JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1993, p. 986–989
0095-1137/93/040986-04$02.00/0

Vol. 31, No. 4

Comparison of Nucleic Acid Hybridization and Nucleic Acid Amplification Using Conserved Sequences from the 5′ Noncoding Region for Detection of Bovine Viral Diarrhea Virus

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Received 30 October 1992/Accepted 30 December 1992

Primers and probes derived from conserved sequences located in the 5′ noncoding region of pestiviruses were evaluated for detection of bovine viral diarrhea virus. With these reagents, hybridization and polymerase chain reaction tests detected 62 of 90 and 90 of 90 bovine viral diarrhea virus isolates, respectively. A quick lysis method for preparing RNA for use in polymerase chain reaction amplification also was evaluated.

Bovine viral diarrhea virus (BVDV), an economically important pathogen of cattle, is a frequent contaminant of fetal bovine serum (FBS) and cell cultures (2, 14, 19, 21, 25, 31). This virus has a single strand of positive-sense RNA and is classified in the pestivirus genus within the family *Flaviviridae* (10). It is serologically related to the two other members of this genus, hog cholera virus (HCV) of swine and border disease virus (BDV) of sheep (8, 9, 22). Accurate detection of BVDV is important for control of disease in cattle and for detection of viral contamination in FBS and cell cultures. Currently, most diagnostic tests for BVDV rely on monoclonal or polyclonal antibodies to detect viral antigens. Because of serological cross-reaction among pestiviruses, these tests may fail to differentiate among pestiviruses (8, 9, 22). Also, antigenic variation within groups of pestiviruses may affect the performance of these tests (1a, 3, 7). Nucleic acid-based diagnostic tests for BVDV are an alternative to immunological tests and have shown promise in preliminary studies (4, 5, 12, 13, 15–17, 24, 28–30, 33, 34). Before a nucleic acid-based test is adopted for diagnostic use, it must be shown that the test can detect a broad range of BVDVs. Previous studies have demonstrated that genomic variability among BVDVs affects the performance of nucleic acid-based tests (4, 5, 12, 15, 16, 24, 28, 32, 34). Additionally, the time, complexity, and cost of sample preparation must be minimized. Finally, because programs to eradicate some pestiviruses from national herds already have been implemented or will be implemented in the near future, differentiation of BVDVs from other pestiviruses is desirable.

Because genomic variability can affect BVDV detection, our purpose was to design a nucleic acid-based reagent for use in nucleic acid hybridization or polymerase chain reaction (PCR) amplification that would detect a wide range of BVDVs. Nucleic acid probes and primers were selected from the 5′ noncoding region of pestiviral genomes. Selection was based on conservation among published base sequences from 4 pestiviruses (Fig. 1) (6, 18, 20, 27) and partial sequences from 20 other pestiviruses (1). Probes and primers were evaluated as potential diagnostic reagents with a large number of BVDV isolates (n = 90).

The BVDV isolates (Table 1) were propagated as described elsewhere (28). The FBS used for tissue culture was found free of adventitious BVDV and antibodies against BVDV as described previously (2). All BVDVs originated in the United States except for two viruses isolated from fetal calf serum from New Zealand.

Detection by hybridization of oligomer probes was tested first. RNA was prepared by centrifugation through a CsCl gradient as described by Qi et al. (26). Slot blots of isolated RNA were generated as described previously (16). An aliquot representing 5% of the total RNA prepared from a 75-cm^2 flask of infected cells was used for each blot. Positive and negative controls consisted of total RNA from cells infected with BVDV-NADL and from uninfected cells, respectively. Two oligomer probes, one complementary to sequences conserved among BVDVs (Fig. 1, sequence H-2) and one complementary to a sequence conserved among all pestiviruses (Fig. 1, sequence H-1), were used for hybridization. The hybridization procedure used has been described (16) and required 100% homology between oligomers and target sequences for a positive signal. Of the 90 BVDV isolates tested, 48 were detected with both oligomer probes, 14 were detected with only the pestivirus complementary probes, and 28 were not detected with either probe (Table 1). This detection rate was similar to that reported by other laboratories using different oligomer probes (15, 16). Lower-stringency conditions might have resulted in detection of more viruses. However, high-stringency conditions were incorporated into the original design of the test to allow differentiation of BVDV from other pestiviruses. Because some BVDVs were not detected with this test, differentiation of BVDV from other pestiviruses was not attempted.

