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Detection of Human Enteric Viruses in Oysters by In Vivo and In Vitro Amplification of Nucleic Acids

HYENMI CHUNG,† LEE-ANN JAYKUS,‡ AND MARK D. SOBSEY*

Department of Environmental Sciences and Engineering University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7400

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This study describes the detection of enteroviruses and hepatitis A virus in 31 naturally contaminated oyster specimens by nucleic acid amplification and oligonucleotide probing. Viruses were extracted by adsorption-elution-precipitation from 50-g oyster samples harvested from an area receiving sewage effluent discharge. Ninety percent of each extract was inoculated into primate kidney cell cultures for virus isolation and infectivity assay. Viruses in the remaining 10% of oyster extract that was not inoculated into cell cultures were further purified and concentrated by a procedure involving Freon extraction, polyethylene glycol precipitation, and Pro-Cipitate precipitation. After 3 to 4 weeks of incubation, RNA was extracted from inoculated cultures that were negative for cytopathic effects (CPE). These RNA extracts and the RNA from virions purified and concentrated directly from oyster extracts were subjected to reverse transcriptase PCR (RT-PCR) with primer pairs for human enteroviruses and hepatitis A virus. The resulting amplicons were confirmed by internal oligonucleotide probe hybridization. For the portions of oyster sample extracts inoculated into cell cultures, 12 (39%) were positive for human enteroviruses by CPE and 6 (19%) were positive by RT-PCR and oligoprobing of RNA extracts from CPE-negative cell cultures. For the remaining sample portions tested by direct RT-PCR and oligoprobing after further concentration, five (about 16%) were confirmed to be positive for human enteroviruses. Hepatitis A virus was also detected in RNA extracts of two CPE-positive samples by RT-PCR and oligoprobing. Combining the data from all three methods, enteric viruses were detected in 18 of 31 (58%) samples. Detection by nucleic acid methods increased the number of positive samples by 50% over detection by CPE in cell culture. Hence, nucleic acid amplification methods increase the detection of noncytopathic human enteric viruses in oysters.

Human enteric viruses are a major cause of shellfish-associated enteric disease (25). While the fecal coliform group of bacteria is widely accepted as the criterion for the sanitary quality of shellfish and their harvesting waters, it does not reliably predict the occurrence of enteric viruses (9). In the absence of proven virus indicators, investigators have attempted the direct detection of enteric viruses in shellfish. Traditional methods to detect and quantify human enteric viruses in shellfish involve extraction and concentration of the viruses from the tissue matrix followed by their isolation and quantitation by using susceptible, live laboratory hosts (32). The use of cell cultures relies on the development of visible cytopathic effects (CPE) caused by the infectious virus. These assays are cumbersome, costly, and slow. Furthermore, no single-cell culture system is susceptible to all human enteric viruses, some human enteric viruses replicate in cell culture without the production of apparent CPE (23), and many of the epidemiologically important enteric viruses cannot be propagated in any cell culture (32).

Alternative virus detection methods such as nucleic acid hybridization are not capable of detecting the low levels of virus contamination anticipated in shellfish (33, 39). However, virus detection methods based on in vitro enzymatic amplification of target nucleic acid sequences, such as PCR, provide great sensitivity as well as specificity. The application of this technology to the detection of human enteric viruses in shellfish is limited by the large volume and inhibitory quality of virus concentrates from shellfish. Several investigators have reported PCR methods for virus detection in shellfish (2, 7, 10), but these methods have not been applied to the detection of naturally occurring enteric viruses in field samples of shellfish having endemic levels of contamination. Furthermore, no direct comparison between cell culture and molecular methods for virus detection in shellfish has been reported. Recently, Lees et al. (18–20) described a method to detect enteric viruses in shellfish and applied this method to a small number of sewage-polluted field samples in the United Kingdom. However, no such studies have been reported in the United States. The purpose of this research was to apply and compare nucleic acid amplification and cell culture methods for the detection of human enteric viruses in field oyster samples from the North Carolina coast of the United States.

