A Sensitive and Reliable Reverse Transcriptase PCR–Enzyme-Linked Immunosorbent Assay for the Detection of Human Pathogenic Viruses in Bivalve Molluscs

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ABSTRACT

A colorimetric method, reverse transcriptase PCR with an enzyme-linked immunosorbent assay (RT-PCR-ELISA) was evaluated for ease of use, reliability, and sensitivity when detecting known human pathogenic virus present in shellfish, using a traditional polyethylene precipitation or immunocapture virus concentration method. The newly developed ELISA method could successfully detect enteroviruses and noroviruses in artificially and naturally contaminated shellfish. Overall, ELISA was shown to be a robust and sensitive method, which had a detection limit of 10 to 100 50% tissue culture infective dose enterovirus per gram of Crassostrea gigas (Pacific oyster) digestive gland and whole Mytilus edulis (common blue mussel). The technique was easily established in a new laboratory and required no specialized equipment. The method had a high sample throughput capable of screening 96 samples per run, making the technique extremely time efficient. RT-PCR-ELISA is a safe, quick, reliable technique, which has the potential for use as a standard virus detection method.

Edible bivalve molluscs have long been associated with bacterial and viral disease outbreaks. During the late 19th and early 20th centuries, the association between the two became well documented in several countries, with numerous cases of illness attributable to the consumption of contaminated shellfish (2, 12, 28). Through their filter-feeding process, bivalve molluscs can retain and concentrate human pathogens, which may be present in their natural habitat due to direct or indirect sewage contamination. Traditionally, these shellfish species, including oysters, mussels, and clams, are consumed raw or lightly cooked. This, together with the fact that the shellfish are consumed whole, can make the consumption of bivalve molluscs a potential hazard to public health.

In 1925, the National Shellfish Sanitation program was developed in the United States, with the intention of preventing shellfish associated disease caused, primarily, by enteric bacteria (10). Bacterial measures, such as the total and fecal coliform count, were introduced as indicators of sewage contamination, and this led to the establishment of bacteriological standards for shellfish and their harvesting waters, and reinforced the need for alternative indicator organisms and improved virus detection methods (11).

Recently, research has focused on the development of reverse transcriptase PCR (RT-PCR)–based methods for detecting hepatitis A and noroviruses (NoVs) in shellfish; however, inhibitory substances in the sample can interfere with nucleic acid polymerase enzymes. Inhibitors, in feces, sewage, and shellfish (which present a particular problem), include humic acid, metal ions, polysaccharides, and glycogen (3–5, 20, 32, 44). In an attempt to overcome this problem of PCR inhibition, research has focused on improving virus extraction–concentration and nucleic acid purification methods.

Immunocapture (IC) RT-PCR has become a promising technique for isolating and concentrating viruses in clinical and environmental samples. IC methods use antibodies to isolate, concentrate, and purify intact viral particles from a sample for subsequent RNA extraction and RT-PCR detection (9, 13, 19, 27, 34, 42, 45, 47). More recently, research has focused on the development of real-time PCR methods, including the incorporation of SYBR green and dual-labeled probes into PCR products and TaqMan to detect virus in various sample matrices (6, 17, 21–23, 26, 32, 36, 37, 41, 45). Sensitive real-time methods have been used successfully to detect NoVs in clinical specimens; however, detection in shellfish has been limited, with only four studies to date reporting successful detection (21, 33, 36, 37). Despite the development of several relatively sensitive detection methods (1, 4, 5, 14, 20, 24, 25, 31, 39, 43), a standard virus-detection method has yet to be established.

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Such a method would need to be sensitive, reliable, easy to perform, and technically feasible in the standard laboratory.

Using poliovirus as a model, we have developed a colorimetric method, RT-PCR with an enzyme-linked immunosorbent assay (ELISA), termed RT-PCR-ELISA, which has been used successfully to detect nucleic acid amplification. Nucleic acid sequences, labeled during PCR amplification, are detected using an ELISA format, combining the sensitivity of PCR signal amplification with the specificity of ELISA antibody detection. To achieve detection of PCR products by ELISA, digoxigenin (DIG)-dUTP was randomly incorporated into the nucleic acid segment during amplification. Through biotinylation of one PCR primer, resulting biotinylated, DIG-dUTP-labeled products can be captured on streptavidin-coated wells of a microtiter plate and undergo subsequent immunoenzyme detection. Using poliovirus-spiked shellfish tissue, the sensitivity of a typical polyethylene glycol (PEG) precipitation method for virus isolation and concentration was evaluated using RT-PCR-ELISA, and compared with an IC procedure (IC-RT-PCR-ELISA). The RT-PCR-ELISA was then refined to allow norovirus detection in naturally contaminated samples.