Detection by PCR amplification was evaluated next. The same sequences were not used for hybridization probes and PCR primers (Fig. 1) because the hybridization probe sequences did not meet the criteria used for selecting PCR primers. The downstream primer used a sequence conserved among pestiviruses while the upstream primer was conserved only among BVDV sequences (Fig. 1). Total RNA was prepared by the same CsCl gradient protocol (26) used

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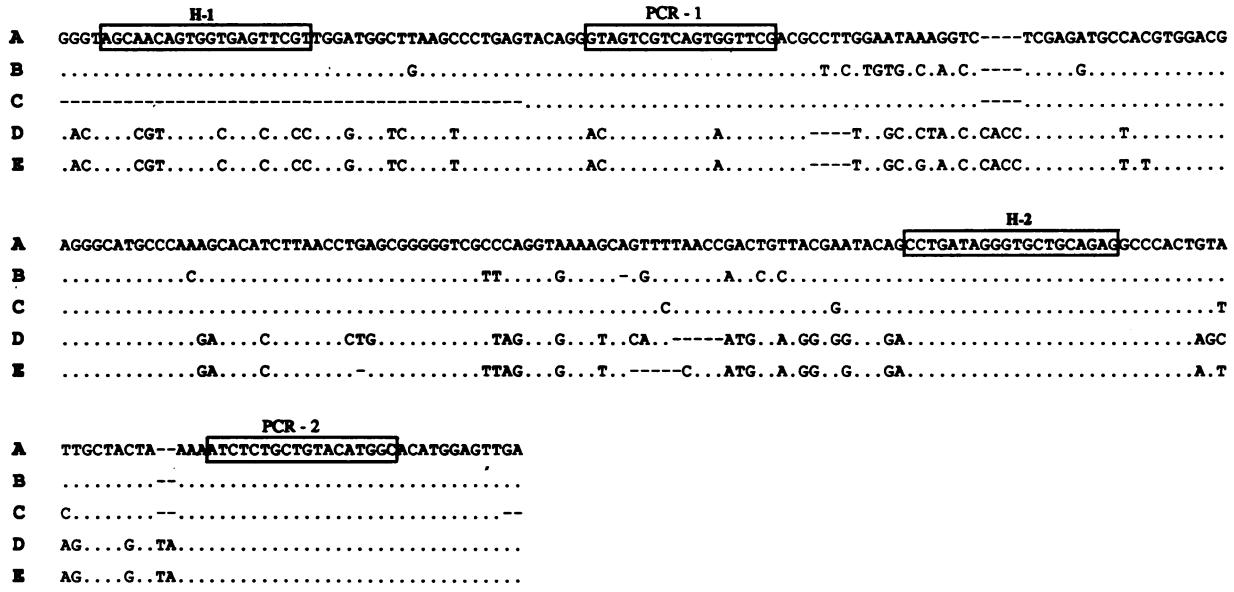

FIG. 1. Nucleotide sequence of BVDV-NADL (A) (6), BVDV-Osloss (B) (27), HCV-Alfort (D) (18), and HCV-Brescia (E) (20) in the 5′ noncoding region from which hybridization probes (H-1 and H-2) and PCR primers (PCR-1 and PCR-2) were selected. The sequence of the cDNA clone, derived from BVDV-Singer, used to confirm PCRs is shown (C). Dots indicate homologous sequences, dashes indicate insertions, and the dashes at the beginning of line C indicate sequences not included in the clone.

in the evaluation of the hybridization test. Aliquots representing 5% of the total RNA extracted from a 75-cm^2-flask culture were used for reverse transcription and subsequent PCR amplification. Reverse transcription and PCRs were done with a commercially available kit (GeneAmp RNA PCR kit; Perkin-Elmer Cetus, Norwalk, Conn.) according to the manufacturer's directions, except for the inclusion of both upstream and downstream PCR primers in the reverse transcription reaction. Our cDNA yields were greater when both primers were present, possibly because of the presence of negative-strand viral RNA generated during replication. The PCR amplification consisted of 25 cycles of 93°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a 10-min extension at 72°C. Total RNAs from BVDV-NADL-infected and uninfected cells were included in each amplification as positive and negative controls, respectively. PCR products were analyzed by gel electrophoresis and confirmed as BVDV by slot blotting onto nylon membranes (26) which was followed by hybridization with a BVDV cDNA probe (Fig. 1, line C). Hybridization and washes were done at 55°C as described earlier (28). All 90 BVDV isolates were detected by PCR amplification (Table 1). The apparent amount of PCR amplification product was not uniform for all the BVDV isolates tested (Fig. 2). This might reflect various amounts of viral RNA present in samples, inefficient reverse transcription, or genomic heterogeneity.

