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Detection of Hog Cholera Virus and Differentiation from Other Pestiviruses by Polymerase Chain Reaction

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Reverse transcription coupled with the polymerase chain reaction (RT-PCR) was used for the detection and differentiation of pestiviruses. For this purpose, one primer pair was selected from a highly conserved region of the genome of pestiviruses. Using these primers (PEST 1-PEST 2), DNA fragments of between 72 and 74 bp could be amplified from all pestivirus isolates tested. In order to differentiate hog cholera virus (HCV) from bovine viral diarrhea virus (BVDV) and border disease virus (BDV), we selected a primer pair from a conserved region in the genome of HCV strains that differed from that sequenced in the genome of BVDV strains. By using these primers (HCV 1-HCV 2), a DNA fragment of 478 bp could be specifically amplified from HCV isolates. By these means, viral RNA was detected in extracts of lymph node, spleen, tonsil, and lung. Such extracts were used directly for RT-PCR without prior RNA isolation. We also performed multiplex PCR by using both the PEST 1-PEST 2 and HCV 1-HCV 2 primer pairs in a single reaction. This allowed the differentiation of HCV from BVDV and BDV in one step. To assess the sensitivity of the method, RT-PCR was compared with virus propagation in tissue culture and subsequent detection by immunofluorescence staining. The results show that RT-PCR is useful for the rapid detection and differentiation of pestiviruses.

Pestiviruses, which have recently been classified as a genus of the family *Flaviviridae* (28), are important pathogens in veterinary medicine. They are responsible for substantial economic losses in cattle, swine, and sheep farming. The three members of the genus *Pestivirus* are hog cholera virus (HCV), bovine viral diarrhea virus (BVDV), and border disease virus (BDV), which cause hog cholera or classical swine fever in pigs and wild boar, bovine viral diarrhea in domestic and wild ruminants, and border disease in sheep, respectively. On the basis of their behaviors in cell culture, two biotypes each of BVDV and BDV are recognized, cytopathogenic and noncytopathogenic (10, 11, 16). The pestivirus genome consists of single-stranded RNA that has positive polarity and is about 12.5 kb in length. The genomes of HCV strains Alfort and Brescia (18, 21) and BVDV strains Osloss and NADL (6, 23) have been sequenced. The overall proportion of identical nucleotides between HCV and BVDV is approximately 70% (18).

Although pestiviruses exhibit defined clinical manifestations in their main hosts, pestiviruses possess a similar host spectrum (1). Hence, HCV can be transmitted to cattle (7), while BVDV naturally infects pigs (27), sheep (2), goats (1), and many wild ruminants (9, 22).

Pestivirus infection is diagnosed by immunofluorescence (IF) and immunoperoxidase techniques in infected organ samples and is confirmed by virus isolation (4). Differentiation of HCV from other pestiviruses is done by using monoclonal antibodies against the major envelope protein (20, 29). Recently, the polymerase chain reaction (PCR) technique (24) has provided new opportunities for the detection of pathogens in clinical material. There appear to be a number of potential advantages from using this method for pestivirus diagnosis. Apart from its rapidity, PCR provides a simple procedure with high specificity and sensitivity. Actually, it is very important to be able to differentiate between HCV and BVDV infections in pig herds, since outbreaks of hog cholera in many countries are controlled by stamping out, while such rigorous measures are not taken in the case of BVDV infections. No diagnostic method for distinguishing between BDV and BVDV is available (2).

Detection of pestiviruses by reverse transcription (RT) and then PCR (RT-PCR) has been reported by several investigators (3, 13, 14, 17, 25, 26). Here we demonstrate the amplification by RT-PCR of a genomic fragment common for all pestivirus strains and isolates tested. For subsequent differentiation of HCV from other pestiviruses, a fragment which was specific only for HCV was amplified. Also, both steps, i.e., detection and differentiation, can be performed in a single reaction (multiplex PCR). Because we carried out RT-PCR without prior RNA extraction, our approach provides an extremely simple and rapid procedure for the routine diagnosis of pestivirus infections.

MATERIALS AND METHODS

Cells. Porcine kidney cells (PK-15) were used for virus propagation and for indirect IF staining to detect and titrate HCV. Cells were maintained in Eagle's minimal essential medium supplemented with 5% (vol/vol) fetal calf serum previously screened by a virus neutralization test for the absence of anti-HCV and anti-BVDV antibodies.

HCV isolates. Supernatants from infected cells were prepared by using the virus strains Alfort/187 and Glentorf (obtained from B. Liess, Hannover, Germany) and virus strain CAP (obtained from H. Laude, Institut National de la Recherche Agronomique, Joey-en-Josas, France). Briefly, PK-15 cells were infected at a multiplicity of infection of approximately 0.01 50% tissue culture infective dose per cell and were incubated for 3 days. Virus was extracted by repeated freezing and thawing of the cells, and the cell debris was separated from the supernatant by centrifugation.