MATERIALS AND METHODS

Virus strain. The Hep-2C cell line was used to grow the poliovirus type I Sabin strain (PV I), kindly provided by Dr. G. Clements, Regional Virus Laboratory, Gartnavel General Hospital, Glasgow, UK. The cells were grown in 75-cm² tissue culture flasks containing Dulbecco’s minimal essential medium supplemented with 10% newborn calf serum and 1% penicillin-streptomycin, and were incubated at 37°C in an atmosphere of 5% (vol/vol) CO₂. Virus stocks were prepared by inoculating subconfluent Hep-2C monolayers with original virus stock. After a 30-min adsorption period at room temperature, the virus-infected monolayers were overlaid with 2% serum and Dulbecco’s minimal essential medium. When complete virus-induced cytopathic effect was observed (usually after 2 to 3 days incubation), the cells and medium were frozen at −70°C. After thawing the medium was removed from the flasks and centrifuged at ~180 × g for 5 min. Virus stocks were then prepared by dispensing 1 ml of supernatant into vials and storing at −70°C. Virus stock titers were determined at the start and end of the study by microtitration assay, using the Karber formula (15).

Serial 10-fold dilutions of PV I stocks, ranging from 1 × 10⁶ to ten 50% tissue culture infective dose (TCID₅₀), were prepared in phosphate-buffered saline (PBS) and used to artificially contaminate oyster and mussel samples. Stool specimens from sporadic outbreaks of gastroenteritis were used as positive controls for RT-PCR amplification. Samples had previously been well characterized and shown to contain either enteroviruses (EVs) or NoVs by electron microscopy and RT-PCR. After positive identification of NoVs in naturally contaminated shellfish samples, results were confirmed by sequencing.

Shellfish samples. Seven oysters samples (C. gigas) and 11 mussel samples (M. edulis) were obtained from the Fisheries Research Services, Aberdeen, Scotland, and depurated in artificial seawater, at 4°C for 1 week prior to use. A static-based depuration system was used. Seawater was changed after 24 h, then every 48 h thereafter.

Shellfish were scrubbed under clean, running water and opened with a sterile shucking knife. For whole-shellfish samples, shellfish flesh and liquor from approximately 10 oysters or approximately 15 mussels were collected. In addition, the digestive glands were dissected from approximately 15 oysters. Shellfish samples were homogenized at ~80°C.

Artificial contamination and virus elution. One-gram aliquots of oyster and mussel homogenate were seeded with 150 µl of 10-fold poliovirus dilutions ranging from 1 × 10⁰ to 10 TCID₅₀. After 15 min of incubation at room temperature, a four-fold volume of 50 mM glycine buffer (pH 9.5) was added to each sample. Samples were shaken at room temperature for 30 min and then centrifuged at 10,000 × g at 4°C for 15 min. Viruses were concentrated from samples, using either a modified PEG precipitation method (16), or an IC method, as described.

Virus concentration and RNA purification. Samples were processed using a modified version of the Häfliger et al. method (16), PEG 6000, as a 50% (wt/vol) solution in PBS, was added to the supernatant to a final concentration of 8%, and the sample mixed for a minimum of 1 h at 4°C. Precipitated virus was pelleted by centrifugation at 15,600 × g at 4°C for 20 min, and the PEG pellet resuspended in 400 µl of 5 M guanidine hydrochloride. The suspension was vortexed for 1 min, incubated for 10 min, room temperature, slow rotation, and then centrifuged at 10,000 × g for 10 min. The supernatant was carefully removed and used for RNA purification.