MATERIALS AND METHODS

Oyster samples. Oyster samples (*Crassostrea virginica*) were collected between May 1991 and May 1992 from Calico Creek, a tidal estuarine creek near Morehead City, N.C., that receives the chlorinated secondary effluent from the sewage treatment plant of the community. Two stations were sampled: station 2, which is approximately 1 km below the discharge, and station 3, which is at the mouth of Calico Creek and about 2 km below the discharge (Fig. 1). Calico Creek and adjacent waters of the lower Newport River estuary are closed to shellfish harvesting, although areas of the river both above and below Calico Creek are open to harvesting. Oysters were collected manually, cleaned under running tap water, shucked, and homogenized. Oyster homogenate was shipped to this laboratory at refrigeration temperatures and stored at −80°C until processed.

Enteric virus concentration from oysters by adsorption-elution-precipitation. A modified adsorption-elution-precipitation procedure (34) was used for virus concentration. Briefly, 50 g of oyster homogenate was diluted sevenfold with cold distilled water, and the viruses were adsorbed to the meat by reducing the conductivity to <2,000 ppm and the pH to 5.0. After centrifugation at 1,700 × *g*

* Corresponding author. Phone: (919) 966-7303. Fax: (919) 966-4711.

† Present address: National Institute of Environmental Research (NIER), 613-2 Bulkwang, Eunpyung, Seoul 122, Korea.

‡ Present address: Department of Food Science, North Carolina State University, Raleigh, NC 27695-7624.

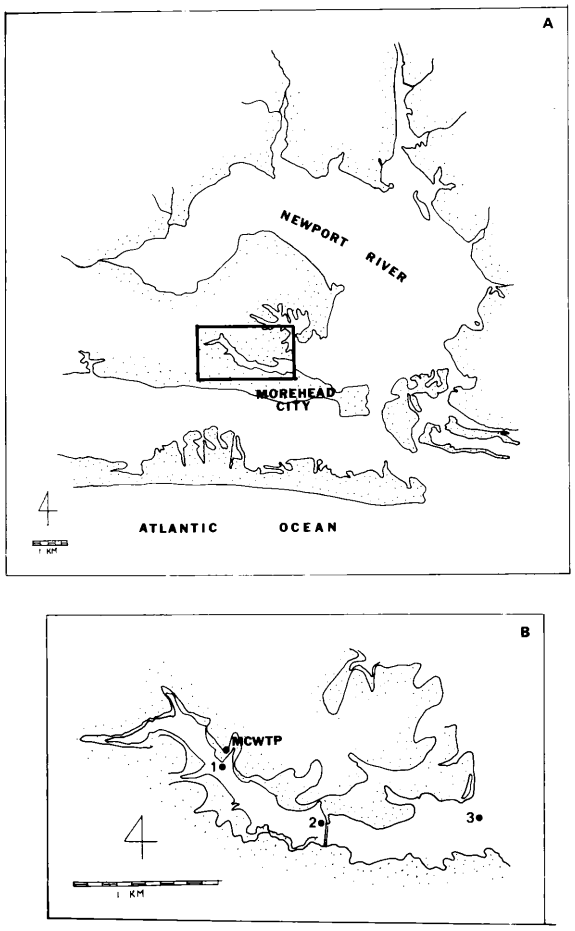

FIG. 1. Calico Creek sampling area MCWTP, Morehead City Wastewater Treatment Plant. (A) General area. (B) Detail from panel A showing the sampling stations.

and 4°C for 15 min, the solids-adsorbed viruses were eluted by resuspension in 350 ml of 0.05 M glycine–0.14 M NaCl with subsequent pH adjustment to 7.5. Shellfish solids were removed by centrifugation at 1,700 × *g* and 4°C for 15 min, and viruses in the supernatant were concentrated by acid precipitation at pH 4.5. The resulting floc was sedimented by centrifugation at 1,700 × *g* and 4°C for 15 min and resuspended in 10 ml of 0.1 M disodium phosphate buffer (pH 9.3 to 9.5), and the pH was adjusted to 7.0 to 7.5. Cytotoxic components of the sample were precipitated by addition of Cat-Floc T to a final concentration of 0.1%, and the precipitate was removed by centrifuging at 3,000 × *g* and 4°C for 20 min. Recovered supernatants were supplemented with gentamicin and kanamycin to final concentrations of 50 and 250 μg/ml, respectively, and incubated at room temperature for 2 h. The final concentrates were stored at −80°C until used for assay or subjected to further treatment.

