Detection of Hepatitis A Virus RNA in Oyster Meat

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Detection of low concentrations of viruses in shellfish is possible with nucleic acid amplification by PCR. Hepatitis A virus (HAV) has been detected in oyster meat by reverse transcription-PCR (RT-PCR). We developed a method to identify HAV RNA by RT-PCR of total RNA extracted from oyster meat contaminated by adsorption, bioaccumulation, or injection. With dot blot hybridization detection of amplicons from the RT-PCR, rapid screening of a large number of samples is feasible. As few as 8 PFU of HAV/g of oyster meat can be detected.

Shellfish are readily contaminated with viruses present in water containing sewage because of the concentrating effect of filter feeding. In the United States, bacterial standards have been employed to designate shellfish beds considered safe for harvest (23). However, indicator bacterial counts have been negative when virus was detected in experimentally contaminated or commercially harvested shellfish (10, 11, 16, 18, 31), and outbreaks of disease have occurred with these harvest controls in place (9, 17, 22, 25). Examination of shellfish for viral contamination has generally relied on cell culture. However, wild-type hepatitis A virus (HAV) is not readily grown in cell culture but requires long periods of adaptation and antigen assays for virus detection, since no cytopathic effect is produced (7, 20).

Identification of unamplified viral nucleic acid is an alternative means of virus detection in shellfish but has required 10^3 to 10^4 infectious particles per 20 g of meat (34). Shellfish contaminated with enterically transmitted viruses may contain only 0.2 to 224 infectious particles/100 g of meat (32). However, reverse transcription of RNA and amplification of cDNA by PCR (RT-PCR) have provided the sensitivity to detect HAV in shellfish, either by immunocapture of virus particles or by direct amplification of viral RNA (2, 8, 9, 12, 14, 15, 19, 33). We have developed a method to detect HAV RNA in shellfish by using RT-PCR and hybridization after extraction of total RNA from oyster meat.

Virus and cells. HAV strain HM-175, cytopathic clone 24A, was propagated and assayed by plaque formation in FRhK-4 cells as previously described (6). Stock virus was used at 5×10^7 to 10×10^7 PFU per ml and was sonicated and diluted in phosphate-buffered saline (PBS) to achieve the desired virus concentrations.

Oyster contamination. Shucked oysters from Washington State or Gulf of Mexico coastal areas were obtained from local markets. HAV was adsorbed to oyster meat by a modification of a previously described method (26, 28, 29). Meat was homogenized in cold sterile water (Abbott Diagnostics, North Chicago, Ill.) at a ratio of 1 g per 5 ml of water in a Waring blender at low speed for 15-s intervals (cumulative time, up to 2 min), followed by the addition of HAV at the desired concentration. After brief homogenization to disperse the virus,

the pH was adjusted to 4.5 to 5.0 with 6 N HCl, and the mixture was incubated at room temperature for 15 to 60 min, after which the homogenate was centrifuged at $2,000 \times g$.

Bioaccumulation of HAV was performed in a 4-liter aquarium with four to six live oysters obtained from local markets or shipped from Louisiana. Synthetic sea salt (Instant Ocean; Aquarium Systems, Mentor, Ohio) was dissolved in deionized water, and the salinity was adjusted to 16 ppt by refractive index measurement. Fecal solids (normal chimpanzee stool at 1 g/liter) and/or algal wafers (Kyorin Co., Himeji, Japan) were added to the aerated sea water. Oysters were observed for viability prior to addition of HAV at a final concentration of 10^3 or 10^4 PFU/ml, and the animals were kept in the aquarium for 16 to 24 h. Prior to removal of the meat for homogenization, the oyster shells were thoroughly cleaned with 95% ethanol and tap water.

Intact oysters were directly injected with 20 µl of HAV or PBS via a micropipette tip inserted into four to five sites in the visceral area. After adsorption for 90 to 120 min at room temperature, total RNA was extracted from 10 to 12 g of meat (one or two oysters) homogenized in an Omnimixer (Ivan Sorvall, Dupont Co., Wilmington, Del.).