A total of 16 supernatants containing the following virus isolates were obtained from various laboratories and were used directly for RT-PCR: Behring, Oldenburg PA 4932,

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TABLE 1. Nucleotide sequence, melting temperature of the primers used for PCR, and length of the expected PCR product

Primer[a]	Sequence	Melting temp (°C)	Product (bp)
PEST 1 (sense)	5'-CCTGATAGGGTGCTGCAGAG-3'	64	74[b]
PEST 2 (antisense)	5'-TCAACTCCATGTGCCATGTAC-3'	62	
HCV 1 (sense)	5'-CCGTGACCGTGGTAGGGGAAA-3'	76	478
HCV 2 (antisense)	5'-ATTTGGTCTTCGAGGCGCAGCA-3'	68	

[a] Locations of the primers in relation to the genome of HCV Alfort (18): PEST 1, positions 300 to 319; PEST 2, positions 373 to 353; HCV 1, positions 9242 to 9262; HCV 2, positions 9719 to 9698.
[b] Produces a 72-bp product in some strains.

Siegburg 1989, Rhön 1989, Austria 1990, Baker A, and Riems (kindly provided by R. Ahl, Tübingen, Germany); D 1047, D 1049, and D 798 (kindly provided by Y. Leforban, Ploufragan, France); and Sch179, Sch186, Sch193, 907/2, 907/3, and 907/1 (kindly provided by J. Dahle, Hannover, Germany).

Similarly, 28 organ samples derived from either wild boars or domestic pigs were obtained from four laboratories in Austria, France, and Germany. All but two of these samples were from animals with a positive HCV diagnosis. Extracts were prepared from the following organs: lymph node, spleen, and lung obtained from a wild boar infected with isolate D1047 (kindly provided by Y. Leforban, Ploufragan, France); pig tonsil samples SP 128/90, SP 2417/90, SP 2605/90, SP 6640/90, SP 6639/90, SP 9875/90, SP 11741/90, SP 15860/90, SP 8253/89, SP 8328/89, SP 8537/89, SP 10366/89, SP 17367/89, and SP 17368/89 (kindly provided by R. Krassnig, Vienna, Austria); pig spleen samples Vi 184/4/90, Vi 364/90, Vi 633/90, and Vi 365/90 (kindly provided by J. Dahle, Hannover, Germany); and pig spleen samples Vi 4810/90, Di 2244-45/90, Di 1627/90, Di 1626/90, Di 903/90, Di 3013/90, and Di 4280/90 (kindly provided by R. Ahl, Tübingen, Germany). The latter two samples had a negative HCV diagnosis and were included by R. Ahl as blind negative controls.

BVDV isolates. A total of 10 cell culture supernatants containing BVDV isolates were included in the present study. BVDV strains NADL, Oregon, and New York-1, as well as the isolates R56/74, R4306/78, 1935/72, R4282/78, and Vaccine-Bern, were kindly provided by C. Hertig, Bern, Switzerland. Isolate Vaccine-Bern has been used as an experimental vaccine (12). Supernatants containing isolates V725/88 and D1040 were obtained from J. Dahle, Hannover, Germany, and Y. Leforban, Ploufragan, France, respectively.

BDV isolates. Cell culture supernatants containing the BDV strain Weybridge and the isolates S137, 135661, 59387, and 175375 were kindly provided by S. Edwards, Central Veterinary Laboratory, Weybridge, England.

Preparation of organ extracts. Organ material (1 g) was added to phosphate-buffered saline (PBS; pH 7.5; 10 ml) in a corex tube, and the mixture was homogenized (Polytron; Kinematica) for 1 min at high speed and was then centrifuged (7,740 × *g*, 20 min) to pellet the cell debris. A fresh sterile blade was used for each sample. The supernatant was either divided into aliquots and frozen or used directly for RT-PCR.

Primer design. Primers were designed to detect either all pestiviruses or HCV specifically. The sequences of two HCV strains, Alfort (18) and Brescia (21), and two BVDV strains, Osloss (23) and NADL (6), were aligned by using the GCG package (8). Regions of complete identity between all four sequences were identified, and a general primer pair (PEST 1-PEST 2) was selected from the 5'-nontranslated region of the genome. Similarly, regions of complete identity between the two HCV strains but of different identities when compared with the BVDV sequences were used to design a HCV group-specific primer pair (HCV 1-HCV 2) from the genome region encoding the nonstructural viral proteins. The expected sizes of the PCR products were 72/74 and 478 bp, respectively. The sequences of the primers are shown in Table 1. Their positions on the pestivirus genome are indicated in Fig. 1 and Table 1.