Alternatively, viruses were concentrated using an IC procedure. Dynabeads were coated with purified mouse anti-poliovirus blend monoclonal antibody (MABB8566, Chemicon Intl., Inc., Temecula, Calif.) according to the manufacturer’s instructions (Dynal UK Ltd., Bromborough, UK). Oyster and mussel homogenate samples were incubated with coated beads for a minimum of 30 min at 4°C, with slow rotation. The coated magnetic beads were then recovered using a magnet, and the fluid was removed by aspiration. Captured virus was eluted from the beads by the addition of 200 µl of elution buffer (0.1 M glycine-HCl [pH 2.8], 0.15 M sodium chloride). Samples were incubated at room temperature for 5 min, and the beads recovered using the magnet. The elution step was repeated, and the combined eluates used for RNA purification.

Viral RNA was extracted from concentrated samples using a commercially available kit (QuickPrep Micro mRNA Purification Kit, Amersham Pharmacia Biotech, Piscataway, NJ). The manufacturer’s instructions were followed, and the purified RNA was eluted 10 µl of diethylpyrocarbonate-treated water.

cDNA synthesis and PCR amplification. A one-step RT-PCR reaction kit (Ready-To-Go RT-PCR Beads, Amersham Pharmacia Biotech) was used for the study. The manufacturer’s instructions were followed. Briefly, 43.5 µl of diethylpyrocarbonate-treated water was added to the tube and the bead dissolved. Four microliters of RNA (corresponding to 1/10th the total volume of RNA), 10 pmol of poliovirus specific primers (Polio 1–Biotinylated: sense GGACT TCGGTTACGACAGG and Polio 2: antisense CAACCCCGGATGTAGCTTGG, which amplify a 248-bp sequence from the 5’ nontranslated region of the poliovirus genome), EV primers (Ent1: sense CGGTTACCTTGTAGCTGCT GT and Ent2: antisense ATTTGCACATAAGCAGCCCA, which amplify a 540-bp region of the noncoding region of the EV genome) (K. Henshilwood, personal communication) or NoV primers (GH: sense AGCCNTGAAATNATGTT and SM31: antisense CGATTTCATCATCACCATA, which amplify a 190-bp re-
tion of the RNA polymerase gene of NoV GII) (14, 38), and 0.5 nmol of DIG-dUTP were added individually to each bead prep, bringing the final volume to 50 μL. The reactions were incubated at 42°C for 30 min and then 15 min at 95°C to inactivate the RT.

PCR amplification was performed for 35 cycles, each cycle consisting of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, with a final extension of 72°C for 7 min. A negative RT-PCR control, with sterile water as template, was included in every amplification reaction. Reactions were performed in duplicate, and replicate experiments were performed.

PCR products were purified using the QIAquick PCR Purification kit (Qiagen GmbH, Hilden, Germany) before further analysis. Manufacturer's instructions were followed, and the purified DNA was eluted in a 50-μL volume of 10 mM Tris-HCl, pH 8.5. Five microliters of DNA was analyzed by electrophoresis through a 1.5% ethidium bromide agarose gel and visualized under UV light. The remaining 45 μL of DNA was screened by ELISA to compare sensitivity with ethidium bromide gel electrophoresis.

**Nested amplification.** When screening naturally contaminated samples, a second-round (nested) PCR was performed after the first round of PCR amplification. For nested amplification, 1.5 μL of the amplified DNA from the first PCR was added to a fresh PCR mixture containing 2 μL of dNTP mix (200 μM dATP, dCTP, and dGTP and 190 μM dTTP); 5 μL 10 × NH4;2, 2 μL MgCl2; 1 pmol forward and reverse primers (neENT1-Biotinylated: sense TCCGGCCCCCTGAAATGGCCTA and neENT2: antisense GAA ACACGGACACCCAAAGTA, which amplify a 123-bp sequence) (K. Henshilwood, personal communication), or NI NoV-Biotinylated: sense AATGGTCATCAGCCTGCTGCT and E3: antisense ATCTCATCATCAAC (which amplify a 113-bp sequence) (14); 0.5 nmol of DIG-dUTP (reaction nested only); and 36 μL of sterile water. After an initial 3-min denaturation period at 95°C, 0.5 U of Taq DNA polymerase was added to the tube. PCR amplification was then performed for 35 cycles, each cycle consisting of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, with a final extension of 72°C for 7 min. PCR products were then purified, and screened as previously described.