Whole virus extraction. After homogenization in water (Abbott Diagnostics), oyster meat contaminated by adsorption or by bioaccumulation was divided into aliquots for either whole-virus extraction or total-RNA extraction. For whole-virus extraction, pelleted homogenized oyster meat was resuspended in 0.05 N glycine–0.14 N NaCl buffer (pH 9.5) to elute HAV (26). After adjustment of the pH to 7.5, the virus-rich supernatant was treated twice with equal volumes of trichlorotrifluoroethane (Fisher Chemicals, Pittsburgh, Pa.), and the extracts were combined and tested by immunocapture RT-PCR (IC-RT-PCR) and/or the plaque assay.

RNA extraction. Total RNA was extracted in a one-step method (5) using guanidium-phenol (RNAzol B) or guanidium-phenol-urea-detergent (Ultraspec; Biotecx Laboratories, Inc., Houston, Tex.). The experiment shown in Fig. 1 was carried out with RNAzol B, while results shown in Fig. 2 were obtained with Ultraspec. The oyster meat pellet obtained after homogenization in water was further homogenized in 10 to 20 ml of RNAzol B per g of meat or in 5 to 10 ml of Ultraspec after bioaccumulation. When contaminated by injection, oyster meat was homogenized in Ultraspec without homogenization in water. With either RNAzol B or Ultraspec, 1/10 volume of chloroform was added to the homogenate, followed by vigorous mixing. After brief incubation at 4°C followed by centrif-

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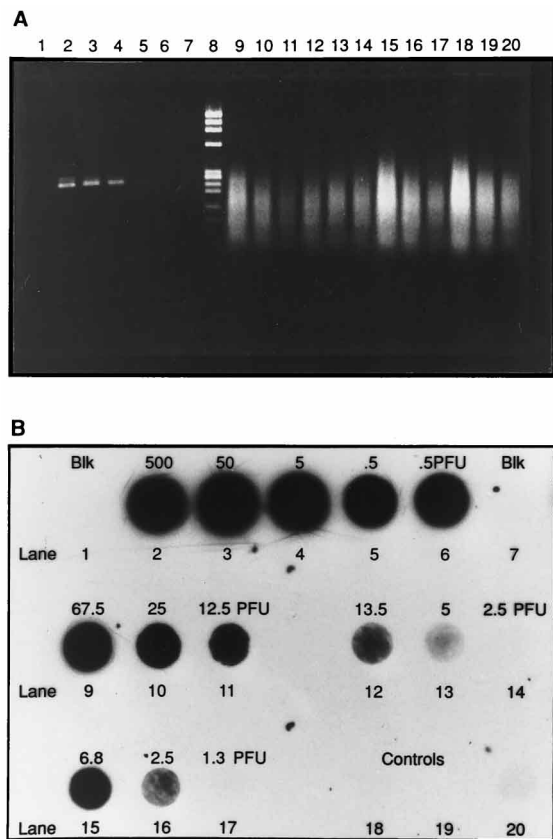
FIG. 1. RT-PCR analysis of total RNA extracted from oyster meat contaminated with HAV at 5,000, 1,000, or 500 PFU per 5 g. (A) Electrophoresis in 2% agarose gel and ethidium bromide staining. Lanes 1 and 7, RT-PCR blank; lanes 2 to 6, IC-RT-PCR of serial dilutions of cell culture-derived HAV; lane 8, φX174 *Hae*III fragments; lanes 9 to 20, amplicons from 25-, 10-, and 5-μl aliquots of RNA extracted from oyster meat (lanes 9 to 11, 5,000 PFU; lanes 12 to 14, 1,000 PFU; lanes 15 to 17, 500 PFU; lanes 18 to 20, no virus). (B) Analysis of amplicons by dot blot and ECL hybridization. The number below each dot represents the corresponding lane from panel A. The number above each dot is the PFU of input virus per RNA aliquot.