The shellfish concentrate volume from initial adsorption-elution-precipitation was 10 to 30 ml per 50-g oyster sample. Up to 90% of the concentrate was analyzed for culturable enteric viruses by cell culture infectivity. The remaining 10% of the sample, ranging from 1 to 2 ml, was further purified and concentrated by trichlorotrifluorethane (Freon) extraction, polyethylene glycol (PEG) precipitation, and adsorption-elution-precipitation with the novel protein-precipitating agent Pro-Cipitate. For seven samples (site 2, collected on 18 November 1991 and 2 March, 13 April, and 10 May 1992; Site 3, collected on 18 November 1991 and 2 March and 10 May 1992), there was sufficient volume of the original oyster homogenate to further concentrate the extract from 50 g by Freon extraction, PEG precipitation, and Pro-Cipitate adsorption-elution-precipitation.

Virus concentration from oyster extract by Freon extraction, PEG precipitation, and Pro-Cipitate adsorption-elution-precipitation. Aliquots of each oyster extract sample were further processed for virus concentration and purification to enable reverse transcriptase PCR (RT-PCR) amplification and detection by oligoprobe hybridization (16). Samples were Freon extracted twice with equal volumes of sample and solvent. The resulting supernatant was adjusted to pH 7.3 to 7.4 and 0.3 M NaCl and supplemented with PEG 8000 to a final concentration of 6% (wt/vol). After overnight incubation at 4°C to precipitate the viruses, the precipitate was recovered by centrifugation at 6,000 × *g* and 4°C for 20 min and resuspended in one-seventh the original volume with 50 mM Tris–0.2% Tween 20 (pH 8.0). Viruses in resuspended precipitates were eluted for 30 min at room temperature with occasional vortexing, and after centrifugation for 15 min at 10,000 × *g* at room temperature, the supernatant was retained.

Viruses in the supernatants were further purified and concentrated by being precipitated with an equal volume of Pro-Cipitate (Affinity Technology, Inc., Parsippany, N.J.). After centrifugation for 15 min at room temperature and 13,800 × *g*, precipitated viruses were eluted in 50 mM Tris–0.2% Tween 20 (pH 9.0) with gentle rotation for 1 h at room temperature. The excess Pro-Cipitate was removed by centrifugation at 6,000 × *g* for 20 min at 15°C. Viruses in Pro-Cipitate eluants were reconcentrated by a second precipitation with 10% PEG–0.3 M NaCl. After 2 h at 4°C, the samples were centrifuged at 6,000 × *g* and 4°C for 20 min, and the precipitate was resuspended in one-seventh the original volume with 50 mM Tris–0.2% Tween 20 (pH 8.0). Aliquots (10 μl) of final concentrate were analyzed for viruses by RT-PCR.

Cell culture assay. All cell culture assays were performed in secondary African green monkey kidney (sAGMK) cells, Buffalo green monkey kidney (BGMK) cells, and/or a continuous cell line of fetal rhesus monkey kidney-derived (FRhK-4) cells. Cell cultures were grown to confluence in Eagle's minimum essential medium containing 10% fetal calf serum, 15 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulfonic acid (HEPES) buffer, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 0.11% sodium bicarbonate, and antibiotics (250 μg of kanamycin per ml and 50 μg of gentamicin per ml).

Aliquots (1.0 to 1.5 ml) of oyster extracts were inoculated onto drained confluent layers of sAGMK cells in 75-cm^2 tissue culture flasks and allowed to adsorb for 90 min at 37°C, with the inoculum being redistributed over the cell layers every 20 min. The sample inocula were removed after the adsorption period and subsequently reinoculated into BGMK cells under the same adsorption conditions. Inoculated cultures were washed with serum-free Eagle's minimal essential medium after removal of the inoculum and then supplemented with maintenance medium that was the same as the growth medium except for a reduced (2%) fetal calf serum concentration. Inoculated cultures were incubated at 36.5°C and observed periodically for CPE for 2 weeks, after which they were frozen and thawed. For the cultures that were negative for CPE, 20% of pooled cell lysates of both sAGMK and BGMK cells were inoculated into sAGMK cells or FRhK-4 cells and incubated for a further 2 weeks as a blind passage. Cell lysates from flasks showing CPE were freeze-thawed once, filtered through sterile 0.2-μm-pore-size membrane filters (Acrodisc; Gelman Sciences, Ann Arbor, Mich.), and then reinoculated into fresh BGMK or FRhK-4 cells for confirmation of CPE. Maintenance medium in all cell cultures was replaced weekly during the incubation periods.

