Presumptive Diagnostic Differentiation of Hog Cholera Virus from Bovine Viral Diarrhea and Border Disease Viruses by Using a cDNA Nested-Amplification Approach

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Hog cholera virus (HCV), bovine viral diarrhea virus (BVDV), and border disease virus (BDV) are closely related pestiviruses. BVDV and BDV are found worldwide but seldom cause disease in swine. In contrast, HCV has been eradicated from swine in several nations but poses a potentially devastating threat to them because of its great virulence. Rapid differential diagnosis of HCV from BVDV and BDV infections in swine is vital for detection of the possible reintroduction of HCV into national herds from which it has been eradicated. Nested polymerase chain reactions (PCRs) for each of two pestiviral genomic segments are described. Amplification of the relatively conserved 5' genomic terminus identified 59 of 61 HCV, BVDV, and BDV isolates generically as pestiviruses. Nested amplification of the second region was designed to differentiate HCV from BVDV and BDV by exploiting relatively conserved differences in the nucleotide sequences that encode the major envelope glycoprotein. This second PCR correctly identified 36 of 36 diverse HCV isolates while failing to recognize any of 25 BVDV and BDV isolates. Multiple restriction fragment length analyses confirmed the identities of both external and nested PCR products. The two sets of PCRs may help confirm the generic identity of most pestiviruses and may permit presumptive differential diagnosis of HCV from BVDV and BDV.

**Materials and Methods**

**Viruses.** A diverse collection of 36 HCV, 20 BVDV, and 5 BDV strains was assembled (see Table 1). All of the HCV strains were identified by using HCV-specific MAbs after first biologically cloning them to ensure freedom from adventitious BVDV and BDV. Growth, cloning, and MAb analysis methods have already been described in detail (1, 7, 17). BVDV and BDV were also propagated and biologically cloned as previously described (10).

**RNA extraction and cDNA synthesis.** Total RNA was prepared from virus-infected cell cultures by using an acid phenol-guanidine isothiocyanate extraction procedure (4). Titrations were performed to ensure a minimum of 10^5 to 10^6 viral 50% tissue culture infective doses in the material extracted. Ten percent of each RNA extract was used in separate 20-μl cDNA syntheses (8) of the 5'-terminal and gp53 genomic segments with a commercial reagent system (Superscript System; Bethesda Research Laboratories, Gaithersburg, Md.). The downstream (3') PCR primers (Fig. 1) for those regions were used to initiate cDNA syntheses.

**PCR target selection and primers.** The relevant regions of the pestivirus 5'-terminal and gp53-encoding sequences are shown in Fig. 1, as are the nested priming sites and the restriction enzyme cleavage sites used to confirm PCR product identity. The sizes and restriction fragment compositions of the predicted PCR products are outlined in Fig. 2. Primers for the gp53 PCR were selected to complement conserved HCV gp53 targets while simultaneously complementing the homologous BVDV sequences as poorly as possible. Primers for the 5' terminus PCR were selected for complete complementarity to the published HCV sequences

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and, within that constraint, to hybridize as well as possible with the two known BVDV sequences. The sequences of the primer used are listed below.

**Primer sequences.** Each of the following primers is written in the 5'-to-3' orientation and identified with the letter placed at the underlined priming sites shown in Fig. 1. The 5' genomic terminus primers used were as follows: A, 5'-CTGGGTGGTCTAAGTCCTGA-3'; B, 5'-GGACAGTCGTCTAGCTAAGTCTGA-3'.

**FIG. 1.** Computer alignment of selected sequences within the 5'-terminal and gp53-encoding regions of HCV strain Alfort (13), HCV strain Brescia (16), BVDV strain NADL (6), and BVDV strain Osloss (18). Nucleotide numbering is that used for publication of the HCV strain Alfort sequence. For each of the two gene segments amplified, A and D delimit the external PCR 5' and 3' priming sequence sites, respectively, while B and C similarly define the nested PCR 5' and 3' priming sites used. *HaeIII*, *XhoI*, and *PstI* restriction sites used to analyze PCR products are indicated by arrows.