**ELISA detection.** Microtiter wells were coated with 150 μL of streptavidin (0.5 μg/mL) at least 1 h prior to use, and then washed twice with PBS-Tween (0.05%, vol/vol) (PBST). Wells were blocked with PBST containing 2% Marbled dried milk (PBSTM) for 1 h at 37°C and then washed three times with PBST. Forty-five microliters of PCR product and 105 μL of PBST were incubated in each well for 30 min, with shaking, at room temperature to allow streptavidin-biotin binding. Wells were washed five times with PBST and then 150 μL of anti-digoxigenin–horse-radish peroxidase (HRP) conjugate in PBSTM (0.15 U/mL) was added to each well. After 30 min of shaking at room temperature, the wells were washed six times with PBST. One hundred microliters of tetramethylbenzidine, the substrate for HRP, containing 0.06% hydrogen peroxide was added per well, and the reaction finally stopped by the addition of 50 μL of 1 M sulfuric acid. Wells were read in a microtiter plate reader at 450-nm absorbance.

An absorbance of 0.2 or greater was indicative of a positive PCR reaction. The 0.2 threshold value was artificially set to allow the ELISA method to be standardized and to ensure that false-positive results from nonspecific signals did not occur. It also simplified data interpretation, with values above this threshold reported as being positive, and values below being negative.

**Cloning and sequencing.** RT-PCR–positive norovirus amplicons were separated from all unincorporated oligonucleotide primers and nucleotides using Chromaspin 100 columns (Clontech Laboratories, Inc., Mountainview, Calif.), ligated into a pGem vector and transformed into Escherichia coli JM109 competent cells (genotype: endA1, recA1, gyrA96, thi, hsdR17 (tK-, m-) relA1, supE44, Δ(lac-proAB), [F’traD36, proAB, lacIqZAM15]) (pGem T Vector System, Promega, Madison, Wis.). For blue–white screening of recombinants, transformed cells were plated onto Luria-Bertani plates containing 100 μg/ml ampicillin, 0.5 mM IPTG, and 40 μg/ml X-gal. White colonies from each sample were screened for inserts, using colony PCR. A minimum of five positive clones from each sample were further purified using microspin 30 k columns (Filtron Technology Corp., Northborough, Mass.). Both DNA strands were sequenced with the ABI Prism Big Dye terminator cycle sequencing system (Applied Biosystems, Foster City, Calif.) and analyzed on an ABI genetic analyzer.

**Colonel PCR.** To determine whether colonies contained inserts of the correct size, white or pale-blue colonies were screened using colony PCR. Colony PCR master mixes were made and contained final concentrations of the following: 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.25 mM MgCl2, 0.2 mM each dNTP, and 20 pmol of each primer, pTAG 3′ TGG AAA ACG ACG GCC AGT GAA and pTAG 5′ GCT ATG ACC ATT ACG CCA A (K. Henshilwood, personal communication). Nuclease-free water was added to a final volume of 49 μL.

The PCR reaction mix was inoculated using a fine loop, which had touched the edge of a large white or pale-blue colony. Amplification reaction conditions included one cycle of 96°C for 10 min, followed by 30 cycles of 94°C for 1 min, 37°C for 1 min, and 72°C for 1 min, with a final extension step of 72°C for 10 min. The PCR products were visualized by gel electrophoresis on a 2% agarose gel (NuSieve 3:1, Flowgen Instruments, Ltd., Sittingbourne, UK) containing 0.5 μg ml−1 ethidium bromide per 25 ml agarose. Reactions positive for NoV were seen as a 313-bp band (200 bp from the vector plus size of insert).

**Sequence analysis.** Sequence data analysis was performed using the MegAlign and EditSeq components of the Lasergene software (DNA Star).

**RESULTS**

**Comparison of gel electrophoresis and ELISA sensitivity with shellfish samples processed using a PEG precipitation method and the IC method.** Using poliovirus-seeded shellfish samples, the sensitivity of ELISA-based signal generation was compared with the sensitivity of amplified PCR product detection by gel electrophoresis. Duplicate extractions and amplification reactions were performed, and on average, experiments were repeated three times. A shellfish sample inoculated with sterile water and a negative RT-PCR reaction were included as controls.

