells infected with CP viruses (except A3 in NCP virus) using p80 as the hybridization probe. The letter-number coordinates correspond to the sample numbers in Table 2. Normal BT cells were used as a control (DI). Autoradiography was carried out for 7 h.

After incubation overnight at 60°C, the nitrocellulose filter was washed twice in 2×SSC, 0.5% SDS at room temperature for 5 min; and once in 1×SSC, 0.5% SDS at room temperature for 15 min; and once in 0.1×SSC, 0.5% SDS at 60°C for 15 min. Autoradiography was performed at -70°C with Kodak AR X-ray film and intensifying screens.

RESULTS

The p80 probe detected to 23/24 (96%) of the BVDV-CP strains, but only 16/37 (43%) of the NCP strains (Figs. 1-4). The p54 probe detected 22/24 (92%) of the BVDV-CP strains, but only 5 of the 37 (13%) NCP strains (Figs. 1-4). The gp53 probe detected 18/24 (75%) of the BVDV-CP strains, but only 7/37 (20%) of the NCP strains. The gp62 probe detected 19/24 (79%) of the BVDV-CP strains, but only 9/37 (24%) of the NCP strains (Tables 2,3 and Figs. 1-4).

Fig. 2. Dot blot results using p54 as the hybridization probe. Samples were the same as those shown in Fig. 1 (except A3 is NCP virus). Autoradiography was carried out for 8 h.

The results clearly show that p80 and p54 were the most effective probes at identifying viruses of CP strains. p80, p54, gp62 and gp53 varied in efficiency of hybridization to individual NCP strains. In particular, p54, gp62, and gp53 hybridized poorly to each of the NCP isolates. Only one CP isolate (C8) did not hybridize to any of the probes (Figs. 1–4).

Normal BT cells included on dot blot as a negative control showed no detectable hybridization signal (Figs. 1, 2 and 3). This indicated no nonspecific binding of the probe under high stringency hybridization conditions.

For the CP strains, adequate signal strengths were present in the autoradiographs 5–7 h after exposure of the hybridized DNA to film. In contrast, the NCP strains required 16–18 h exposure to achieve adequate signal strengths. Yet, virus titer (10^6 to 10^7 viral particles/ml) was about the same among the different strains (data not shown).

Fig. 3. Dot blot results of NCP viruses (except E7 is CP virus) using p80 as the hybridization probe. The letter-number coordinates correspond to the sample numbers in Table 2. Normal BT cells were used as a control (G3 and G5). Autoradiography was carried out for 16 h.

TABLE 2

Results of using four different cDNA probes for the detection of BVD virus¹

Sample	Results obtained by			
	gp62	gp53	p54	p80
<i>Cytopathic</i>				
A1	+	+	+	+
A2	+	+	+	+
E7	+	+	—	+
A4	+	+	+	+
A5	+	+	+	+
A6	+	+	+	+
A7	+	+	+	+
A8	+	+	+	+
B1	+	+	+	+
B2	+	+	+	+
B3	+	+	+	+
B4	+	+	+	+
B5	+	+	+	+
B6	+	+	+	+
B7	+	+	+	+
B8	+	+	+	+
C1	—	+	+	+
C2	+	—	+	+
C3	—	—	+	+
C4	—	±	+	+
C5	+	—	+	+
C6	—	±	+	+
C7	+	—	+	+
<i>Noncytopathic</i>				
E1	+	+	+	+
E2	+	+	+	+
E3	±	+	+	+
E4	+	+	—	+
E5	+	+	+	+
E6	+	+	—	+
A3	+	+	+	+
E8	+	+	—	+
F1	—	+	±	+
F2	—	—	—	+
F3	—	—	—	+
F4	—	—	—	+
F5	—	—	—	+
F6	—	—	—	+
F7	—	—	—	+
F8	—	—	—	±
G1	—	—	—	+

¹Twenty-one of 37 NCP strains which were undetectable with any of the probes tested are not listed in Table 2.

Fig. 4. Dot blot results of NCP viruses (except E7 is CP virus) using the following hybridization probes: gp53 (blot a), gp62 (blot b), and p54 (blot c). Autoradiography was carried out for 18 h.

TABLE 3

Summary of four probes used for identifying the BVD infected cells

Probes	NCP ¹ detection	CP ² detection	Total detection
gp62	7/37	19/24	26/61
gp53	9/37	18/24	27/61
p54	5/37	22/24	26/61
p80	16/37	23/24	39/61

¹Total identified NCP is 16/37 (21 unidentified).

²Total identified CP is 23/24 (1 unidentified).

DISCUSSION

The objective of this experiment was to develop a dot blot assay suitable as a diagnostic test for BVDV. Four hybridization probes were developed representing genomic regions encoding the viral proteins, gp62, gp53, p54, and p80. All probes were derived from cloned cDNA of the NADL CP strain. These four genes represented greater than 40% of the NADL genome. These probes were tested against 61 field-collected strains of BVDV. Of these 61 samples, 37 were NCP and the remaining 24 samples were CP. A summary of how effective these four probes were at identifying the BVDV infected cells is

given in Tables 2 and 3. Based on the results of Table 2, use of probes other than p80 did not result in detection of additional NCP strains, presumably because there is not complementary detection among the probes. Thus, p80 was the best choice for BVDV detection among the probes tested.

The detection rate was much lower for NCP strains than for CP strains. The p80 probe detected 64% and the other three probes about 43% of the NCP strains. The failure to detect 36% of the NCP strains with any of the tested probes developed from the CP strain indicates a significant variation in sequences and the necessity of developing additional probes for highly conserved segments of NCP strains to allow identification of all of NCP BVDV. Currently, we are sequencing the genomes of two NCP strains and these sequences may show which regions are highly conserved and would likely serve as sequence for probe development and NCP detection. By employing the appropriate combination of CP and NCP genomes cDNA sequences as probes, it may be possible to use the dot blot assay as a general BVD diagnostic test for all BVDV strains.

In the current dot blot hybridization procedures, the cells containing target BVDV RNA were directly affixed to the nitrocellulose filter. By overcoming the need to purify RNA and the problems associated with this procedure, this method leads to an acceleration of sample preparation. In addition, this method facilitates working with many sample simultaneously, reducing the amount of required sample materials. Thus, automated mass screening of cattle is more feasible and would allow for control programs and epidemiologic studies to be done.

It is interesting to note that both p80 and p54 had identical detection rates for CP strains. Yet with NCP strains, p80 detected on 16/37 and p54 detected only 4/37 – the lowest detection rate among all probes. This low detection rate may indicate that the p54 region is the least conserved segment between CP and NCP strains (Collett et al., 1989). Whether this region is involved with mutation or insertion events that make CP and NCP sequences so much different in this region needs further investigation (Meyers et al., 1989).

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