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Rapid and Efficient Extraction Method for Reverse Transcription-PCR Detection of Hepatitis A and Norwalk-Like Viruses in Shellfish

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As part of an effort to develop a broadly applicable test for Norwalk-like viruses and hepatitis A virus (HAV) in shellfish, a rapid extraction method that is suitable for use with one-step reverse transcription (RT)-PCR-based detection methods was developed. The method involves virus extraction using a pH 9.5 glycine buffer, polyethylene glycol (PEG) precipitation, Tri-reagent, and purification of viral poly(A) RNA by using magnetic poly(dT) beads. This glycine–PEG–Tri-reagent–poly(dT) method can be performed in less than 8 h on hard-shell clams (*Mercenaria mercenaria*) and Eastern oysters (*Crassostrea virginica*) and, when coupled with RT-PCR-based detection, can yield results within 24 h. Observed sensitivities for seeded shellfish extracts are as low as 0.015 PFU of HAV and 22.4 RT-PCR$_{50}$ units for Norwalk virus. Detection of HAV in live oysters experimentally exposed to contaminated seawater is also demonstrated. An adaptation of this method was used to identify HAV in imported clams (tentatively identified as *Ruditapes philippinarum*) implicated in an outbreak of food-borne viral illness. All of the required reagents are commercially available. This method should facilitate the implementation of RT-PCR testing of commercial shellfish.

Hepatitis A virus (HAV) and Norwalk-like viruses (NLVs) are environmentally stable, positive-stranded RNA viruses that are readily transmitted via the fecal-oral route. Shellfish, being aquatic filter feeders, readily bioconcentrate these viruses. As a result, consumption of virus-contaminated shellfish represents a significant health threat to shellfish consumers; as well as an economic threat to the seafood industry. Although 120 enteric viruses have been found in human sewage, the viral illnesses most frequently associated with shellfish consumption in Europe and the United States are HAV and genogroup I and II NLVs (25). Recently, NLVs have emerged as the most common food-borne pathogen in the United States (28). Approximately 1.4 million cases of HAV-mediated illness occur worldwide (16), with approximately 83,000 cases occurring within the United States per annum (28). However, the potential for widespread viral outbreaks from contaminated shellfish is great, as evidenced by an outbreak of HAV in Shanghai, China, resulting in approximately 300,000 illnesses (14).

Shellfish waters in the United States are classified as approved, conditional, restricted, or prohibited for shellfish harvesting based primarily on the monitoring of fecal coliform levels in shellfish-growing waters. While these coliform standards are generally effective in blocking feces-contaminated shellfish from the marketplace, these standards offer no indication of viral contamination that may persist for a month or longer within shellfish or estuarine sediments after coliform bacterial counts have returned to acceptable levels (9). Furthermore, point source discharge of human waste from commercial and recreational vessels can result in viral contamination of approved shellfish beds without observation of increases in fecal coliform counts in marine water samples (4, 19).

There is a clear need for a practical test for viral contamination of shellfish. Unfortunately, wild-type HAV strains are difficult to propagate (often without apparent cytopathic effects) and methods for NLV propagation in vitro are unknown. Consequently, reverse transcription (RT)-PCR-based detection of viral nucleic acid represents the quickest and most practical means of detecting NLV and HAV within shellfish tissues. Although seemingly straightforward, successful RT-PCRs from samples derived from shellfish present formidable challenges, which prevent direct testing as a practical means of preventing shellfish-borne viral illness. These difficulties have been attributed to the presence of humic substances, large amounts of glycogen, and the properties of shellfish extracts (2, 17, 24, 37).

Current methods described for the extraction of enteroviruses from shellfish samples are cumbersome, often requiring several days to perform and involving many steps, including multiple polyethylene glycol 8000 (PEG) precipitations, pH changes, flocculant applications, and the use of Freon (trichlorotrifluoroethane) (2, 3, 8, 11, 12, 20, 21, 23, 36). Although the average infectious dose of Norwalk virus (NV) or HAV is not known, it may be less than 100 virions (6). Therefore, an effective testing method needs to successfully extract and detect limited quantities of virus. In this report, we describe a rapid 1-day) extraction-and-detection procedure which results in efficient RT-PCR amplification of limited quantities of HAV and NV in shellfish extracts. This glycine–PEG–Tri-reagent–poly(dT) extraction method (GPTT method) readily extracts viruses in samples derived from virus-seeded shellfish homogenates, from live shellfish exposed to virus-contaminated seawater, and in wild shellfish implicated in an outbreak of food-borne viral illness.

