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Reverse Transcriptase-PCR Assay for Detection of Hog Cholera Virus

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A reverse transcriptase-PCR strategy was developed for the detection of hog cholera virus. Hog cholera virus template was amplified from tissue culture fluids and from tissues and blood of infected pigs, but not from samples containing other pestiviruses. Restriction endonuclease analysis identified samples as historic or recent isolates.

Hog cholera virus (HCV), bovine viral diarrhea virus (BVDV), and border disease virus (BDV) constitute the *Pestivirus* genus, which has recently been classed as a member of the *Flaviviridae* (2, 17). Pestiviruses contain a single positive-strand RNA, approximately 13 kb in length, which includes a sole large open reading frame (4, 9, 11). Structural proteins are processed from the 5′ end, while nonstructural proteins are found at the 3′ end of the polyprotein (3).

Pestiviruses are associated with economically important diseases of animals. Acute HCV infection is characterized by high mortality, with pigs exhibiting clinical signs of fever, inappetence, diarrhea, and terminally, purplish discoloration of the extremities (15). Chronic infections involve periods of anorexia, fever, and diarrhea. Infection of pregnant sows may lead to abortion or birth of mummified, stillborn, or weak piglets.

Both BVDV and BDV are endemic in many countries, while HCV disease is a reportable disease in many regions which are attempting to eradicate or prevent virus entry into the swine population (1, 10, 12). BVDV and BDV also replicate in swine; hence, the discrimination between BVDV-BDV and HCV is of paramount relevance to control procedures. The current method of detecting pestiviruses relies on propagation in tissue culture and subsequent detection of antigens by immunofluorescence or immunoperoxidase staining with HCV- or BVDV-BDV-specific monoclonal antibodies (5, 18).

Several groups have described reverse transcriptase (RT) and subsequent PCR (13) protocols for the detection and discrimination of HCV and BVDV-BDV, utilizing primers derived from the structural (8, 14) or nonstructural (19) genes. This communication describes a primer design based on the p125 nonstructural gene region of the Alfort and Brescia HCV strains (9, 11) for RT-PCR detection of HCV.

Viral stocks of BVDV, BDV, and HCV, originating from a broad geographic range, were propagated in bovine kidney, sheep chorionic plexus, and PK-15 cell cultures, respectively. Virus titers were determined in microtiter plates, by an immunoperoxidase antigen detection scheme (5, 18), and expressed as 50% tissue culture infective doses ($TCID_{50}$). Cells were lysed by two freeze-thaw cycles, followed by treatment with 0.1 mg of proteinase K per ml and 1% sodium dodecyl sulfate (final concentration) at 37°C for 1 h. Total cell nucleic acid was extracted with a single treatment with phenol-chloroform-isoamyl alcohol and concentrated by ethanol precipitation in the presence of sodium acetate.

Three-month-old pigs were inoculated oronasally with a British classical swine fever (CSF) or American Bureau of Animal Industry (BAI) strain of HCV and sacrificed 23 (CSF) or 9 (BAI) days postinfection (dpi). Retrieval of nucleic acid from frozen tissue specimens of lung, liver, spleen, and tonsil was performed essentially as described above; however, the tissue lysates were subjected to proteinase K treatment overnight at 56°C. For comparative purposes, tissue RNA was also extracted with an RNA isolation kit (Stratagene, Edmonton, Alberta, Canada). Heparinized blood was collected from pigs inoculated with the CSF or BAI strain at the time of euthanasia and frozen at −70°C, until processed for RT-PCR. After nucleic acid was retrieved by either the phenol or kit method (see above), an aliquot of the preparation was incubated in the presence of heparinase I (Sigma, St. Louis, Mo.) for 2 h at 25°C, as described by Izraeli et al. (7).

Primer sets were designed to hybridize to a portion of the p45-p75 boundary of the p120 gene of the Alfort and Brescia strains (9, 11): HCV-1, nucleotides 5067 to 5087 (5′-GCTC CTGGTTGGTAACCTCGG-3′); HCV-2, nucleotides 5554 to 5574 (5′-TGATGCTGTCACACAGGTGAA-3′). The primers possess melting temperatures of approximately 68°C (HCV-1) and 62°C (HCV-2) (16). BVDV-BDV-specific primers were designed from the same region of the BVDV NADL strain (4), to allow amplification of BVDV-BDV in specificity testing.