Poliovirus type 1 was detected down to 1 × 10⁴ TCID₅₀ when PCR products from whole-oyster samples were screened by ethidium bromide gel electrophoresis (Fig. 1a, i). In comparison, ELISA detected positive products at 1 × 10³ TCID₅₀ (Fig. 1a, ii). When PCR products from whole-mussel samples were screened, positive fragments could be visualized down to 1 × 10³ TCID₅₀ after ethidium bromide gel electrophoresis (Fig. 1b, i). A titer of between 100 and 1,000 TCID₅₀ could be detected by ELISA (Fig. 1b, ii).

When the experiment was repeated using oyster digestive gland samples, detection sensitivity was comparable with that reported for the whole mussel (Fig. 1c, i, and 1c,
Detection of poliovirus in artificially contaminated whole oysters (a), whole mussels (b), and oyster digestive glands (c), using the PEG precipitation virus extraction method as compared with detection using IC in whole mussels (d) and oyster digestive glands (e). (i) RT-PCR products visualized on an ethidium bromide-stained agarose gel. Samples were loaded in duplicate wells. M, molecular marker VI; C, negative reagent control; Sp, control shellfish extract; 10^6 to 10, shellfish extract spiked with 1 × 10^6 to 10 TCID_{50} poliovirus. (ii) Detection of RT-PCR products by ELISA. Dep, depurated shellfish (negative extract control); C, negative reagent control. Line indicative of 0.2 absorbance units (450 nm) detection threshold for positive PCR reactions.

After ethidium bromide gel electrophoresis, positive amplicons could be visualized down to 1 × 10^3 TCID_{50}; however, ELISA could detect positive samples between 10 and 100 TCID_{50}.

The detection sensitivity of gel electrophoresis and ELISA were also compared using shellfish samples, which had been processed using the IC method. Detection sensitivities for whole-mussel homogenate (Fig. 1d) and oyster digestive gland homogenate (Fig. 1e) were the same. When amplified products were run on an ethidium bromide gel and visualized under UV light, amplicons were clearly observed down to a virus titer of 1 × 10^4 TCID_{50}. When assayed by ELISA, detection sensitivity improved 10-fold, with detection at 1 × 10^3 TCID_{50} achieved.

RT-PCR-ELISA screening of natural samples. Five shellfish samples from harvesting sites, with varying degrees of bacterial contamination, were screened for the presence of EVs and NoVs. Samples were processed using the PEG precipitation method for virus extraction, RNA was purified, and RT-PCR performed. Virus titers found in natural shellfish samples are often extremely low; therefore, a nested reaction was performed to increase PCR sensitivity.

Results for gel electrophoresis and ELISA, after a nested round of amplification, are shown in Figure 2. Samples harvested from category A sites (<230 E. coli or <300 fecal coliforms [FCs] per 100 g of shellfish) were negative for both EVs and NoVs when analyzed by gel electrophoresis and ELISA (Fig. 2a, i, and 2b). Positive controls were included in the reaction to confirm that PCR inhibition was not responsible for the negative results. The category C oysters, harvested from waters exposed to sewage contam-
FIGURE 2. Detection of EVs and NoVs, using a nested PCR reaction. Shellfish were harvested from sites with varying degrees of contamination. (a) RT-PCR products visualized on an ethidium bromide–stained agarose gel. Samples were loaded in duplicate wells. (a, i) −, negative reagent control; +, positive PCR control; AA, AB, and AD, mussel samples from a category A site. (a, ii) Cat. C, oysters harvested from category C site; Cat. D, oysters harvested from a category D site. (b) Detection of RT-PCR products by ELISA. A, B, and D, mussel samples from a category A site; Cat. C, oysters harvested from category C site; Cat. D, oysters harvested from a category D site; C−ve, negative control; C+ve, positive control. Data are presented as an average of two replicates ± standard deviation, and is a typical data set from replicated experiments. Line indicative of 0.2 absorbance units (450 nm) detection threshold for positive PCR reactions.

FIGURE 3. Phylogenetic tree illustrating, at the nucleotide level, the genetic relationships of isolates when compared with representative clinical and published strains. The phylogenetic tree was generated using the Clustal V algorithm within MegAlign (DNA Star, Inc.). The tree is based on the alignment of approximately 80 nucleotides within the NoV RNA polymerase region of the genome, excluding PCR primers.