RT-PCR. Virus-infected cell culture supernatant or organ extract (2.5 μl) was added to 2× RT-PCR buffer (15 μl; 50 mM Tris HCl [pH 8.4], 150 mM KCl, 5 mM $MgCl_2$, 0.5 mM [each] deoxynucleoside triphosphates), Ultrapure water (Facola AG, Basel, Switzerland) (17.5 μl), and 2.5 μl of 10 to 20 μM antisense primer (either PEST 2 or HCV 2). The mixture was heated at 90°C for 5 min and was immediately cooled on

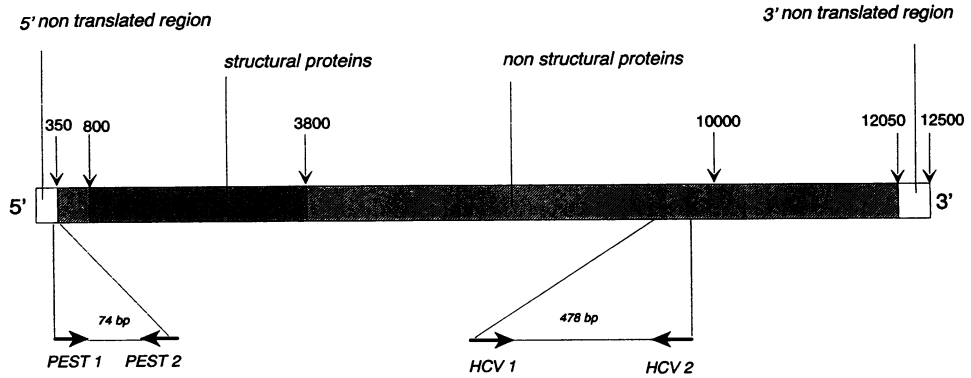

FIG. 1. Schematic representation of the pestivirus genome indicating the position of the primer pairs and the expected lengths of the PCR products.

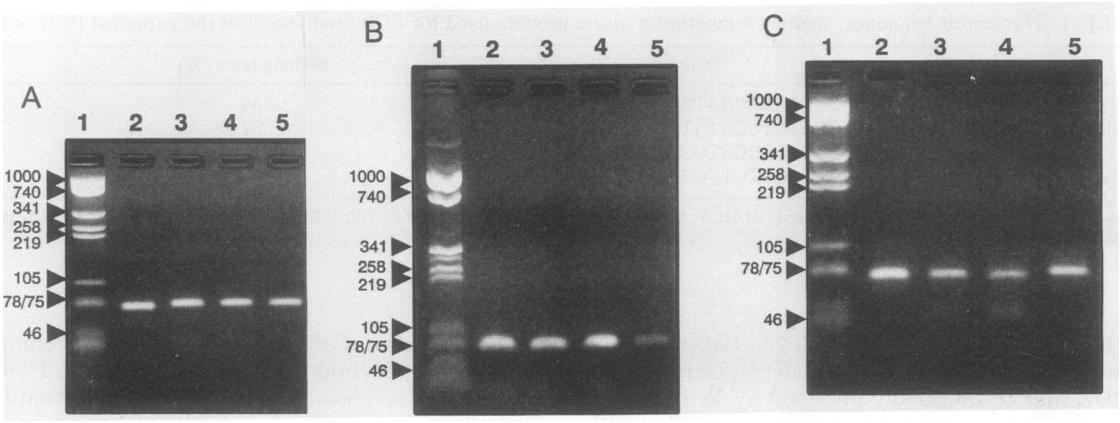

FIG. 2. RT-PCR with primer pair PEST 1-PEST 2. Supernatants from cell cultures infected with HCV (A), BVDV (B), or BDV (C) were used. Lanes 1 in panels A, B, and C are pBluescript II SK$^+$ digested with *Sau*3A-I. Fragment sizes (in base pairs) are indicated on the left of each panel; lanes 2, plasmid-positive control (plasmid pBV-18). (A) HCV Behring (lane 3), Oldenburg (lane 4), and Siegburg (lane 5). (B) BVDV 1935/72 (lane 3), Vaccine-Bern (lane 4), and New York-1 (lane 5). (C) BDV Weybridge (lane 3), 175375 (lane 4), and S137 (lane 5).

ice. Ten units of RNase inhibitor (Boehringer Mannheim, Indianapolis, Ind.) and 50 U of Moloney murine leukemia virus reverse transcriptase (Superscript; GIBCO BRL, Bethesda, Md.) in 2× RT-PCR buffer (5 μl) were added, and the reaction mixture was incubated for 1 h at 37°C. The sense primers (2.5 μl, 10 to 20 μM either PEST 1 or HCV 1) and 0.5 U of *Taq* polymerase (Supertaq; Stehelin, Basel, Switzerland) in 2× RT-PCR buffer (5 μl) were added to the sample. The mixture was overlaid with paraffin oil to avoid evaporation. Amplification was performed for over 35 to 40 cycles in a water bath thermal cycler (Bio-med Thermocycler 60; Gesellschaft für Biotechnologie und Medizintechnik mbH, Theres, Germany). The cycling profiles were 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s for PEST 1-PEST 2 and 95°C for 15 s, 62°C for 60 s, and 72°C for 60 s for HCV 1-HCV 2. Plasmid pBV-18 was used as a positive control. pBV-18 is a partial cDNA clone of BVDV strain NADL, covering the 5′-nontranslated region of the genome. Alternatively, supernatants from cells infected with HCV strain Alfort/187 or BVDV strain NADL served as positive controls. Supernatants from uninfected cells were used as negative controls. In addition, two of the organs included in the study (pig spleen samples Di 3013/90 and Di 4280/90) were derived from animals with a negative HCV diagnosis and thus represented negative controls (see above).