Nucleic acid was added to an RT mix (20 μl final volume) containing 20 to 40 U of RNase inhibitor, 500 ng of random primers, 0.5 mM deoxynucleoside triphosphates (dNTPs), 200 U of Moloney murine leukemia virus RT (BRL, Burlington, Ontario, Canada), 1 mM dithiothreitol, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM $MgCl_2$. A 50-μl layer of mineral oil was added, and the tubes were transferred to a Coy thermal cycler. The mixture was incubated at 37°C for 1 h, and then enzyme denaturation was performed at 95°C for 5 min. With the temperature remaining at 80°C, the PCR mixture was added, resulting in a final volume of 50 μl and final concentrations of 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.8 mM $MgCl_2$, 0.5 mM dNTPs, 50 pmol of HCV- or BVDV-specific

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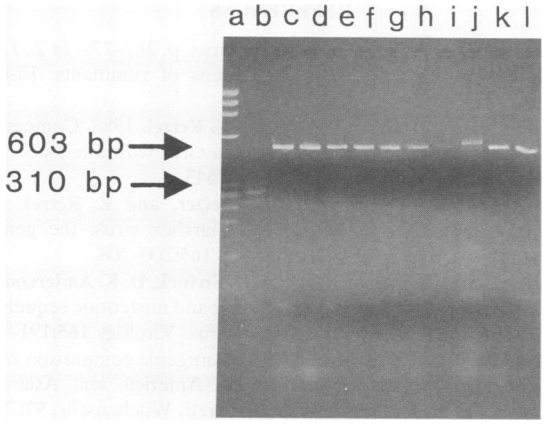

FIG. 1. Representative HCV strains amplified by use of HCV primers. Lane a, ϕX174-*Hae*III size standards; lane b, cell control; lanes c to g, recent strains (lane c, 1821; lane d, Osterode; lane e, Bas-rhin; lane f, V622; lane g, VRI); lanes h to l, historical strains (lane h, Windsor; lane i, Alfort; lane j, Henken; lane k, Baker; lane l, BAI).

primers, and 1.25 U of Amplitaq polymerase (Perkin-Elmer, Nepean, Ontario, Canada). The PCR protocol consisted of 40 cycles of denaturation at 94°C (1 min), primer annealing at 59°C (1 min), and elongation at 72°C (2 min). Amplification products (approximately 20% PCR volume) were analyzed on 3% NuSieve–1% SeaKem agarose containing 0.5 μg of ethidium bromide per ml. Gels were electrophoresed at 50 V for 2 h in Tris-acetate-EDTA buffer, also containing 0.5 μg of ethidium bromide per ml. Bands were visualized with a UV light source and compared with ϕX174-*Hae*III size standards.

Restriction endonuclease analysis (REA) protocols involved digesting PCR products for 2 h at 37°C with three restriction endonucleases (*Ava*II, *Mbo*I, and *Nco*I) which were expected to digest the HCV Alfort RT-PCR fragment (9).

A variety of historical (pre-1980s) and recent (1980s) strains of HCV were detected following amplification with HCV-specific primers (Fig. 1; Table 1). Products consistent with the anticipated 508-bp fragment were generated following amplification of all HCV strains tested, except for the Henken strain, in which a band of approximately 550 bp, as well as several other smaller fragments, was consistently noted. On the basis of empirical observations, all isolates were amplified with equal efficiency, with the exception of the Henken strain, which exhibited less RT-PCR product. Following 40 cycles of amplification, the test was capable of detecting <0.01 $TCID_{50}$ of in vitro-propagated HCV (data not shown).

HCV-specific primers did not amplify sequences from tissue culture control samples. In addition, no false-positive reactions for cytopathic (Singer, Oregon, NADL) or noncytopathic (New York) BVDV samples or for noncytopathic (4080, B4491) BDV samples were noted. The presence of an amplifiable template in both BVDV and BDV specimens was confirmed with BVDV-BDV-specific primers (data not shown).

HCV from various internal organs from a pig sacrificed 23 dpi with the CSF strain (Fig. 2) and a pig sacrificed 9 dpi with the BAI strain (data not shown) was detected. PCR fragments were not generated from samples of normal pig tissues. In addition, amplifiable template was present in samples of frozen heparinized blood collected 9 and 23 dpi from pigs inoculated with CSF or BAI, respectively (Fig. 3), provided that the nucleic acid preparation was treated with heparinase I prior to RT-PCR testing.

TABLE 1. RT-PCR and REA of HCV strains

HCV strain	Country of origin[a]	HCV PCR	REA pattern		
			*Ava*II[b]	*Mbo*I[c]	*Nco*I[d]
Historical					
Baker	USA	+	1	1	1
BAI	USA	+	2	1	1
Alfort	France	+	2	1	1
Glentorf	Germany	+	2	1	1
Henken	Holland	+[e]	4	1	4
Windsor	Canada	+	2	1	4
Recent					
1821	Belgium	+	2	2	3
Bas-rhin	France	+	3	2	2
Osterode	Germany	+	4	2	2
V622	Germany	+	3	2	2
Classical	UK	+	3	2	2
VRI	Malaysia	+	3	2	2
EVI	Brazil	+	3	2	2

[a] USA, United States of America; UK, United Kingdom.
[b] Sizes of REA fragments (in base pairs) for the indicated patterns: 1, 268 and 240; 2, 268, 200, and 40; 3, 460 and 48; 4, uncut.
[c] Sizes of REA fragments (in base pairs) for the indicated patterns: 1, uncut; 2, 474 and 34.
[d] Sizes of REA fragments (in base pairs) for the indicated patterns: 1, 456 and 52; 2, 306, 150, and 52; 3, 306 and 202; 4, uncut.
[e] A slightly larger PCR product was noted (see the text).