From the five samples tested, three distinct isolates were obtained. The genetic relationship of the three identified NoVs is shown in a phylogenetic tree in Figure 3. Due to the nature of the primers used in the study, only GII sequences were observed. Separation of genogroup I and genogroup II strains can be clearly observed, and all three isolates show significant sequence similarity with GII genogroup strains. Sequence A showed 94.7% identity with the Mur1-1997-JP (accession no. AB019269). Sequence B showed 98.7% identity with the Richmond strain sequence (accession no. AF414419), and sequence C showed 97.4% identity with the Grimsby clinical isolate strain sequence, most closely related to the Lordsdale strain sequence (accession no. X86557).

DISCUSSION

Assays, based on PCR amplification, can be used to detect viruses in environmental samples. But in order to be effective, the assay must be specific, sensitive, and reliable, with rapid delivery of results.

Interference by inhibitors has presented a major problem when developing virus-detection methods. Several traditional extraction methods have been used to successfully detect virus in contaminated shellfish, but these are often time-consuming, cumbersome, and involve multiple steps, increasing the risk of contamination and RNA degradation. These methods extract and concentrate all viral fragments, leading to potential false-positive results when used for shellfish monitoring (1, 4, 5, 14, 24, 25, 31, 39, 43). The use of an IC procedure for virus extraction offers several advantages over more traditional methods, and several studies have reported good detection sensitivities (9, 19, 27, 34, 42, 46, 47).

During this study, we evaluated a colorimetric method RT-PCR-ELISA for ease of use, reliability, and sensitivity
when detecting known human pathogenic virus present in shellfish. Detection sensitivity using a typical PEG precipitation method, and an IC method for virus concentration with a poliovirus model, was established. A sensitivity of 1,000 TCID$_{50}$ poliovirus was reported when samples were concentrated using PEG precipitation and reactions were analyzed by electrophoresis, sensitivity comparable to that previously reported for poliovirus detection in oysters using gel electrophoresis (8). When reactions were analyzed using the ELISA method, detection was possible down to 100 TCID$_{50}$ poliovirus. However, it should be noted that more PCR reaction was analyzed by ELISA in comparison to that screened by gel electrophoresis. When an IC method was used to extract and concentrate virus from the samples, detection sensitivity decreased by approximately 10-fold to between 100 and 1,000 TCID$_{50}$, and 1,000 to 10,000 TCID$_{50}$ poliovirus when samples were screened by ELISA and gel electrophoresis, respectively. This 10-fold difference in detection sensitivity has been reported by others (7, 42, 47).

Several studies have compared detection sensitivity of an IC procedure with more traditional extraction-concentration methods, and similar detection sensitivities of the two methods have been reported (9, 47). However, data from this study indicated that the elution-concentration method was more sensitive than was the IC procedure when detecting poliovirus in artificially contaminated shellfish. It has been suggested that the decrease in detection sensitivity reported when an IC procedure is used may be due to the presence of viral RNA, not associated with intact virus particles (42). Viral RNA free of virions will not be detected by the antibody-capture method.

The successful detection of virus in artificially contaminated samples, using RT-PCR-ELISA, was expanded to include studies involving naturally contaminated shellfish. For sensitive detection of virus in naturally contaminated samples, a nested amplification reaction was an important step in the detection procedure. The introduction of a second amplification step, and the concomitant manipulation of the previously amplified material, could lead to a significant increase of false positives due to cross-contamination. In this study, steps were taken to prevent generation of such false-positive results. Nested PCR reactions were prepared using filter tips, and work was performed within a designated flow hood. The problem of false-positive results could be circumvented in the future by designing a one-step, single, closed-tube system for amplification.

Using the ELISA method, we could successfully detect EVs and NoVs in shellfish samples from a category D site, where harvesting is prohibited. At category D sites, samples are found to exceed the upper levels of contamination specified for approved harvesting waters by microbiological testing standards (>6,000 FCs per 100 g of shellfish); therefore, the positive detection of EV and NoV GII from these samples was not unexpected.