Multiplex PCR. Reactions were performed as described above, except that all four primers, PEST 1-PEST 2 and HCV 1-HCV 2 were included in the PCR. Primers PEST 2 and HCV 2 were used for RT. Amplification was performed over 40 cycles by using the same profile used with the HCV 1-HCV 2 primers alone (95°C for 15 s, 62°C for 60 s, 72°C for 60 s).

Agarose gel electrophoresis. A part of the reaction mixture (10 μl) was combined with loading buffer (2 μl; 1% [wt/vol] sodium dodecyl sulfate, 100 mM EDTA (pH 8), 20% [wt/vol] Ficoll, 0.25% [wt/vol] bromophenol blue, 0.25% [wt/vol] xylene cyanol), and this mixture was analyzed on 2 to 4% agarose gels (NuSieve 3:1; FMC BioProducts, Rockland, Maine) run in TBE buffer (100 mM Tris base, 50 mM boric acid, 2 mM EDTA). Gels contained ethidium bromide for visualization under UV light.

Virus infection and indirect IF assay. Selected HCV isolates were grown on PK-15 cells in slide flasks (SlideFlask; Nunc, Naperville, Ill.). The cell medium was removed, the monolayers were inoculated with 0.5 ml of organ extract (diluted 1:10 in Eagle's minimal essential medium), and the virus was allowed to adsorb to the cells for 2 h at 37°C. The cells were washed twice with PBS (pH 7.5) prior to the addition of serum-free medium. After 3 days, cells were fixed in 85% (vol/vol) acetone in PBS at −20°C for 15 min and washed twice with PBS. Polyclonal anti-HCV antiserum was added, and the antibodies were allowed to bind for 45 min at 37°C before protein A-fluorescein isothiocyanate (Pharmacia P-L Biochemicals, Milwaukee, Wis.) was added and the IF was read. In some cases, the cell culture supernatants harvested from the slide flasks were titrated by using 10-fold dilutions up to a dilution of 10^{-7} in chamber slides (Lab-Teks; Nunc); in other cases, the organ extracts were titrated directly in chamber slides (Lab-Tek) by following the same procedure described above.

RESULTS

Amplification and differentiation of pestivirus RNA in supernatants from virus-infected cell cultures. Conditions for RT-PCR were optimized before the 72- or 74-bp (72/74-bp) fragment could be consistently amplified from virus-infected cell culture supernatants. The most important reaction parameter proved to be the annealing temperature. A number of temperatures for the PEST 1-PEST 2 primer pair were tested and 58°C was selected, which is 4 and 6°C, respectively, below the melting temperatures of the primers. As shown below for the multiplex PCR, annealing at 62°C also gave satisfactory results. Finally, optimization of the cycling steps resulted in a program in which 35 PCR cycles with the primer pair PEST 1-PEST 2 were performed in 1 h and 40 min. Figure 2A shows the 74-bp fragment after RT-PCR with the primer pair PEST 1-PEST 2 with cell culture supernatants containing three different HCV isolates. Figure 2B shows the corresponding amplification products from three supernatants derived from cells infected with BVDV isolates. Finally, Figure 2C shows the results obtained with supernatants containing three different BDV strains. Plasmid pBV-18 served as a positive control, and the supernatant from uninfected cells served as a negative control (data not shown). The genome of BVDV NADL exhibits a deletion of two nucleotides in the amplified region when compared with

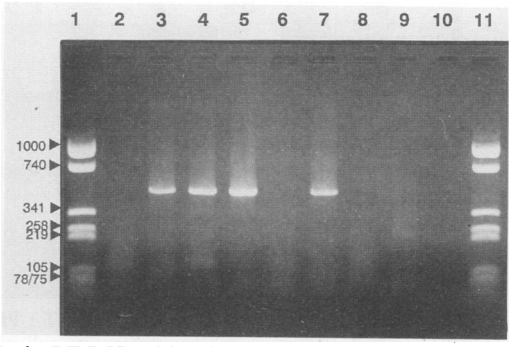

FIG. 3. RT-PCR with primer pair HCV 1-HCV 2. Supernatants from cell cultures infected with HCV, BVDV, and BDV were used. Lanes 1 and 11, pBluescript II SK+ digested with *Sau*3A-I. Fragment sizes (in base pairs) are indicated on the left. Lane 2, BVDV 1935/72; lane 3, HCV Glentorf; lane 4, HCV Oldenburg; lane 5, HCV Siegburg; lane 6, BVDV R4306/78; lane 7, HCV Alfort/187; lane 8, BVDV NADL; Lane 9, BDV 135661; lane 10, negative control (supernatant from uninfected cell culture).

the reported HCV sequences, and thus, pBV-18 gives rise to a fragment of only 72 bp.