MATERIALS AND METHODS

Virus stocks and titration. NV strain 8FIIa (18) was obtained from human stool produced during a volunteer study involving NV. A virus stock was produced by diluting the stool 10-fold in Dulbecco's minimum essential medium (Gibco BRL, Gaithersburg, Md.), centrifuging it at 16,200 × *g* for 20 min, and

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serially filtering it through Millex 0.45-μm (HV) and 0.1-μm (VV) low-protein-binding filters (Millipore Corp., Bedford, Mass.). One-milliliter aliquots were frozen at −80°C.

HAV was obtained from the American Type Culture Collection as VR-1402, a cell culture-adapted, cytopathic clone of strain HM-175 that was originally designated HM-175/18f(22). This clone produces readily visible plaques in fetal rhesus monkey kidney cells (FRhK-4). The HAV stock was titered by plaque assay as described by Richards and Watson (31). Plaques were enumerated for each dilution in duplicate. Three independent trials yielded an average virus titer of 9×10^6 PFU/ml. By adapting the methods of Reed and Muench (30), an RT-PCR 50% end point ($RT\text{-}PCR_{50}$) was determined for HAV and NV using serial 10-fold dilutions of virus stocks, the Qiagen one-step RT-PCR kit, and primer sets 2949-3192 for HAV and M5-M3 for NV (primers are described below). Three independent serial dilutions were made in RNase-free H_2O, and three RT-PCR samples were assayed per dilution. For RT-PCR of HAV, RT was done at 50°C for 30 min, Taq activation for 15 min was done at 95°C, and 40 cycles of annealing at 60°C for 1 min, extension at 72°C for 1 min, and denaturation at 95°C for 30 s were performed. The final cycle was 2 min of annealing at 60°C and a 10-min extension at 72°C. For NV, the same conditions were used, except that the PCR annealing temperature was 56°C.

Shellfish. All of the live and shucked oysters (*Crassostrea virginica*) and live clams (*Mercenaria mercenaria*) tested were obtained from local seafood markets. Live oysters were contaminated by exposure to water containing HAV. Individual oysters, observed to be pumping, were placed in separate 10-gallon aquaria containing 20 liters of natural seawater at room temperature within a large, custom-designed biohood. Nine thousand PFU of HAV was mixed into each tank. The individual oysters were removed from the virus-contaminated water after 16 h, shucked, and frozen at −80°C until analyzed. Imported clams, believed to be Manila clams (*Ruditapes philippinarum*), were provided by Jerold Mulnick and Richard Manney (U.S. Food and Drug Administration, import alert 16–50). These clams were imported from China and were implicated in an outbreak of viral illness in New York State. They were packaged as cooked clams frozen on the half shell, although they appeared raw.

Virus extraction and concentration. Oyster and clam homogenates were prepared for seeding with NV and HAV by using approximately 25 g of shucked, frozen shellfish stored at −80°C. After thawing, shellfish were blended with 175 ml of glycine buffer, pH 9.5 (0.1 M glycine, 0.3 M NaCl), at 20°C by using a laboratory blender (model 31BL91; Waring, New Hartford, Conn.) at the high setting for 3 min. Thirty milliliters of shellfish extract was seeded with serial 10-fold dilutions of virus ranging from 15 to 0.15 PFU for HAV and 224,000 to 22.4 $RT\text{-}PCR_{50}$ units of NV. The seeded extract was then incubated for 30 min at 37°C and clarified by centrifugation at $15{,}000 \times g$ at 4°C. Viral particles were precipitated from the supernatant by using an equal volume of 16% PEG (Sigma Chemical Co., St. Louis, Mo.) with 0.525 M NaCl. After precipitation for 1 h on ice, samples were centrifuged at $10{,}000 \times g$ for 5 min at 4°C.

For extraction and concentration of virus from live, artificially contaminated oysters, single oysters (approximate volume of 10 ml) were blended in 90 ml of glycine buffer. Thirty milliliters of extract was then clarified by centrifugation and PEG precipitated as described above.