Positive GII NoV amplicons were sequenced to confirm identity and also to allow us to investigate the diversity of GII NoV strains contaminating the oysters. A phylogenetic tree illustrating the genetic relationships of sequences obtained during this study with published sequences and clinical isolates is shown in Figure 3. Three different NoV GII sequences were identified in oysters harvested from a category D site, suggesting that the harvesting waters were contaminated with various NoV GII strains. Contamination of samples by more than one NoV sequence has previously been reported during monitoring studies of shellfish harvesting areas (17, 29, 30).

EVs were also identified in the category D shellfish samples. This was not altogether surprising, as EVs are commonly shed from the intestinal tract and are frequently detected in harvesting waters. The association of NoVs and EVs has previously been documented, and the detection of both these viruses in the shellfish was suggestive of a mixed infection (30). Due to time and cost restraints of the study, we were unable to sequence these positive samples.

In this study, we evaluated an IC method of virus extraction using a poliovirus model. Virus extraction was simple, rapid, and required fewer steps than existing extraction-concentration methods, greatly reducing the risk of contamination from RNases. Despite an observed small decrease in detection sensitivity when an IC procedure was used, the combination of IC with PCR amplification should increase the likelihood of detecting intact virus particles. Due to the nature of the antibody-antigen interaction, only virus particles with functional epitopes will be recognized by the antibody, and hence, captured from the sample. A PCR-positive result without an IC step only confirms that viral RNA was present in the sample. Despite the obvious advantages of IC, use of the method for NoV detection would need to be evaluated further as due to the extreme genetic diversity of these viruses, it could be argued that false-negative results may arise due to a lack of cross-reactivity between certain isolates and the antibody used.

Ethidium bromide gel electrophoresis after RT-PCR amplification is a routine method for determining successful nucleic acid amplification. It is an inexpensive detection method that requires no specialized equipment, can be performed in most laboratories, and requires a relatively short assay time. Despite the advantages of gel electrophoresis, the method uses reagents that are potentially carcinogenic, and the positive identification of faint amplicons can be difficult. During the study, a maximum of 30 samples could be screened per gel. Gel electrophoresis took approximately 1.5 min to run; hence, 1.5 min was required for each sample tested. The ELISA method was consistently as sensitive, if not marginally more so, than was gel electrophoresis and could process more samples in a given time (96 samples in approximately 1 h, 50 min). Purification of PCR samples was required prior to ELISA detection to eliminate a background signal observed in ELISA negative controls. This additional step did increase assay cost by approximately $2 as well as assay time; however, overall the technique had a fast sample throughput, and no specialized equipment was required.

Recently research has focused on developing real-time PCR methods to detect viruses in various sample matrices (6, 18, 21–23, 26, 33, 36, 37, 41, 45). Real-time PCR can screen samples rapidly, does not require postamplification...
analysis, and has increased test specificity through the inclusion of a hybridization procedure. However, the method does present some drawbacks compared with existing detection methods including the need for stringent design and optimization of primer and probe combinations prior to use, and the requirement for specialized, expensive equipment. It has also been reported that the presence of amplification inhibitors can present a major problem when assessing NoV levels in shellfish extracts, using a real-time system (33).

The future application of real-time systems for virus detection in shellfish is promising; however, at present current methods must be evaluated further before real-time PCR can be employed as a standardized virus detection method.

In summary, the RT-PCR-ELISA method is a rapid, sensitive method, which can routinely detect the presence of virus in a range of sample matrices (35). Modifications to assay procedures were not required, even when various complex shellfish sample matrices were tested, and as part of this study, the method was easily reestablished in a different laboratory to allow detection of virus in naturally contaminated samples. No specialized laboratory equipment was required to perform the assay, and all reagents used were easy to obtain and not harmful. The determination of a positive reaction was easy, even by eye, and assay format permitted the simultaneous processing of 96 samples. With a total detection time of 1 h 50 min, a screening time of approximately 1 min per sample could be achieved.

Taken together, results from this study suggest that the RT-PCR-ELISA may be standardizable as a virus detection method for shellfish-screening programs. In particular, the simplicity of ELISA makes it ideal for use as a prescreening method.

The inclusion of an IC step would permit the detection of intact viral particles alone. The IC method was simple to perform, required minimal equipment, and may be more applicable for on-site testing. A combination of IC, nested PCR amplification, and ELISA detection may be the way forward when considering the implementation of a standardized method.

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