Since the individual melting temperatures of primers HCV 1 (76°C) and HCV 2 (68°C) are quite different, the annealing temperature was selected empirically. An annealing temperature of 62°C produced the most consistent results. Figure 3 shows the amplification products of four HCV isolates, three BVDV isolates, and one BDV isolate by using the HCV 1-HCV 2 primer pair. A 478-bp band was observed in all four HCV lanes; no bands could be seen in the lanes representing BVDV and BDV, indicating the specificity of the primer pair. Also, no bands were observed in the lane representing supernatant from uninfected cells. Table 2 summarizes the results obtained with 34 different pestivirus isolates. The 72/74-bp fragment could be amplified in all 34 supernatants from cell cultures infected with these viruses, whereas RT-PCR with the primer pair HCV 1-HCV 2 generated a 478-bp fragment only for the 19 samples containing HCV but not for those containing BVDV and BDV.

Amplification and differentiation of pestivirus RNA in organ extracts of HCV-infected animals. A total of 26 organ extracts expected to contain HCV as well as two blind negative samples were prepared as described above, and 2.5 μl of a 10^{-1} dilution in 10 mM Tris HCl (pH 8.3) was used directly for RT-PCR. Amplification products with primer pairs PEST 1-PEST 2 and HCV 1-HCV 2 of three different organ extracts (lymph node, spleen, and lung) from a single animal infected with HCV isolate D 1047 are shown in Fig. 4. The results for all extracts tested are summarized in Table 3.

All but two extracts proved to be positive when analyzed with primer pair PEST 1-PEST 2. Initial testing with the HCV 1-HCV 2 primer pair resulted in only 8 of 28 extracts producing the expected 478-bp fragment. Those extracts remaining HCV 1-HCV 2 negative were inoculated on PK-15 cells. The supernatants were titrated in 10-fold dilutions up to 10^{-7} and were assayed for virus by indirect IF. Testing of the supernatants from the titrations by PCR resulted in an additional eight positive samples, leaving 12 samples negative for the HCV 1-HCV 2 primer pair. Of these 12 PCR-negative samples, seven exhibited a positive IF at a dilution of 10^{-1} or 10^{-2}, whereas two extracts (Di 903/90 and SP 2605/90) were positive at a dilution of 10^{-5}. In these two cases, a radioactive nucleotide was added to the PCR mixture, and products of the expected size were observed (data not shown). Continued passage of the negative samples showed no improvement in the IF titer, indicating that the virus replicated very poorly in cell culture. In one sample (SP 17368/89), no antigen could be detected by IF, whereas

TABLE 2. Amplification of pestivirus RNA in virus-infected cell culture supernatants

Virus	Strain or isolate	Amplification by primer pairs: PEST 1-PEST 2	HCV 1-HCV 2	PEST 1-PEST 2/HCV 1-HCV 2[a]
HCV	Alfort/187	+	+	ND[b]
HCV	CAP	+	+	ND
HCV	Glentorf	+	+	ND
HCV	Behring	+	+	+/+
HCV	Siegburg 1989	+	+	+/+
HCV	Rhön 1989	+	+	+/+
HCV	Baker A	+	+	+/+
HCV	Riems	+	+	+/+
HCV	Oldenburg PA 4932	+	+	+/+
HCV	Austria 1990	+	+	+/+
HCV	Sch179	+	+	+/+
HCV	Sch186	+	+	+/+
HCV	Sch193	+	+	+/+
HCV	907/1	+	+	+/−
HCV	907/2	+	+	+/+
HCV	907/3	+	+	+/+
HCV	D 1047	+	+	ND
HCV	D 1049	+	+	ND
HCV	D 798	+	+	ND
BVDV	D 1040	+	−	ND
BVDV	1935/72	+	−	+/−
BVDV	Vaccine-Bern	+	−	+/−
BVDV	New York-1	+	−	+/−
BVDV	R56/74	+	−	+/−
BVDV	Oregon	+	−	+/−
BVDV	R4306/78	+	−	+/−
BVDV	R4282/78	+	−	+/−
BVDV	NADL	+	−	+/−
BVDV	V725/88	+	−	+/−
BDV	Weybridge	+	−	+/−
BDV	135661	+	−	+/−
BDV	S137	+	−	+/−
BDV	59387	+	−	+/−
BDV	175375	+	−	+/−

[a] Tested by Multiplex PCR.
[b] ND, not determined.