Purification of CPE-positive cell culture lysates for RT-PCR. CPE-positive cell culture lysates were first extracted with an equal volume of Freon. The extracted lysates were further purified by exclusion chromatography on Sephadex G-25 (Sigma Chemical Co., St. Louis, Mo.) spin columns. Spin columns were prepared in 1-ml syringes by the method of Sambrook et al. (27), except that silane-treated glass wool (Supelco, Bellefonte, Pa.) was used as the column support. The columns were centrifuged at 400 × *g* and room temperature for 4 min. Lysate volumes of 100 μl were passed through columns which had been equilibrated with 50 mM Tris buffer (pH 8.0). Aliquots (10 μl) of column-purified lysate were used in RT-PCR.

RNA isolation from cells of CPE-negative cultures. Intracellular RNA was extracted from CPE-negative cell cultures by the method of Shieh et al. (30). Briefly, the cells were harvested by scraping and concentrated by centrifugation, and the intracellular RNA was extracted by treatment with Nonidet P-40 and proteinase K. Following phenol-chloroform extraction, RNA was precipitated and washed with ethanol and resuspended in 10 mM Tris–0.1 mM EDTA (TE) buffer (pH 7.0). Isolated RNA was quantitated spectrophotometrically at 260 nm (Spectronic 1201; Milton Roy Co., Rochester, N.Y.) and held at −80°C until used. Samples of 200 ng were used in RT-PCR.

PCR primers and oligoprobes. The oligonucleotide primer and probe sequences for enteroviruses and hepatitis A virus (HAV) used in this study have been described previously (28). The highly conserved 5′ untranslated region of the enteroviruses was used as the target for the synthesis of a 197-bp panenterovirus cDNA (5′ primer, CCTCCGGCCCCTGAATG; 3′ primer, ACCGGATG GCCAATCCAA; internal oligoprobe, TACTTTGGGTGTCCGTGTTTC). For HAV, the genomic region corresponding to the VP1-VP3 capsid protein junction was the target for a 192-bp cDNA (5′ primer, CAGCACATCAGAAAGGTG AG; 3′ primer, CTCCAGAATCATCTCCAAC; internal oligoprobe, TGCTCC TCTTTATCATGCTATG). The downstream or antisense 3′ primers are complementary to the positive-sense virion RNA, and the upstream 5′ primers are homologous to the positive-sense viral RNA. Internal oligomer probes were synthesized in the positive-sense orientation so that they hybridize only with cDNA or PCR products and not with viral genomic positive-sense RNA.

RT-PCR for viruses in sample concentrates. RT-PCR was done with the Gene-Amp kit (Perkin-Elmer/Cetus Corp., Norwalk, Conn.) as specified by the manufacturer, except that the reaction volumes for reverse transcription were increased from 20 to 30 μl to accommodate a 10-μl sample and 50% PEG 4000 (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was added to achieve a final concentration of 2.5% PEG per tube to prevent RT-PCR inhibition. Viral RNA was denatured or released from virions by heating reaction mixtures at