Isolation of viral RNA. After PEG precipitation, the pellet was resuspended in 5 ml of Tri-reagent (Sigma) by vigorous vortex mixing and repipetting. After a 5-min incubation at 20°C, each sample was transferred to a 15-ml polypropylene centrifuge tube and 1.2 ml of chloroform was added. Samples were vigorously vortexed for 30 s and then incubated at room temperature for 5 min. Samples were centrifuged at $12{,}000 \times g$ for 5 min. The top aqueous layer, containing the RNA, was precipitated by addition of 0.5 volume (approximately 2.5 ml) of isopropanol for 5 min at 20°C, followed by centrifuging at $5{,}000 \times g$ for 5 min. The resulting white pellets were washed with cold 75% ethanol, and each pellet was then resuspended in 300 μl of RNase-free water. To facilitate rapid resuspension, samples were heated to 90°C and vortexed. Four hundred microliters of 1 × RNA binding buffer (20 mM Tris-HCl [pH 7.5], 1.0 M LiCl, 2 mM EDTA) was added, and the samples were subjected to vortexing for 30 s, followed by heating to 65°C for 3 min and addition of 100 μl of Dynabeads-oligo$(dT)_{25}$ (Dynal, Oslo, Norway). Samples were rocked gently for 30 s and placed in a magnetic bead attractor (Stratagene, La Jolla, Calif.) for 1 min. The supernatant was removed and discarded. The magnetic beads (pellet) containing the viral RNA were washed by resuspension with 500 μl of 2× RNA binding buffer and rotated at 8 rpm (model 4152110; Barnstead/Thermolyne, Dubuque, Iowa) for 5 min at room temperature. Tubes were placed on the magnetic bead attractor for 1 min, and then the supernatant was removed and the tube contents were resuspended in washing buffer (10 mM Tris-HCl [pH 7.5], 0.15 M LiCl, 1 mM EDTA). This process was repeated three times. Samples were then resuspended in 100 μl of RNase-free H_2O and heated to 90°C for 2 min to liberate the viral RNA from the Dynabeads, followed by magnetic extraction to pellet the Dynabeads. RT-PCR was performed with 10-μl aliquots of the eluate.

Primers and RT-PCR. RT-PCR was performed on shellfish extracts by using gene-specific primers and the one-step RT-PCR kit from Qiagen (Valencia, Calif.) in accordance with the procedures recommended by the manufacturer with 10 U of cloned RNase inhibitor (Gibco-BRL). This kit utilizes a proprietary buffer, two reverse transcriptases, and a hot-start *Taq* polymerase. For HAV, primers originally described by Robertson et al. (32) and Normann et al. (29), (+)2949 5′ TATTTGTCTGTCACAGAACAATCAG 3′ and (−) 3192 5′ AGG AGGTGGAAGCACTTCATTTGA 3′, were used at a final concentration of 0.1 μg/50-μl sample or approximately 0.25 μM for each primer. RT-PCR was performed at 50°C for 30 min, followed by a 15-min *Taq* activation step at 95°C. Forty cycles were performed by using a 60°C annealing temperature for 1 min, 1 min of extension at 72°C, and 30 s of denaturation at 95°C. For the final cycle, the annealing time was extended to 2 min and the final extension was performed for 10 min. A 267-bp amplicon was sequenced and confirmed to encode a portion of the HAV genome.

To verify the positive HAV test for imported Chinese clams seized in an outbreak, nested primers (dkA24 [5′ CTTCCTGAGCATACTTGAGTC 3′] and dkA25 [5′ CCAGAGCTCCATTGAACTC 3′] were designed by using the amplicon sequence generated with +2949 and −3192. These nested primers generate a 200-bp amplicon. Previously amplified sequences were diluted 1/10,000 and reamplified by using the dkA24 and dkA25 primers at a concentration of 0.1 μg/50-μl reaction mixture or 0.3 μM each, the Qiagen one-step RT-PCR kit, and an initial *Taq* activation step of 15 min at 95°C, followed by 40 cycles of annealing at 50°C for 1 min, extension for 1 min at 72°C, and denaturation at 95°C for 30s.

For NV strain 8FIIa, primers M5 (5′ CACCACCATAAACAGGCTG 3′) and M3 (5′ AGCCTGATAGAGCATTCTTT 3′), originally described by Matsui et al. (27), were used at a concentration of 0.1 μg/50-μl reaction mixture or approximately 0.3 μM for each primer. Touchdown RT-PCR (15) was performed as follows: RT at 50°C for 30 min, followed by PCR with 3 initial annealing cycles at 60°C and then reducing the annealing temperature by 0.5°C increments every 3 cycles to 56°C, which was used for the final 28 cycles. Extension reactions were performed for 1 min at 72°C, and denaturation cycles were at 95°C for 30 s. Primer pair M3-M5 produces a 224-bp amplicon. Sequence analysis confirmed that this amplicon encoded portions of NV strain 8FIIa.

Negative RT-PCR controls were performed by using (i) eluate extracted from uncontaminated oysters and (ii) RT-PCR cocktails with RNase-free H_2O in place of eluate. Positive RT-PCR controls were performed by using 1 μl of HAV stock, 10 U of cloned RNase inhibitor, 8 μl of RNase-free H_2O, or 9 μl of NV stool filtrate with 1 μl (10 U) of RNase inhibitor, followed by heating to 99°C for 5 min to release the viral RNA from its capsid (33). All of the primers used were synthesized by Midland Certified Reagent Co. (Midland, Tex.). After the RT-PCRs, amplified nucleic acids were visualized by polyacrylamide gel electophoresis (PAGE) using 4 to 20% gradient gels (Bio-Rad, Hercules, Calif.) and ethidium bromide staining.