Cell culture HAV or oyster eluates were incubated in anti-HAV immunoglobulin G-coated microcentrifuge tubes at 42°C for 2 h and washed with PBS plus 0.5% bovine serum albumin. Immunocaptured virus was heated at 95°C for 5 min in buffer containing deoxynucleotide triphosphates and the negative-sense primer. Reagent concentrations in the RT-PCR mixture were 20 mM Tris (pH 8.3), 50 mM KCl, 2.0 mM MgCl$_2$, 0.2 mM deoxynucleotide triphosphates, and 200 ng of each primer. After snap freezing and thawing, 40 U of RNAsin (Boehringer Mannheim, Inc.), 25 U of reverse transcriptase (avian myeloblastosis virus; Boehringer Mannheim or Perkin-Elmer, Norwalk, Conn.), and 5 mM dithiothreitol were added, and the mixture was incubated for 1 h at 42°C, followed by incubation for 5 min at 95°C. *Taq* polymerase (2.5 U; Perkin-Elmer or Boehringer Mannheim) and 200 ng of positive-sense primer were added, and the reaction mixture was carried through 40 cycles of denaturation at 95°C for 35 s, annealing at 55°C for 60 s, and extension at 72°C for 75 s. RT-PCR of extracted RNA was performed under the same conditions as for IC-RT-PCR.

PCR products were separated by electrophoresis in 2% agarose gels followed by ethidium bromide staining or were transferred to Biotrans nylon membranes (ICN Biochemicals, Irvine, Calif.) and probed by the same methods as for dot blot hybridization (see below). Biotinylated DNA fragments (Bioventures, Murphreesboro, Tenn.) were used for size comparison in gels analyzed by hybridization.

Hybridization. Dot blot hybridization was performed after denaturation of 10 μl of amplicon in 400 μl of 0.4 N NaOH and 25 mM EDTA (3). Denatured samples were loaded onto a nylon membrane with a dot blot apparatus (Schleicher and Schuell, Keene, N.H.) and nucleic acid fixed by UV cross-linking (Stratalinker; Stratagene, La Jolla, Calif.). A biotinylated oligoprobe internal to the primers and complementary to HAV cDNA (21) was used for hybridization at 54°C (10°C below the T_m) in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.1% sodium dodecyl sulfate. After

ugation at 12,000 × g, RNA was precipitated from the upper aqueous layer after addition of an equal volume of isopropanol and cooling on ice for 15 to 90 min. RNA was washed twice with 75% ethanol, dried under a vacuum, and resuspended in 1 mM EDTA after RNAzol B extraction or in diethyl pyrocarbonate-treated water (Biotecx Laboratories, Inc.) after Ultraspec extraction. RNA obtained with RNAzol B was reprecipitated in 0.2 M NaCl and 2 volumes of ethanol overnight at −20°C, while RNA obtained from Ultraspec was not reprecipitated with ethanol except in the experiment for which results are shown in Fig. 2A. RNA concentrations were determined from the optical density at 260 nm.

RNA was amplified by RT-PCR or further purified by centrifugation in a microconcentrator with a molecular-weight cutoff of 30,000 (Centricon; Amicon, Beverly, Mass.) or by Sephadex G-50 column chromatography (1) (Quick Spin; Boehringer Mannheim Co., Indianapolis, Ind.).

HAV RNA detection. HAV RNA was detected following RT-PCR (21) using a negative-sense primer directed at nucleotides 2390 to 2414 (5′-GGAAATGTCTCAGGTACTTTCTT TG-3′) and a positive-sense primer directed at nucleotides 2167 to 2192 (5′-GTTTTGCTCCTCTTTATCATGCTATG-3′). IC-RT-PCR was performed as described previously (27).
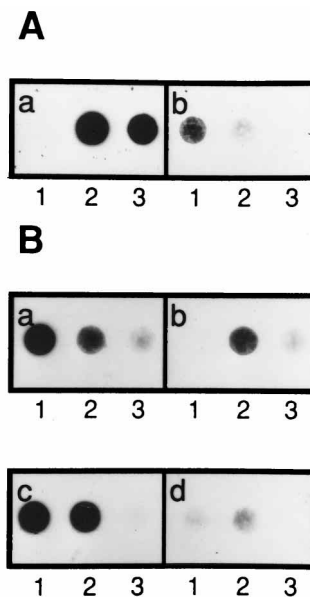