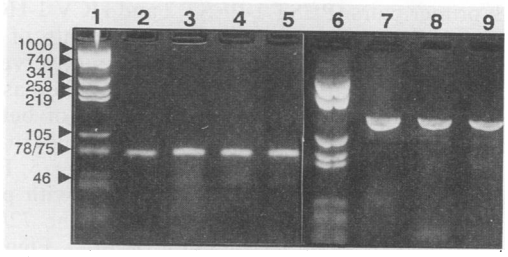

FIG. 4. RT-PCR with primer pairs PEST 1-PEST 2 (lanes 2 to 5) and HCV 1-HCV 2 (lanes 7 to 9). Organ extracts containing HCV were used. Lane 1, pBluescript II SK+ digested with *Sau*3A-I. Fragment sizes (in base pairs) are indicated on the left. Lane 2, pBV-18 (positive control); lane 3, HCV D 1047, lymph node; lane 4, HCV D 1047, spleen; lane 5, HCV D 1047, lung; lane 6, marker DNA; lane 7, HCV D 1047, lymph node; lane 8, HCV D 1047, spleen; lane 9, HCV D 1047, lung.

TABLE 3. Amplification of pestivirus RNA in HCV-infected organ extracts

Strain or isolate	Organ	Amplification by primer pair:		IF[a]
		PEST 1-PEST 2	HCV 1-HCV 2	
Vi 184/4/90	Spleen	+	−[b]	10^{-2}
Vi 364/90	Spleen	+	+	ND[c]
Vi 633/90	Spleen	+	+	ND
Vi 365/90	Spleen	+	+	ND
Vi 4810/90	Spleen	+	−[b]	10^{-2}
Di 2244-45/90	Spleen	+	+	10^{-2}
Di 1627/90	Spleen	+	−[b]	10^{-1}
Di 1626/90	Spleen	+	+	ND
Di 903/90	Spleen	+	−[b]	10^{-5}
Di 3013/90	Spleen	−	−[b]	Negative
Di 4280/90	Spleen	−	−[b]	Negative
D 1047	Lymph node	+	+	ND
D 1047	Spleen	+	+	ND
D 1047	Lung	+	+	ND
SP 9875/90	Tonsil	+	+[b]	10^{-5}
SP 11741/90	Tonsil	+	+[b]	10^{-3}
SP 15860/90	Tonsil	+	+[b]	10^{-6}
SP 128/90	Tonsil	+	+[b]	10^{-7}
SP 2417/90	Tonsil	+	−[b]	10^{-1}
SP 2605/90	Tonsil	+	−[b]	10^{-5}
SP 8253/89	Tonsil	+	−[b]	10^{-1}
SP 8328/89	Tonsil	+	+[b]	10^{-6}
SP 8537/89	Tonsil	+	+[b]	10^{-6}
SP 17368/89	Tonsil	+	−[b]	Negative
SP 17367/89	Tonsil	+	−[b]	10^{-1}
SP 10366/89	Tonsil	+	+[b]	10^{-5}
SP 6639/90	Tonsil	+	+[b]	10^{-5}
SP 6640/90	Tonsil	+	−[b]	10^{-2}

[a] Dilution up to which IF was observed.
[b] Amplification after passage on PK-15 cells. The corresponding IF was performed on the passaged supernatants.
[c] ND, not determined.

by RT-PCR the 72/74-bp pestivirus-specific fragment was observed. The two samples (Di 3013/90 and Di 4280/90) which were negative in the PCR with each of the two primer pairs were also negative in the IF test.

Sensitivity. To assess the sensitivity of pestivirus detection by RT-PCR in comparison with detection by IF staining with polyclonal antibodies, a series of 10-fold dilutions up to a concentration of 10^{-6} for HCV isolates Siegburg and Oldenburg (supernatant of infected cell culture) and HCV strain D 1047 (extract) was prepared. With these samples, RT-PCR with both primer pairs (PEST 1-PEST 2 and HCV 1-HCV 2) and IF staining were performed. Equal amounts of virus dilution were used for the two tests. IF was observed for samples with concentrations of 10^{-2} to 10^{-4}, whereas PCR products could be detected at concentrations of between 10^{-3} and 10^{-5}. The RT-PCR data obtained with supernatants containing HCV strain Oldenburg are given in Fig. 5. Figure 5A shows the amplification products obtained with primer pair PEST 1-PEST 2. The band representing the 72/74-bp fragment can still be seen at a dilution of 10^{-4}. Figure 5B shows the result obtained with primer pair HCV 1-HCV 2. The 478-bp fragment was generated up to a concentration of 10^{-4}. IF was positive up to a concentration of 10^{-3} (data not shown).