CAGTAGTTCCG-3'; C, 5'-CTGCAGCACCCTATCCAGTTCC-3'; D, 5'-TCAACTCTCTGTCAGCTGTA-3'. The gp53 segment primers used were as follows: A, 5'-ATATATGCTCAAGGCGAGT-3'; B, 5'CTCTGTCATATATATCTAGAC-3'; C, 5'-CATTTCCTTTATAGGTCATC-3'; D, 5'-ACAGCAGTAGTATCCATTCTCTT-3'.

**PCR and post-PCR procedures.** Each 20-μl cDNA product was diluted to 100 μl with a commercial PCR reagent mixture (8, 20) to which the appropriate 5' PCR primer had been added (GeneAmp Reagents Kit, Perkin Elmer Cetus, Inc., Norwalk, Conn.). External amplifications were accomplished during 35 cycles of 94°C for 25 s, 60°C for 1 s, and 74°C for 35 s. Nested amplifications were performed in a similar manner, by using 1 μl of the external amplification product, but the annealing temperature was raised to 63°C. Aliquots (20 μl) of all reaction mixtures were electrophoresed in 3% agarose gels (Seaplaque; FMC Bioproducts, Inc., Rockland, Maine) which were photographed under UV illumination following ethidium bromide staining. Additional aliquots were incubated for 2 h with the appropriate restriction enzymes (Fig. 1 and 2) and then also analyzed by gel electrophoresis.

**RESULTS**

All but 2 of the 61 pestiviruses reacted as predicted (Table 1 and Fig. 2A) in the 5' terminus PCR. Both exceptions were BVDV isolates, and these repeatedly failed to yield either external or nested PCR products. The nested 5'-terminal segments of the other 59 viruses were amplified with the production of the expected PCR products, both before and after restriction endonuclease digestions, as exemplified in Fig. 3 and 4. Amplified HCV gp53 virus segments also appeared as predicted, both before and after (Fig. 3 and 4) restriction digestion. None of the BVDV or BDV isolates yielded the predicted gp53 PCR products. Twenty of these failed to
support any detectable amplification; five (Fig. 5) permitted amplification of a discrete and much larger fragment that was refractory to \textit{HaeIII} digestion. Table 1 summarizes the amplification results.

**DISCUSSION**

The differential identification system functioned as predicted, except for the two BVDV isolates previously mentioned. The gp53 primer pairs have slightly lower calculated melting temperatures (2), \textit{T_m}, than the 5′-terminus primers. The gp53 primers for both the external and nested PCRs also contain more mismatches with the two homologous known BVDV sequences than do their 5′-terminus primer counterparts (Fig. 1). It is perhaps a combination of these factors which enables this differential procedure to function.

**TABLE 1.** Historical data and results of 5′ terminus and gp53 gene segment amplifications of HCV, BVDV, and BDV

<table>
<thead>
<tr>
<th>Virus type</th>
<th>No. of isolates tested</th>
<th>Dates of isolations</th>
<th>Location(s) of isolations</th>
<th>PCR amplification result*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5′ terminus segment</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>External segment</td>
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<tr>
<td></td>
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<td></td>
<td>External segment</td>
</tr>
<tr>
<td>HCV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Archival isolates</td>
<td>10</td>
<td>1951–1976</td>
<td>United States, Canada</td>
<td>10</td>
</tr>
<tr>
<td>Recent isolates</td>
<td>11</td>
<td>1980–1989</td>
<td>Western Europe</td>
<td>11</td>
</tr>
<tr>
<td>Recent isolates</td>
<td>3</td>
<td>1980s</td>
<td>Malaysia</td>
<td>3</td>
</tr>
<tr>
<td>Recent isolates</td>
<td>7</td>
<td>1980–1992</td>
<td>Brazil, Mexico, Central America</td>
<td>7</td>
</tr>
<tr>
<td>Attenuated vaccine strains</td>
<td>5</td>
<td>1963–1972</td>
<td>Taiwan, France, United States, Mexico</td>
<td>5</td>
</tr>
<tr>
<td>BVDV</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Laboratory reference strains</td>
<td>4</td>
<td>1961–1980</td>
<td>United States</td>
<td>4</td>
</tr>
<tr>
<td>Diagnostic isolates</td>
<td>16</td>
<td>1975–1990</td>
<td>United States</td>
<td>14</td>
</tr>
<tr>
<td>BDV diagnostic isolates</td>
<td>5</td>
<td>1985–1990</td>
<td>United States</td>
<td>5</td>
</tr>
</tbody>
</table>

* Values indicate number of isolates yielding predicted specific amplification product.