FIG. 2. (A) Dot blot hybridization of RT-PCR amplicons from total RNA extracted from naturally contaminated oysters. The quantity of RNA in RT-PCR was 10 μl (dots 1), 5 μl (dots 2), or 1 μl (dots 3). The level of HAV in seawater was 10^4 (a) or 10^3 (b) PFU/ml. (B) Detection of HAV RNA in oysters directly injected with 5,000 (a), 1,000 (b), 500 (c), or 100 (d) PFU of HAV. The quantity of RNA in the RT-PCR mixture was 10 μl (dot 1), 5 μl (dot 2), or 1 μl (dot 3).

washing with $2\times$ SSC plus 0.1% sodium dodecyl sulfate, the membrane was incubated with strepavidin-horseradish peroxidase conjugate (Vector Laboratories, Burlingame, Calif.) at room temperature for 15 min prior to the final wash (3). Hybridization products were detected by epichemiluminescense (ECL; Amersham, Arlington Heights, Ill.) with X-ray film (X-Omat; Eastman Kodak, Rochester, N.Y.) exposed initially for 2 to 5 min and again for 10 to 120 min, depending upon the intensity of the first exposure.

Virus recovery. Recovery of infectious virus by organic solvent extraction of adsorbed and eluted virus was determined by plaque assay. In four experiments in which input virus was 10^4 to 10^5 PFU per 50 g of homogenized oyster meat, the mean efficiency of virus recovery was 41% (range, 30 to 50%). In oysters contaminated by feeding in seawater, there was a consistent uptake of HAV, with uptake increasing with an increasing concentration of virus in seawater. HAV at a seawater concentration of 10^3 PFU/ml yielded 5.8×10^2 PFU/g of oyster meat, and 1.71×10^3 PFU/g was detected in animals fed in water containing 10^4 PFU/ml.

Sensitivity of RT-PCR. Serial dilutions of cell culture-derived HAV were used to determine the sensitivity of RT-PCR and dot blot hybridization. IC-RT-PCR consistently detected 0.5 PFU (Fig. 1B, lanes 5 and 6), and as few as 0.09 PFU was detected on occasion (data not shown). However, when RNA was isolated with RNAzol B, the level of detection was as much as 10-fold lower (data not shown).

HAV (5×10^6 PFU) was adsorbed to 100 g of homogenized oyster meat, and virus was eluted as described above. Analysis by IC-RT-PCR required a 1:4 dilution of the final eluate in order to detect a PCR product by hybridization. However, the intensity of the dot blot hybridization did not correlate with the recovery of infectious virus, which suggested the presence of inhibitors. Eluate that contained 85 PFU of HAV repeatedly produced an intensity on dot blot hybridization consistent with <0.5 PFU of cell culture-derived HAV (data not shown).

RNA detection after adsorption. Rather than develop methods to remove the apparent PCR inhibitors obtained during virus elution, we examined the sensitivity of PCR amplification using total RNA extracted from oyster meat. HAV was adsorbed to 5-g aliquots of oyster meat (5,000, 1,000, and 500 PFU per aliquot), and total RNA was extracted with RNAzol B and purified by membrane filtration. A range of volumes (5, 10, or 25 µl) of each RNA preparation were analyzed by RT-PCR with ethidium bromide staining and dot blot hybridization (Fig. 1). Ethidium bromide staining did not identify an appropriate-size amplicon from the oyster meat RNA (Fig. 1A, lanes 9 to 20), compared to the detection of an amplicon of 5 PFU of cell culture-derived HAV (Fig. 1A, lane 4). The wide smear of ethidium bromide-stained material seen in lanes 9 to 20 varied in intensity with different oysters or lots and with the quantity of RNA analyzed. When analyzed by dot blot hybridization, HAV RNA equivalent to 100 PFU/g was initially detected (Fig. 1B, lanes 15 to 17), and in subsequent experiments 40 PFU/g was detected (data not shown). For cell culture-derived HAV, 0.5 PFU was consistently detected with dot blot hybridization (Fig. 1B, lanes 5 and 6). This method was found to be 10-fold more sensitive than ethidium bromide staining, equal to or more sensitive than Southern blot detection (data not shown), and equal to second-round PCR detection (data not shown).