Multiplex PCR. To combine detection and differentiation of pestiviruses, the two primer pairs were used together in a single reaction. The same cycling profile described above for

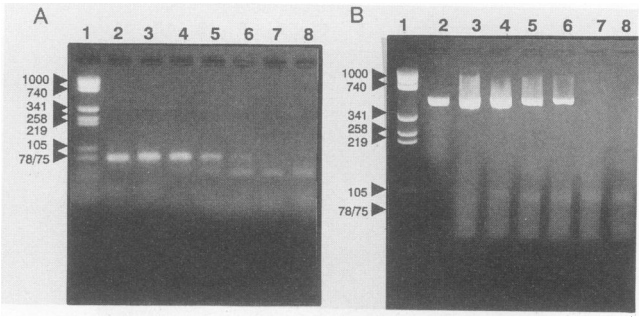

FIG. 5. Sensitivity of RT-PCR with primer pairs PEST 1-PEST 2 (A) and HCV 1-HCV 2 (B). A supernatant from a cell culture infected with HCV Oldenburg PA 4932 (10-fold dilutions up to 10^{-6}) was used. (A) Lane 1, pBluescript II SK$^+$ digested with *Sau*3A-I. Fragment sizes (in base pairs) are indicated on the left. Lane 2, HCV Oldenburg (undiluted supernatant); lane 3, 10^{-1} dilution; lane 4, 10^{-2} dilution; lane 5, 10^{-3} dilution; lane 6, 10^{-4} dilution; lane 7, 10^{-5} dilution; lane 8, 10^{-6} dilution. (B) Lane 1, pBluescript II SK$^+$ digested with *Sau*3A-I. Fragment sizes (in base pairs) are indicated on the left. Lane 2, HCV Oldenburg PA 4932 (undiluted supernatant); lane 3, 10^{-1} dilution; lane 4, 10^{-2} dilution; lane 5, 10^{-3} dilution; lane 6, 10^{-4} dilution; lane 7, 10^{-5} dilution; lane 8, 10^{-6} dilution.

primer pair HCV 1-HCV 2 was used. Figure 6 shows a multiplex PCR performed on five HCV isolates, three BVDV isolates, and three BDV isolates. As expected, both the 72/74- and the 478-bp fragments were amplified simultaneously with HCV-containing cell culture supernatants (lanes 2 to 6), but only the 72-bp fragment showed up for the BVDV and BDV samples (lanes 7 to 12). Table 2 shows the results obtained with supernatants containing HCV, BVDV, and BDV isolates. The use of the multiplex PCR described here enables multiple determinations to be performed in a single reaction.

DISCUSSION

A number of other workers have used RT-PCR to detect pestiviruses (3, 13, 14, 17, 25, 26). The effort in the present study, however, mainly focused on BVDV. In this report we described a primer pair that is suitable for use in the

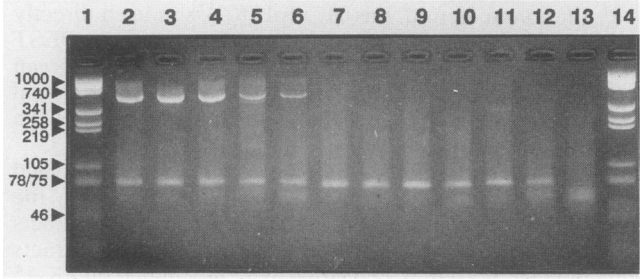

FIG. 6. Multiplex PCR with primer pairs PEST 1-PEST 2 and HCV 1-HCV 2. Supernatants from cell cultures infected with HCV, BVDV, and BDV were used. Lanes 1 and 14, pBluescript II SK$^+$ digested with *Sau*3A-I. Fragment sizes (in base pairs) are indicated on the left. Lane 2, HCV Oldenburg PA 4932; lane 3, HCV Siegburg 1989; lane 4, HCV Rhön; lane 5, HCV Riems; lane 6, HCV Baker A; lane 7, BVDV NADL; lane 8, BVDV Oregon; lane 9, BVDV Vaccine-Bern; lane 10, BDV Weybridge; lane 11, BDV 1355661; lane 12, BDV S137; lane 13, negative control (supernatant from uninfected PK-15 cells).

detection of pestiviruses in general and a primer pair that allows for the specific differentiation and identification of HCV.

We selected primers on the basis of nucleic acid homology alone, without taking into account the protein-coding regions of the genome. The 5′-nontranslated region of pestivirus genomes contains stretches of high sequence homology, and hence, the PEST 1-PEST 2 primer pair was selected from this region. Given the high degree of homology in this area, it is not unexpected that the sequence of our PEST 2 primer is identical to that of primer P3 of Boye et al. (3), except that it lacks the four 3′ nucleotides. Primer $PBVD_3$ of Schroeder and Balassu-Chan (25) is also derived from this region of the genome. The PEST 1 primer is 85% homologous to the corresponding sequence in the 5′-untranslated region of hepatitis C virus (15), which was recently classified as a member of the *Flaviviridae* (28). Similarities between flaviviruses and pestiviruses have previously been shown at the amino acid level (5, 19). Nevertheless, we expect no reaction of these primers with flavivirus sequences because a sequence related to the PEST 2 primer was not detected in the flavivirus sequences that we compared.