Testing of Chinese clams. The GPTT procedure was modified for testing of imported Chinese clams implicated in an outbreak of viral illness. Modification was necessary because of the small amount of virus present. One dozen frozen, uncooked clams on the half shell were mixed with 100 ml of glycine buffer at 37°C for 20 min to facilitate thawing. After removal of shells, meats were blended and incubated at 37°C as described previously. Six aliquots of approximately 40 ml each were pelleted at $15{,}000 \times g$ at 4°C. Supernatants were divided into equal parts, and PEG precipitation was performed by using an equal volume of 16% PEG with 0.525 M NaCl. After PEG precipitation for 1 h on ice, viral particles were concentrated by centrifugation at $10{,}000 \times g$ for 5 min at 4°C. The resulting 12 individual PEG pellets were resuspended in 2.5 ml of Tri-reagent by vigorous vortex mixing and repipeting. After a 5-min incubation at room temperature, four pellets resuspended in Tri-reagent were combined into three tubes and 2 ml of chloroform was added to each of the three combined tubes. Samples were vigorously vortexed for 30 s, followed by incubation at 20°C for 5 min. The Tri-reagent–chloroform mixture was partitioned by centrifugation at $12{,}000 \times g$ for 5 min. The top aqueous layer, containing viral and oyster RNAs, was precipitated by the addition of 0.5 volume (approximately 5 ml) of isopropanol and incubation for 5 min at 20°C, and the RNA was pelleted by centrifuging at $3{,}000 \times g$ for 15 min. Pellets were washed with cold 75% ethanol, and each tube was resuspended in 600 μl of RNase-free water. To facilitate rapid resuspension, samples were heated to 90°C prior to resuspension. Eight hundred microliters of RNA binding buffer was added to each of the three aliquots, and the mixture was transferred to microcentrifuge tubes. Samples were vortexed for 30 s and then heated to 65°C for 3 min. One hundred microliters of Dynabeads-oligo$(dT)_{25}$ was added and mixed with the RNA extract. Samples were rocked gently for 30 s and placed in a magnetic extractor for 1 min. The supernatant was removed and

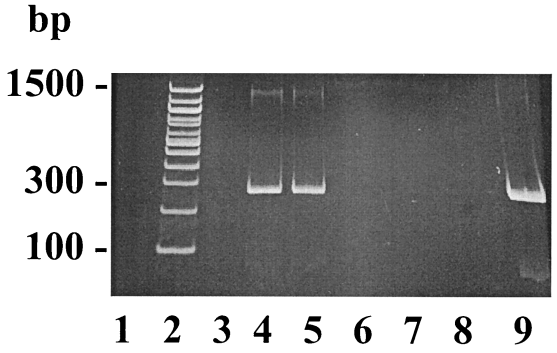

FIG. 1. Eastern oyster extract and HAV. Thirty milliliters of oyster tissue homogenate was prepared as described in Materials and Methods. The homogenate was seeded with dilutions of HAV. Viral RNA was extracted by the GPTT procedure, followed by one-step RT-PCR and PAGE analysis of 10% of the total RNA extracted. Lanes: 1, RNase-free H_2O substituted for oyster extract (negative control); 2, 100-bp molecular size ladder; 3, blank; 4, 1 μl of a 1:600 dilution of HAV (15 PFU); 5, 1 μl of a 1:6,000 dilution of HAV (1.5 pfu); 6, 1 μl of a 1:60,000 dilution of HAV (0.15 PFU); 7, 1 μl of a 1:600,000 dilution of HAV (0.015 PFU); 8, nonseeded oyster RNA extract; 9, 99°C-denatured HAV (positive control).

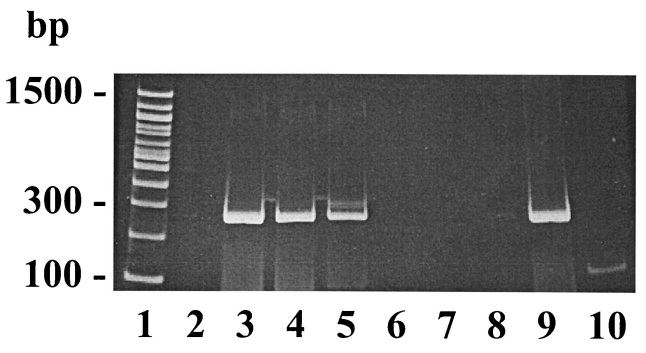

FIG. 2. Hard-shell clam extract and HAV. Thirty milliliters of clam homogenate was prepared as described in Materials and Methods. The homogenate was seeded with dilutions of HAV. Viral RNA was isolated by GPTT extraction, followed by one-step RT-PCR and PAGE analysis of 10% of the total extracted RNA. Lanes 1, 100-bp molecular size ladder; 2, blank; 3, 1 μl of a 1:600 dilution of HAV (15 PFU); 4, 1 μl of a 1:6,000 dilution of HAV (1.5 PFU); 5, 1 μl of a 1:60,000 dilution of HAV (0.15 PFU); 6, 1 μl of a 1:600,000 dilution of HAV (0.015 PFU); 7, 1 μl of a 1:6,000,000 dilution (0.0015 PFU) of HAV; 8, blank; 9, 99°C-denatured HAV (positive control); 10, nonseeded RNA extracted from clams.