Sequence comparisons (5, 10, 15, 22) and cDNA probe (19) and oligonucleotide hybridization analyses (10) have demonstrated considerable genetic heterogeneity among BVDV isolates. Few, if any, genomic regions are universally conserved, and overall nucleotide sequence conservation within the BVDVs is estimated to be only 74% (5). We selected the relatively conserved 5′-terminus of the pestiviral genome as a generic pestiviral marker, although it is not perfectly conserved (Fig. 1). In light of BVDV heterogeneity and the selection of 5′ terminus PCR primers to be completely homologous to the two known HCV RNA sequences, the failure to detect two of the BVDVs should not be surprising.

Previous reports (3, 10, 19, 22) document the difficulty in developing oligonucleotides, cDNA probes, and PCR primers.

**FIG. 3.** Products of pestiviral 5′ terminus and gp53 gene segment amplification. A recent (1992) HCV isolate from Central American swine was used in this example. Lanes: 1, 231-bp external product of 5′-terminal segment PCR; 2, 149-bp nested product generated by amplification of the product shown in lane 1 with internal primers; 3, 308-bp external product of the gp53 gene segment; 4, 172-bp nested product within the first-stage gp53 amplified segment; 5, molecular size markers (sizes are shown in base pairs on the right).

**FIG. 4.** Restriction enzyme analysis of HCV PCR products. Panels: A, 5′ terminus amplified segments; B, gp53 amplified segments. The Ames strain of HCV was used in this example. Fragment sizes agree with those predicted in Fig. 2. Panel A lanes: 1 and 5, intact external and nested PCR products; 2 and 6, external and nested PCR products following \textit{PstI} digestion; 3 and 7, restriction fragments produced by \textit{XhoI} digestion. Fragment sizes (in base pairs) are shown on the left, and molecular size markers (lanes 4 and 8) are shown on the right. Panel B lanes: 1 and 4, intact external and nested gp53 PCR products; 2 and 5, \textit{HaeIII} digestion products with sizes shown on the left and size standards (lanes 3 and 6) shown on the right.
ers capable of identifying all pestiviruses. Cell cultural
isolation methods have proven to be rapid, reliable, and
sensitive for generic detection of these viruses (17). The
PCR, therefore, may offer no compelling advantage for that
purpose. However, we have demonstrated the ability of
the PCR to presumptively differentiate HCV from other pestivi-

Several investigators have reported PCR-based diagnostic
tests for BVDV and HCV (3, 11, 12, 20, 21). None of those
procedures were designed or evaluated for differential diag-
nostic use. Although ruminant pestiviruses are found world-
wide, several nations have achieved HCV control or eradi-
cation. For these nations, rapid differentiation of HCV from
BVDV and BDV is an urgent necessity. The genetic heter-
genetics of pestiviruses and the mutational proclivities of
single-stranded RNA viruses must be appreciated. Within
that context, however, the PCR method described here may
be used to presumptively differentiate HCV from BVDV and
BDV infections of swine.

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FIG. 5. External amplification of the 5′-terminal (lanes 1 to 6)
and gp53 (lanes 9 to 14) gene segments of six pestiviruses and an
uninfected cell culture-negative control (lanes 7 and 15). For each
specimen, 5-μl aliquots of extracted RNA were amplified separately
with primers specific for 5′ and gp53 sequences. Amplifications were
performed simultaneously with aliquots of the same master reagent
mixture. Lanes: 1 and 9, recent HCV isolate from Mexico. 2 to 6 and
10 to 14, United States-origin BVDV diagnostic isolates. Both HCV
and BVDV supported amplification of the specific 5′ product, but
only HCV yielded the specific gp53 PCR product. Nested PCR of
the products shown revealed a similar absence of expected gp53-
specific products for only BVDV (data not shown). Lane 8 con-
tained molecular size markers (sizes are in base pairs).