RNA detection after bioaccumulation. Total RNA was extracted with RNAzol B from oysters contaminated by feeding in seawater (5×10^4 to 1×10^3 PFU of HAV per ml) and was analyzed by RT-PCR. Appropriately sized amplicons were visualized by ethidium bromide staining and were also detected with dot blot hybridization (data not shown). When oysters were contaminated with 10^3 PFU of HAV/ml of seawater, 5 µl of total RNA produced an amplicon representing 4 PFU, based on a comparative endpoint titration (Fig. 2A, panel b). The concentrations of total RNA extracted from oysters were 2.4 mg/ml for those contaminated at 10^3 PFU/ml and 3.8 mg/ml for those contaminated at 10^4 PFU/ml. Initial A_{260}/A_{280} ratios for the final RNA extract were 1.50 to 1.37, and these ratios ranged from 1.65 to 1.80 after membrane treatment, although in some experiments high-purity RNA was obtained with the initial ethanol precipitation. Occasionally, when a larger volume of RNA extract was used in RT-PCR, an inhibitory effect was observed; it was overcome by use of smaller volumes (Fig. 2A, panel a).

RNA detection after direct injection. Direct injection of HAV was used to produce the low levels of virus contamination expected to be found in shellfish naturally feeding in contaminated waters. RT-PCR and dot blot analysis of total RNA extracted from oyster meat with Ultraspec or RNAzol B could detect 40 PFU of HAV/g of oyster meat (data not shown). After concentration and purification by quick-spin column chromatography of Ultraspec-extracted RNA, detection at the level of 8 PFU/g was achieved (Fig. 2B, panel d).

These experiments showed that analysis of extracted RNA by RT-PCR and dot blot hybridization provided a rapid and sensitive means to detect HAV in shellfish contaminated by various methods and eliminated the purification steps required for virus isolation. Direct RNA extraction reduced the opportunity for virus loss during purification, and the sensitivity of RT-PCR with dot blot hybridization was comparable to that of IC-RT-PCR. We detected the equivalent of 8 PFU of HAV/g of oyster meat, and the highest sensitivity was achieved when RNA was purified by column chromatography (1) or when smaller volumes of RNA extract were tested. These findings suggest that not all inhibitors of RT-PCR were removed by solvent extraction and alcohol precipitation of RNA or that excess nonviral RNA competed with viral RNA in the PCR. A further increase in sensitivity might be achieved by additional RNA purification, the removal of background oyster RNA (12), or limiting the extraction of RNA to only the oyster digestive tissues (2).

The 41% recovery of infectious HAV obtained by adsorption and elution from oyster meat was similar to that reported by other investigators (14). However, detection of wild-type virus from unconcentrated environmental samples is not feasible because of low virus concentrations and the long adaptation required for cell culture growth (7). Even methods that detect nucleic acid from virus particles isolated from shellfish do not establish infectivity (8). IC-RT-PCR can detect nucleic acid from intact viral particles that may not be infectious, since the ratios of noninfectious to infectious HAV particles are 58:1 to 79:1 for cell culture-adapted virus and 2×10^5:1 for wild-type virus and may differ by host conditions (8, 13). However, since free HAV RNA would most likely be degraded outside of an intact virion in the marine environment, its detection in shellfish would indicate intact virus particles.

In this study, the level of HAV detected after total RNA extraction and PCR amplification was comparable to that with PCR amplification of RNA obtained from whole virus (19). Detection of very low levels of virus (0.2 PFU/g of meat) has been described for a virus concentration method that required 2 days in addition to the time required for RT-PCR amplification and detection (15). The HAV RNA extraction and detection require only 2 days, can be performed in most laboratory settings, use commercially available reagents, and have the potential advantage of being used for multiplex PCR to

detect a number of pathogenic viruses known to contaminate shellfish, in addition to bacteriophage indicators (30). If examination of the oysters for whole virus or bacterial indicators is required, the samples can be homogenized and divided before RNA extraction.

That HAV infection is associated with contaminated shellfish, raw vegetables, and water is well established (4, 9, 24). However, studies to detect HAV RNA in environmental samples have not quantitated virus. Since the minimum infectious dose of HAV for humans has not been determined, studies to detect HAV RNA in environmental samples do not provide an estimate of the health significance, except when they are associated with disease transmission (9). Rapid, quantitative methods for viral nucleic acid detection are needed to define the public health utility of this method of shellfish certification.

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