discarded. The magnetic beads (pellet) containing the viral RNA were washed with 500 μl of 2× binding buffer and rotated for 5 min at 20°C. Tubes were placed on the magnetic extractor for 1 min; this was followed by removal of the supernatant and rinsing with wash buffer. This process was repeated three times. Samples were then resuspended in 33 μl of RNase-free H_2O, and the three samples were pooled. The combined sample was then heated to 90°C for 2 min to liberate the viral RNA; this was followed by magnetic extraction to pellet the Dynabeads. RT-PCR was performed by using 10 μl of this sample, HAV primers (+)2949 and (−)3192, and nested primers dkA24 and dkA25. Samples positive by RT-PCR and nested PCR were sequenced for absolute confirmation of HAV and compared with currently used laboratory strains to preclude sample contamination by laboratory strains.

RESULTS

Seeded shellfish extracts. Oyster and clam homogenates were seeded with dilutions of HAV or NV and subjected to GPTT extraction. Results for HAV-seeded oysters and clams are shown in Fig. 1 and 2. For oyster extracts, RNA purified from homogenate seeded with 1 μl of a 1:6,000-diluted virus stock gave a positive RT-PCR product (Fig. 1) corresponding to approximately 1.5 PFU of HAV. One-$\log_{10}$ higher sensitivities were occasionally observed in oysters (data not shown). For clam extract, RNA purified from homogenate seeded with 1 μl of a 1:60,000 dilution gave a positive RT-PCR result (Fig. 2). This corresponds to approximately 0.15 PFU. Since 1/10 of the RNA extracted from these samples (10 of 100 μl) was tested, specific tests actually detect the equivalent of 0.15 PFU of viral RNA extracted from seeded oyster extract and 0.015 PFU for seeded clam extract, respectively.

For NV, determination of PFU is not possible since the virus has not been successfully cultured. However, testing of seeded oyster extracts with as little as 0.001 μl or 224 RT-PCR_{50} units of NV stock ($10^{8.35}$ RT-PCR_{50} units/ml) resulted in a positive test when a touchdown PCR procedure was used (Fig. 3). Since 1/10 of the NV RNA extract was tested, this assay detected approximately 22.4 RT-PCR_{50} units.

Live, artificially contaminated oysters. To demonstrate that the extraction method could be performed on live, contaminated shellfish, individual oysters were exposed to 9,000 PFU of HAV and shucked, and viral RNA was extracted as described previously. Serial 10 fold dilutions of oyster extracts containing HAV RNA were evaluated. One microliter of the 100-μl RNA extract tested positive for HAV (Fig. 4). It was not possible to directly determine the fractional amount of HAV taken up by the artificially contaminated oyster. However, if it is assumed that all of the virus added to 20 liters of seawater was ingested by the oyster, then a minimum sensitivity of approximately 27 PFU or 1,500 RT-PCR_{50} units of HAV was observed. This value was derived by extracting 30 of 100 ml

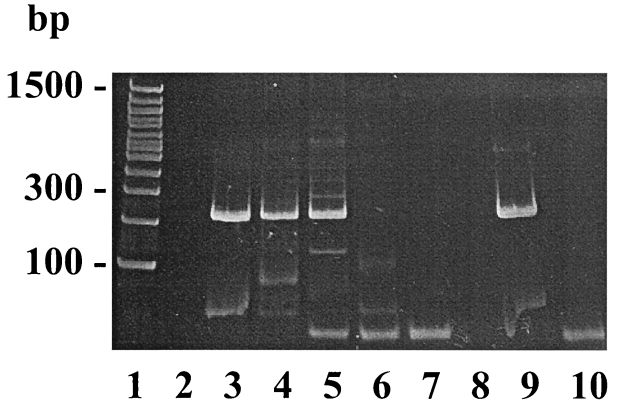

FIG. 3. Eastern oyster extract and NV. Thirty milliliters of oyster homogenate was prepared as described in Materials and Methods. The homogenate was seeded with dilutions of NV, and viral RNA was then extracted by the GPTT procedure, followed by one-step touchdown RT-PCR and PAGE analysis of 10% of the total extracted RNA. Lanes 1, 100-bp molecular size ladder; 2, blank; 3, 1 μl of undiluted NV (224,000 RT-PCR_{50} units); 4, 1 μl of 1:100-diluted NV (2,240 RT-PCR_{50} units); 5, 1 μl of 1:1,000-diluted NV (224 RT-PCR_{50} units); 6, 1 μl of 1:10,000-diluted NV (22.4 RT-PCR_{50} units); 7, RNA extracted from nonseeded oysters; 8, blank; 9, 99°C-denatured NV (positive control); 10, RNase-free H_2O substituted for oyster extract (negative control).