TABLE 1. Detection of human enteric viruses in field oyster samples

Sampling date (mo-day-yr)	Station 2[a]				Station 3[a]			
	MPNCU/100 g[b]	RT-PCR RNA[c]	RT-PCR direct[d]	Combined	MPNCU/100 g[b]	RT-PCR RNA[c]	RT-PCR Direct	Combined
5-6-91	<2	−	−	−	N	N	N	N
5-14-91	8	−	−	+	<2	−	−	−
6-3-91	2	−	−	+	<2	−	−	−
6-17-91	<2	−	−	−	N	N	N	N
6-24-91	<2	−	+	+	N	N	N	N
7-9-91	2	−	(+)	+	<2	−	−	−
7-22-91	<2	−	−	−	<2	−	−	−
8-5-91	5	+	−	+	N	N	N	N
8-26-91	10	−	+	+	N	N	N	N
9-11-91	<2	−	+	+	5	−	−	+
9-30-91	2	−	−	+	<2	−	−	−
11-4-91	<2	−	−	−	<2	−	−	−
11-18-91	N*	+	−×	+	16*	−	−×	+
12-2-91	2	−	−	+	N	N	N	N
1-6-92	<2	+	−	+	2	+	−	+
2-3-92	<2*	−	−	−	<2	−	−	−
3-2-92	<2*	+	−×	+	10*	−	−×	+
4-13-92	<2*	+	(+)×	+	N	N	N	N
5-11-92	>24*	−	−×	+	<3*	−	−×	−
Total positive/total no.	8/18	5/19	5/19	14/19	4/12	1/12	0/12	4/12

[a] +, positive; −, negative; N, no data available; *, cultivated on FRhK-4 cells for secondary passage because of endogenous foamy agent infection of the sAGMK cell line.

[b] 90% of oyster extract analyzed by cell culture except when indicated by an asterisk, which indicates that 10 to 30% of the sample was analyzed because of endogenous retrovirus infection.

[c] <0.04% of cell culture lysate or RNA extracted from lysate was analyzed by RT-PCR.

[d] 10% of the sample was concentrated for direct RT-PCR, and 20% of the final concentrate (3% of the total sample) was analyzed by direct RT-PCR except when indicated by a cross, which indicates that a 50-g oyster homogenate was concentrated and 20% of the concentrate was analyzed by direct RT-PCR. Parentheses indicate that the sample was confirmed by dot blot hybridization only.

99°C for 5 min and then adding RT (2.5 U) and RNase inhibitor (1.0 U). Reverse transcription was done at 42°C for 1 h with random primers, after which the tubes were heated to 99°C for 5 min to inactivate the enzyme. After being chilled, the tubes were supplemented with 5 U of *Taq* polymerase and primer pairs as appropriate. PCR amplification was performed for 40 cycles, each consisting of 95°C for 1.5 min, 55°C for 1.5 min, and 72°C for 1.5 min. A 10- to 15-μl portion (10 to 15%) of the reaction volume was analyzed by electrophoresis at 150 V for 2 h on 2% agarose gels, stained with ethidium bromide, and visualized under UV light.

Oligoprobe hybridization. RT-PCR products were diluted in 2× SSPE (17.53 g of NaCl, 2.76 g of NaH_2PO_4, and 0.74 g of EDTA per liter of distilled water) and blotted on nylon membranes (GeneScreen; DuPont, Boston, Mass.). Alternatively, RT-PCR products were transferred from electrophoresed agarose gels by the method of Southern (27). The DNA was bound by cross-linking with shortwave UV light (Ultraviolet Products, Inc., San Gabriel, Calif.) for 3 to 5 min at a distance of 15 cm. Procedures for digoxigenin-labelled oligoprobe hybridization with cDNA of enteroviruses and HAV have been described previously (28).

RESULTS

Detection of cytopathogenic enteric viruses in cell cultures. Approximately 90% of each sample, corresponding to 45 g of oyster meat, was inoculated into 6 to 17 cell culture flasks (75 cm^2) containing confluent layers of sAGMK or BGMK cells. Of the 31 oyster samples tested, 12 (39%) were positive for CPE by quantal cell culture assay, representing 8 of 18 (44%) of the samples originating from station 2 and 4 of 12 (33%) from station 3 (Table 1). Some cultures displayed presumptive CPE by late in the second week of incubation. However, because of sample cytotoxicity, which may mimic viral CPE, all presumptive CPE-positive cultures were confirmed by blind passage. The levels of enteric virus contamination, as computed by the most-probable-number (MPN) method for a single dilution (31, 34), were 2 to 24 MPN units/100 g of oyster meat. Because of contamination of sAGMK cells by adventitious viral agents, some blind passages were made in FRhK-4 cells as an alternative host. Because portions of some samples were lost to this contamination, concentrations of enteric viruses were computed only from the fraction of sample concentrate successfully assayed (Table 1).