REA was performed to confirm the identity of the RT-PCR products. A general pattern corresponding to the date of original isolation became apparent (Table 1). The REA pattern for Alfort HCV was not consistent with that predicted from published sequence information (9). However, the predicted Alfort HCV map was apparent following digestion of a variety of recent strains, including V622, Bas-rhin, classical, VRI, and EVI isolates. The Alfort REA pattern was identical to those of other historical isolates, such as Glentorf and BAI.

Rapid differential diagnosis that distinguishes between porcine and ruminant pestiviruses is critical in the event of an outbreak. Several groups have reported an RT-PCR design for the discrimination of BVDV-BDV and HCV (8, 19), with use

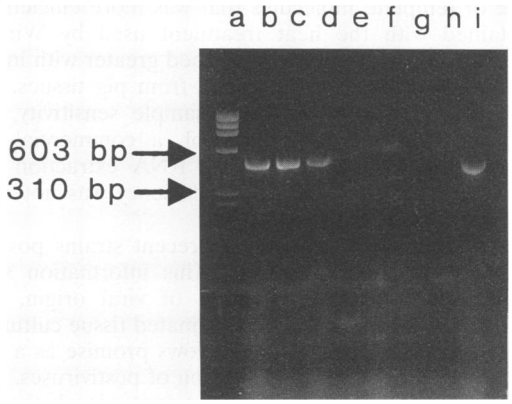

FIG. 2. Detection of CSF-HCV in frozen pig tissues by RT-PCR. Lane a, ϕX174-*Hae*III size standards; lanes b to d, tissues from an infected piglet (lane b, tonsil [200 $TCID_{50}$]; lane c, lung [2×10^4 $TCID_{50}$]; lane d, liver-spleen [200 $TCID_{50}$]); lanes e and f, tissues from an uninfected piglet (lane e, tonsil; lane f, lung-liver-spleen0; lanes g and h, negative and reagent controls; lane i, lane b sample processed with a commercial RNA isolation kit.

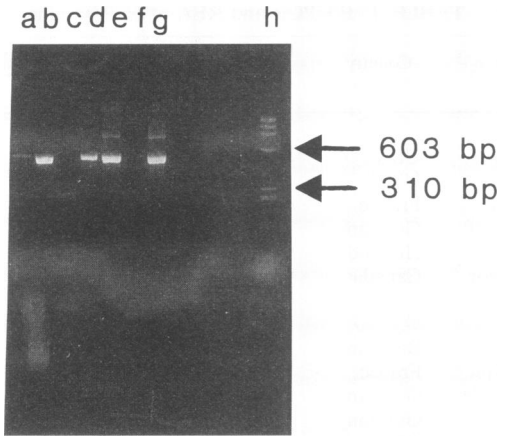

FIG. 3. Detection of HCV in frozen heparinized blood. Lanes a to c, samples processed with a commercial RNA isolation kit (lane a, BAI-inoculated pig; lane b, CSF-inoculated pig; lane c, noninoculated pig); lanes d and e, HCV-spiked blood sample (lane d, sample processed by conventional methods; lane e, sample processed with the commercial RNA isolation kit); lane f, reagent control; lane g, HCV-positive control; lane h, ϕX174-*Hae*III size standards.

of either structural or nonstructural gene regions. This report outlines discriminatory amplification of a nonstructural genome segment for amplification of HCV from tissue culture, pig tissue, or heparinized blood origin. To date, all HCV strains subjected to the RT-PCR protocol described possess the expected 508-bp PCR product, with the exception of the Henken isolate. This amplification product was slightly larger than expected; however, the REA pattern of the 550-bp fragment was similar to that of the Windsor strain. Modification in the p125 genome region in cytopathic BVDV isolates has been reported previously (6). Sequence analysis of the various Henken PCR products would confirm the nature of any mutations in this region, as well as providing an explanation for the appearance of multiple bands following the amplification of this strain.

The sensitivity of the RT-PCR protocol described above appeared to be greater than that reported previously (19). It is expected that the phenol extraction procedure resulted in an exposure of template molecules that was more efficient than that obtained with the heat treatment used by Wirz and coworkers (19). Assay sensitivity seemed greater with in vitro-propagated virus than with template from pig tissues. In an attempt to improve upon in vivo sample sensitivity, while ensuring a rapid and easy protocol, a commercial RNA isolation kit was employed for the RNA extraction steps. However, no increase in RT-PCR efficiency was noted with these modifications (Fig. 2 and 3).

It is apparent that historical and recent strains possessed distinct REA patterns (Table 1). This information should prove useful for the determination of viral origin, in an outbreak or in submissions of contaminated tissue cultures. In conclusion, this RT-PCR protocol shows promise as a rapid, sensitive, and specific test for detection of pestiviruses. These advantages are especially notable with samples in which virus is difficult to demonstrate by conventional isolation techniques.

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