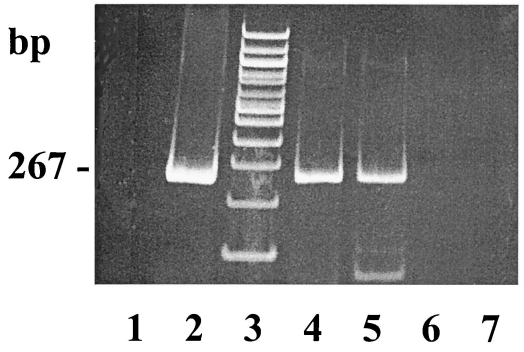

FIG. 4. Live Eastern oysters contaminated with HAV. A live oyster was artificially contaminated with HAV in the laboratory, shucked, and homogenized in glycine buffer. Thirty milliliters (30% of the homogenate) was used to extract viral RNA by the GPTT procedure, followed by one-step RT-PCR and PAGE (4 to 20% gradient) analysis. Lanes 1, RNase-free H_2O substituted for oyster extract (negative control); 2, 99°C-denatured HAV (positive control); 3, 100-bp molecular size ladder; 4, RT-PCR performed with 10 μl (10% of the total) of RNA extracted from an HAV-contaminated oyster; 5, RT-PCR performed with 1 μl (1% of the total) of RNA extracted from an HAV-contaminated oyster; 6, RT-PCR performed with 1 μl of a 1:10 dilution of RNA extracted from an HAV-contaminated oyster; 7, RT-PCR performed with 1 μl of a 1:100 dilution of RNA extracted from an HAV-contaminated oyster. Results were similar for two additional individually processed oyster extracts.

of oyster homogenate and obtaining positive RT-PCR results for 1% (1 of 100 μl) of the purified RNA.

Testing Chinese clams. Clams imported from China and subsequently served at a restaurant in New York State were implicated as the potential vector for viral illnesses. These clams were tested for the presence of HAV RNA by using an adaptation of the RNA extraction method. Initial attempts, using six clams blended in 175 ml of glycine buffer (pH 9.5), followed by extraction of viral RNA from 30 ml of this extract, were unsuccessful. However, when six individual clams were blended in a total volume of 200 ml of glycine buffer and the entire volume was extracted and combined in a final volume of 100 μl, RT-PCR analysis produced a faint positive band corresponding to the appropriate molecular weight (data not shown). Subsequently, 12 clams were homogenized and the entire blended sample was extracted and combined in a final volume of 100 μl. Testing of 10 μl of extracted HAV RNA by RT-PCR gave a strong amplified band at 267 bp consistent with HAV (Fig. 5). This result was confirmed by using nested PCR primers dkA24 and dkA25, which amplified a 200-bp band. This amplicon was sequenced and further confirmed as a strain of HAV which differed from all of the other HAV strains used within our laboratory.

DISCUSSION

Numerous methods of virus RNA extraction from shellfish and detection have been described (1–3, 7, 8, 10, 11, 13, 21, 26, 34–37). Many of these extraction procedures can no longer be performed since Freon has been deemed environmentally unsafe and is no longer manufactured. Only one of these procedures requires less than 24 h to perform (35). Using gene-specific primers for HAV and NV, we demonstrated rapid, efficient RNA extraction and one-step RT-PCR detection of theses viruses in bivalve shellfish. This procedure has been successfully used with Eastern oysters and hardshell clams and adapted for imported Chinese clams. This method facilitates the detection of both HAV and NV RNAs extracted from virus-seeded homogenized shellfish or from live, artificially and naturally contaminated oysters and clams. The total time required to perform the GPTT extraction procedure is approximately 6 to 8 h. When coupled with one-step RT-PCR, an additional 3.5 h is required to perform RT-PCR, followed by 2 h for product analysis by PAGE. This preparation scheme involves the use of commercially available reagents and common laboratory chemicals, such as Dynabeads and Tri-reagent, and does not require specific antibodies, the use of Freon, or expensive instrumentation, other than a centrifuge and a thermocycler. Therefore, this test could be easily implemented by industry and regulatory agencies. Use of a one-step RT-PCR, rather than separate RT and PCR, reduces the handling time required and the risk of potential contamination or pipetting errors compared to multistep protocols.