Detection of enterovirus and HAV RNA in CPE-positive cell cultures by RT-PCR and oligoprobing. RNA extracted from CPE-positive cell culture lysates was subjected to RT-PCR for enteroviruses and HAV after removal of cellular debris and interfering materials by Freon extraction and spin column gel chromatography. As shown in Fig. 2, enterovirus RNA sequences were detected by RT-PCR and oligoprobe hybridization in 7 of 22 cell cultures, representing 6 of the 12 (50%) samples tested. HAV sequences were detected in 2 of the 12 samples by the same methods (Fig. 3). One HAV cell culture was also positive for enterovirus RNA. Overall, 8 of 22 CPE-positive culture flasks representing 7 of 12 (58%) CPE-positive samples were positive for enterovirus or HAV RNA by RT-PCR and oligoprobe hybridization. The identities of the viruses in the CPE-positive cell cultures that were negative for enterovirus or HAV RNA by RT-PCR and oligoprobe hybridization were not determined. It is possible that this CPE was due to other enteric viruses such as reoviruses.

Detection of enterovirus nucleic acid sequences in CPE-negative cell cultures by RT-PCR and oligoprobing. Culture flasks without visible CPE from both CPE-negative and CPE-positive samples were tested for the presence of enterovirus RNA by RT-PCR. The cells from three CPE-negative cell cultures per sample were pooled, and the RNA was extracted, with a final yield of 0.43 to 1.24 μg/ml. When 200-ng quantities of the extracted RNA were subjected to RT-PCR for enteroviruses, enterovirus-specific amplicons were not visible on agarose gels, presumably because of the presence of nonspecific

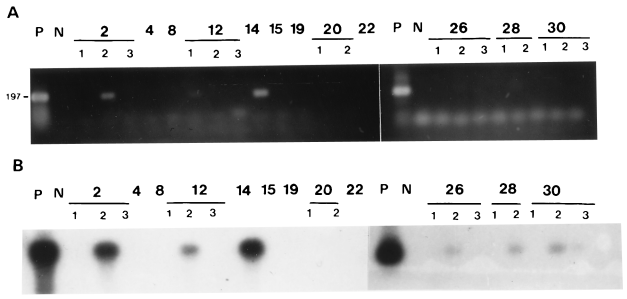

FIG. 2. Detection of enterovirus RNA in CPE-positive cell cultures of field oyster samples by RT-PCR and oligoprobe hybridization. Shown are the photographic results from gel electrophoresis (A) and subsequent oligoprobe hybridization (B) after Southern transfer of RT-PCR products. Lane numbers correspond to the harvest date for oyster samples (month-day-year–station numbers) (see below); small numbers below the sample numbers designate replicate flasks. 2, 5-14-91–2; 4, 6-3-92–2; 8, 7-9-91–2; 12, 8-26-91–2; 14, 9-9-91–3; 15, 9-30-91–2; 19, 12-2-91–2; 20, 8-5-91–2; 22, 1-6-92–3; 26, 11-18-91–3; 28, 3-2-92–3; 30, 5-11-92–2; N, complete reaction cocktail without virus; P, positive control RNA from poliovirus-infected cell culture.

amplification. However, after Southern transfer and oligoprobe hybridization, 6 of 19 CPE-negative samples (32%) were confirmed positive for enterovirus RNA (Fig. 4). Four of the six positive samples were positive only by RT-PCR amplification of extracted viral RNA; the other two samples were also positive by CPE in cell culture.