The success of the PCR test in detection of BVDV encouraged us to further evaluate the test on its ability to differentiate BVDV from other pestiviruses. To this end, HCV isolates (n = 36) were propagated in a porcine kidney cell line (PK-15) that was grown in minimal essential medium (F15 Eagle's medium; GIBCO, Grand Island, N.Y.) supplemented with 5% FBS, 1% sodium pyruvate, and 1% L-glu-

TABLE 1. Results of nucleic acid hybridization and PCR amplification for detection of BVDV

Source or type (no.) of viral isolates	No. of viruses detected with:		
	BVDV probe (H-1)	Pestivirus probe (H-2)	PCR
(90) BVDV			
Bovine tissue (56)	36	42	56
FBS (30)	11	17	30
Deer tissue (1)	1	1	1
Porcine cell line (2)	0	2	2
Bovine cell line, HCV (1)	0	0	1
Porcine tissue, BDV (36)	ND[a]	ND	4
Bovine tissue (10)	ND	ND	1

[a] ND, not done.

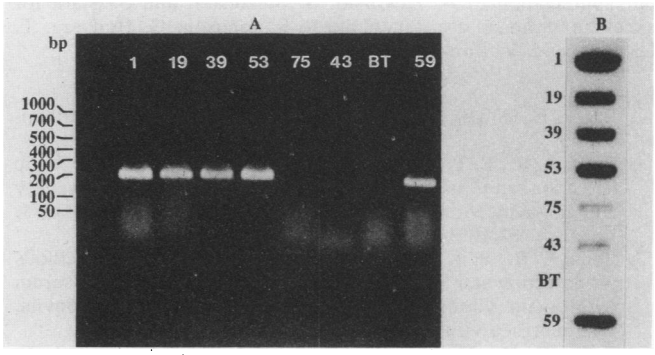

FIG. 2. (A) Example of PCR amplification products separated by electrophoresis in a 1.5% agarose horizontal gel. (B) Example of blotting of amplification products onto nylon membranes and hybridization with a probe derived from BVDV-Singer. The PCR products blotted are the same as those shown in panel A. Numbers refer to individual BVDV isolates from panel A. No. 1 represents the BVDV-NADL positive control, while BT refers to the negative control (PCR results with uninfected bovine turbinate cells). RNA used for the amplifications was prepared by the quick lysis method.

tamine. The HCV originated from North America, Central America, South America, Europe, and Asia. The BDV isolates (n = 10) were propagated in ovine fetal turbinate cells grown in McCoy's medium (GIBCO) supplemented with 10% FBS. The BDV originated in the United States (supplied by M. Sawyer and B. I. Osburn, University of California, Davis). Cell lines used for viral propagation were free of adventitious BVDV as determined by indirect immunoperoxidase or immunofluorescence staining. Total RNA from pestivirus-infected cells was prepared as described previously (26). Differentiation of BVDV from other pestiviruses by use of these primers was unsuccessful, as 4 of 36 HCV isolates and 1 of 10 BDV isolates were amplified (Table 1). However, because BVDV can infect both swine and sheep, it is possible that these isolates may be BVDV and not HCV or BDV.

We also evaluated a quicker, less expensive method for preparing sample RNA (23) that was modified as follows. Confluent monolayers of cells, grown in 25-cm^2 flasks, were inoculated with virus at a multiplicity of infection of 10. After 24 h, cultures were lysed by addition of 0.75 ml of a solution of 2% sodium dodecyl sulfate–200 mM Tris (pH 7.5)–1 mM EDTA. After addition of 225 μl of 4.37 M potassium acetate, the mixture was incubated on ice for 2 min and centrifuged for 5 min at $1.6 \times 10^4 \times g$. Supernatant was extracted with chloroform, and RNA was precipitated by addition of isopropanol. Aliquots representing 25% of the precipitated RNA were used for the PCR test. By this method of preparation, all 90 BVDV isolates were detected by PCR amplification. The simplicity and speed of this method of RNA preparation are favorable for use in a diagnostic setting.

In summary, the hybridization probes tested failed to detect all BVDV. The PCR primers tested did detect all BVDV but failed to differentiate BVDV from all other pestiviruses. We also showed that RNA samples for PCR testing can be produced cheaply and with a minimum of equipment. Further testing will be conducted to optimize test conditions and to adapt the PCR test for use on field samples.

We thank E. Berry (North Dakota State University, Fargo) for access to sequence information and M. Sawyer and B. I. Osburn (University of California, Davis) for BDV isolates. We also express our appreciation to B. Zaworski, S. Steadham, and S. Stark for excellent technical assistance and to S. Johnson, G. Hedberg, T. Glasson, and W. Romp for help in manuscript preparation.

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