The GPTT extraction procedure involves homogenization of shellfish tissues in glycine-NaCl buffer at pH 9.5 to elute viruses from the solids. Following clarification by centrifugation, the supernatant is heated at 37°C for 30 min. We found that incorporation of this step reduced the incidence of spurious priming with the HAV and NV primers (data not shown). We suspect that this is due to enzymatic digestion of oyster RNA with endogenous RNases liberated upon tissue homogenization and that viral RNA encased within viral capsids is protected from these RNases. This 30-min digestion results in significantly smaller pellets after Tri-reagent treatment and subsequent isopropanol precipitation. In the interest of developing a rapid test, a 1-h PEG precipitation was determined to be sufficient for virus recovery. Subsequently, extraction with

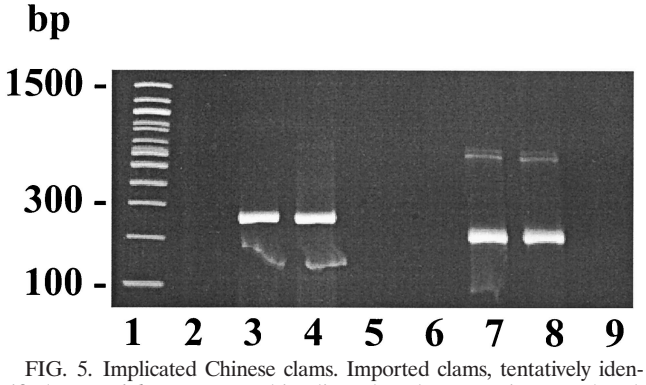

FIG. 5. Implicated Chinese clams. Imported clams, tentatively identified as *R. philippinarum* and implicated as the vector in an outbreak of viral illness, were tested for the presence of HAV. Twelve clams were homogenized in glycine buffer, and viral RNA was extracted from the entire sample as described in Materials and Methods. One-step RT-PCR and PAGE analysis were performed by using 10% of the total RNA extracted. Nested PCR was performed as described in Materials and Methods. Lanes 1, 100-bp molecular size ladder; 2, blank; 3, RT-PCR on RNA extract from the implicated clams; 4, 99°C-denatured HAV (positive control); 5, RNase-free H_2O substituted for oyster extract (negative control); 6, blank; 7, nested PCR of imported clams (performed on the RT-PCR sample shown in lane 3); 8, nested PCR of 99°C-denatured HAV (performed on the RT-PCR sample shown in lane 4); 9, RNase-free H_2O substituted for oyster extract (negative control).

Tri-reagent, a mixture of guanidinium isothiocyanate, phenol, and chloroform (5), was used to directly purify oyster RNA and simultaneously lyse the viral capsids to release viral RNA. After RNA purification by Tri-reagent and isopropanol precipitation, the pellet is dissolved in RNase-free H_2O. We found this difficult to perform at room temperature; however, when the pellet-H_2O mixture is heated to 90°C, the pellet dissolves after a few minutes of vortex mixing. Use of the poly(dT) magnetic beads was incorporated to facilitate improved viral RNA purification and to further ensure the removal of RT-PCR inhibitors. The use of poly(dT) magnetic beads should be applicable to all enterically transmitted members of the *Picornavirus* family (HAV, poliovirus, and coxsackievirus), the *Calicivirus* family (genogroup I and II NLVs and genogroup III Sapporo virus strains), and astroviruses, as well as hepatitis E strains, since these viral genomes are all composed of single-stranded RNA with poly(A) tails.

We view the GPTT method as superior to other methods based on expedience and sensitivity. For HAV, detection of 0.015 and 0.15 PFU equivalents of viral RNA by using 10% of the total RNA extracted from 30 ml (approximately 3.75 g of shellfish tissue) of clam and oyster homogenate is more sensitive than most currently published tests. For example, Atmar et al. (3) reported the detection of 100 PFU of HAV seeded in 1.5 g of stomach and digestive diverticulum extract. Cromeans et al. (7) reported the detection of 8 PFU of HAV per g of oyster meat. Using immunomagnetic capture, Lopez-Sabater et al. (26) detected as little as 10 PFU of HAV in 20 g of oyster meat. Sunen and Sobsey (37), using a clam extraction procedure involving the use of guanidinium isothiocyanate extraction and immunomagnetic capture, reported detecting less than 10 PFU of HAV. Dix and Jaykus (8) reported the detection of 10^3 PFU for HAV and 450 RT-PCR units of NV from 50 g of clams. However, our method may not be as sensitive as that of Goswami et al. (10), who detected 400 HAV RNA particles by using random primed RT-PCR and reported achieving a sensitivity of as little as 10 viral RNA molecules by using oligo(dT) primer for RT.