The HCV 1-HCV 2 primer pair was selected in an area of high HCV homology but low BVDV homology. We also attempted to select a BVDV-specific primer pair using the same rationale, but the pair that we tested exhibited cross-reactivity with HCV and BDV (data not shown).

In considering the potential of RT-PCR as a diagnostic tool, we decided to design a procedure that minimized sample handling. The ability to handle large numbers of samples is crucial in a diagnostic test that is expected to be widely used, and to this end we decided not to purify viral RNA from the samples. This is in contrast to the procedures used by other workers, who have all purified viral RNA prior to PCR (3, 13, 14, 17, 25, 26). Thus, supernatants from virus-infected cell cultures were used directly in the assay, while the organs were homogenized in PBS, the debris was pelleted, and the supernatant was used. The initial heating step to 90°C for 5 min prior to RT is presumably sufficient to break open the viral capsid and to release the viral RNA. This considerably simplifies the procedure for processing samples and is significantly faster than nucleic acid purification.

By using the PEST 1-PEST 2 primer pair with supernatants from virus-infected cell cultures, all 19 HCV, 10 BVDV, and 5 BDV isolates included in the present study were detected. We were also able to detect virus in all 27 extracts from organs of animals infected with HCV. Given the origin of the samples tested, which were derived from many distinct outbreaks in different European countries over a time range of more than 10 years (see the sample designations in Tables 2 and 3), we consider the PEST 1-PEST 2 primer pair to be suitable for use in the routine diagnosis of pestivirus infections in our laboratory.

The HCV 1-HCV 2 primer pair was found to be specific for HCV, while BVDV and BDV were not detected. However, in most instances but not in the experiment whose results are shown in Fig. 4, the HCV 1-HCV 2 primer pair was not as sensitive as the PEST 1-PEST 2 primer pair. This can be seen from the results for the organ extracts. The PEST 1-PEST 2 primer pair was able to detect virus in samples which, in our hands, were negative (Table 3, sample SP 17368/89) or had a low (10^{-1} to 10^{-2}) IF titer for HCV, whereas the HCV 1-HCV 2 primers did not reveal the expected positive result with all HCV samples. The difference in sensitivity could be due to the size of the fragment generated by the PEST 1-PEST 2 primer pair, which is six to seven times shorter than the HCV 1-HCV 2 fragment. This may result in an increased efficiency of amplification in the PCR. Even a small increase in efficiency in the early cycles could lead to a large difference in the observed band. Nevertheless, this does not explain the two negative results obtained with the HCV 1-HCV 2 primer pair when the virus titer was relatively high, as determined by IF (Table 3, extracts Di 903/90 and SP 2605/90). Also, passage of the organ extracts in cell culture did not lead to a positive PCR result in all cases. This could mean that the negative result was caused by a sequence divergence which did not allow proper annealing of at least one of the HCV primers. Furthermore, after repeated passage of these samples in cell culture, virus titers remained low, indicating that these viruses, which are field isolates and therefore are not adapted to growth in cell culture, replicated very poorly. One sample (SP 17368/89) remained negative after passage in cell culture and subsequent IF testing (Table 3). It was the only PCR-positive sample (primer pair PEST 1-PEST 2) which could not be confirmed to contain HCV by IF. These findings clearly show that several independent methods are required to allow a valuable diagnosis. Thus, we always attempt virus isolation in cell culture for those samples which are not positive with both primer pairs (PEST 1-PEST 2 and HCV 1-HCV 2). In contrast, samples which give a positive result with both primer pairs are considered positive for HCV.

We also tested the abilities of the two sets of primers, PEST 1-PEST 2 and HCV 1-HCV 2, to work in a single reaction (multiplex PCR). The results were encouraging, although we observed a decrease in the signal when both primer pairs were used. Thus, a sample that was positive for each of the primer pairs when used separately was negative for HCV 1-HCV 2 when multiplex PCR was performed (Table 2, sample 907/1). We believe that our PCR-based diagnostic approach could be improved by increasing the sensitivity of the test. This could be achieved, for instance, by performing additional testing, such as nested PCR or nucleic acid hybridization. A higher specificity could possibly be obtained by using additional primer pairs specific for HCV. These possibilities must be investigated further before our HCV diagnosis can rely on RT-PCR exclusively. Nevertheless, the ability to perform multiplex PCR on a single sample permits multiple tests to be performed, whereas previously only a single determination was possible.

In the present work we presented a RT-PCR assay for pestiviruses with the ability to differentiate HCV from BVDV and BDV by using a range and number of HCV samples larger than those used in previous reports (3, 26). The procedure requires no RNA extraction, which is a clear improvement over previous work. We also found that the assay had sensitivity comparable to that of virus isolation in cell culture and then antigen detection by indirect IF staining. Consequently, we believe that the RT-PCR presented here will provide a rapid and sensitive diagnostic tool for the detection of pestiviruses and the differentiation of HCV from other pestiviruses.

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