Generally speaking, propagation of HAV is difficult, making quantitation and direct comparison of sensitivities based on PFU counts somewhat problematic. The particle-to-PFU ratios of in vitro-propagated HAV stocks have been estimated to be as high as 1,000 particles per PFU (38). Consequently, it is not surprising that we obtained sensitivities of less than 1 PFU/25-g sample of shellfish tested. The RT-PCR_{50} of our HAV stock was $10^{8.7}$/ml, while the average HAV titer was 9×10^6/ml. The resulting ratio is 55.7 RT-PCR_{50} units/PFU. Although the number of HAV RNA molecules required to give a positive RT-PCR amplification is not known, it is evident that our HAV stock has a particle-to-PFU ratio of at least 40, even if only a single RNA molecule were required for successful RT-PCR amplification.

Quantification of viral contamination in live shellfish is more difficult. However, for testing of oysters contaminated by exposure in 20 liters of seawater, we identified a sensitivity of at least 27 PFU/oyster if all (100%) of the input HAV (9×10^3 PFU) was ingested after a 16-h exposure. For testing of seized imported clams, the amount of HAV present was unknown. Testing of single clams or fractions of the total clam homogenate did not result in a positive test. When 12 clams were tested and all of the homogenate was extracted and pooled, a positive test resulted, directly demonstrating the potential utility of this method.

Direct comparisons of NV detection sensitivity are more difficult, since this virus has not been successfully propagated in vitro and RT-PCR procedures, enzymes, and quantitiation methods are variable. However, we view our seeded-shellfish sensitivities of 22.4 RT-PCR_{50} units per 30 ml of homogenized oysters (3.75 g) as comparable to those reported by others. For example, Dix and Jaykus (8) reported the detection of 450 RT-PCR units of NV from 50 g of clams. Using a seminested RT-PCR procedure, Häflinger et al. (13) detected approximately 33 RT-PCR units from mussels and 3,300 RT-PCR units from oysters. A test by Gouvea et al. (11) recognized 20 to 200 virions of NV strain 8FIIa based on 10^5 to 10^6 particles/ml reported by electron microscopy. However, it is conceivable that this test underestimated the number of particles present, since we found that our NV-containing stool has an apparent titer of approximately $10^{9.35}$ RT-PCR_{50} units/ml. The NV stock was prepared by 1:10 dilution of stool in Dulbecco's minimal essential medium and successively filtered by using 0.45- and 0.1-μm-pore-size filters; the RT-PCR_{50} of this stock was $10^{8.35}$/ml. Our observations that NV stocks may be higher than 10^5 to 10^6 virions/ml are not unique, since an NV stool titer as high as 10^9 RT-PCR units/ml has been reported previously (13).

The challenges in developing a broadly applicable test for detection of food-borne viruses in shellfish are primarily twofold. The first challenge is the ability to rapidly and efficiently extract and purify RNA that is free of RT-PCR inhibitors from shellfish tissues. Our procedure is less labor intensive than other techniques and should permit a single laboratory worker to test 12 shellfish samples in a day. The second challenge is identification of primer sets which are broadly reactive with different viral strains yet do not produce spurious amplified sequences. We have not assessed the inclusivity of our RT-PCR test or to what degree it will detect wild-type field strains likely to contaminate shellfish. The HAV strains have less variable nucleotide sequences than NLV strains. All known HAV strains have the same serotype, and U.S. strains vary by approximately 7.5% at the nucleotide level, with a somewhat greater variability of approximately 15% observed worldwide (32). Therefore, it is conceivable that this test with this primer set will recognize the majority of wild-type HAV strains. However, it is doubtful that the NV primer set M3-M5 will recognize different genogroup I and II NLVs, since these are known to have highly variable genomic sequences.

Some tests utilize extraction of dissected digestive diverticula and shellfish stomachs rather that whole shellfish (2, 8, 21). Ostensibly, this enhanced sensitivity is due to the presence of less RT-PCR inhibitors and an elevated virus concentration. However, a comprehensive understanding of the mechanisms of viral persistence and distribution within shellfish is lacking. The GPTT method uses whole shellfish and concentrates and purifies viral RNA without RT-PCR inhibitors or the need for shellfish dissection.

GPTT RNA extraction provides a convenient, relatively fast, and simple means of extracting HAV and NV from shellfish tissues. It provides RNA relatively free from RT-PCR inhibitors. Additional studies are needed to assess the effectiveness

of this method with shellfish taken from other geographic regions and during different seasons. The validation of this method by other laboratories is necessary and should include a continued assessment of the sensitivity and reproducibility of the technique and an evaluation of the frequency of false-positive and -negative RT-PCR results when it is used for routine testing. The development of primers with enhanced inclusivity and specificity for detecting a broad range of enteric viruses is needed to simplify virus screening efforts for shellfish and other foods.

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