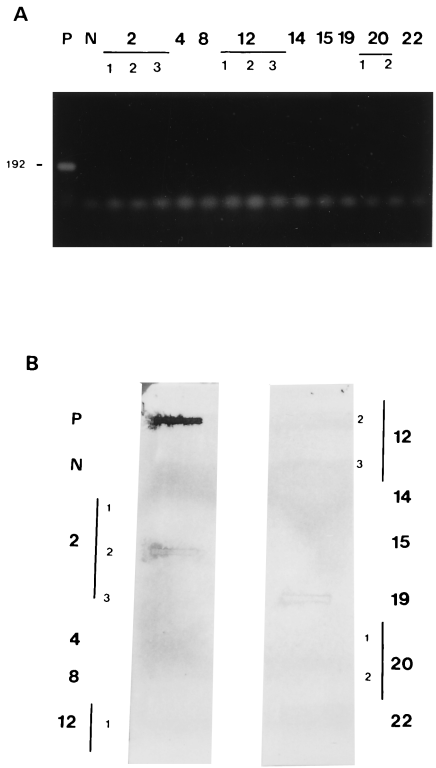

FIG. 3. Detection of HAV RNA in CPE-positive cell cultures of field oyster samples by RT-PCR and oligoprobe hybridization. Shown are the photographic results from gel electrophoresis (A) and subsequent oligoprobe hybridization (B) after dot blotting of RT-PCR products. Lane numbers correspond to the harvest date for oyster samples (month-day-year–station number) (see below); small numbers below the sample numbers designate replicate flasks: 2, 5-14-91–2; 4, 6-3-91–2; 8, 7-9-91–2; 12, 8-26-91–2; 14, 9-9-91–3; 15, 9-30-91–2; 19, 12-2-91–2; 20, 8-5-91–2; 22, 1-6-92–3; N, complete reaction cocktail without virus; P, positive control containing 500 PFU of HAV.

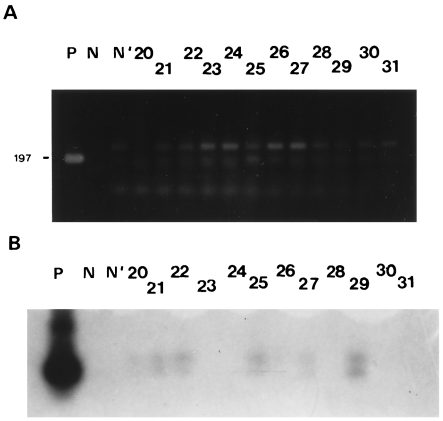

FIG. 4. Detection of enterovirus RNA in CPE-negative cell cultures of field oyster samples by RT-PCR and oligoprobe hybridization. Shown are the photographic results from gel electrophoresis (A) and subsequent oligoprobe hybridization (B) after Southern transfer of RT-PCR products. Lane numbers correspond to the harvest date for oyster samples (month-day-year–station number): 20, 8-5-91–2; 21, 1-6-92–2; 22, 1-6-92–3; 23, 2-3-92–2; 24, 2-3-92-3; 25, 11-18-91–2; 26, 11-18-91–3; 27, 3-2-92–2; 28, 3-2-92–3; 29, 4-13-92–2; 30, 5-11-92–2; 31, 5-11-92–3. N, complete reaction cocktail without RNA; N′, negative control RNA from uninoculated cell cultures; P, positive control RNA from poliovirus-infected cell culture.

Detection of viral RNA by direct RT-PCR. Direct detection of human enteroviruses and HAV by RT-PCR amplification and oligoprobe hybridization was applied to extracts of oyster samples that were further purified and concentrated by Freon extraction, PEG precipitation, and Pro-Cipitate adsorption-elution-precipitation. A total of 13.2 μl of the final extract, corresponding to about 1.7 g of original oyster meat, was analyzed by RT-PCR with each primer pair (panenterovirus and HAV). For the seven 50-g samples, 39.6 μl of the final extract, corresponding to about 8.9 g of original oyster sample, was tested by direct RT-PCR per primer pair. Of the 31 samples, 5 (16%) were confirmed positive for enterovirus RNA by direct RT-PCR and oligoprobe hybridization (Fig. 5). Analysis of RT-PCR products by gel electrophoresis revealed strong bands for two of the positive samples and weak bands for the remaining three. RT-PCR products were confirmed as being enteroviruses by internal oligonucleotide probe hybridization of RT-PCR products as slot blots (data not shown) and Southern transfers (Fig. 5). None of the 31 samples tested by direct RT-PCR and oligoprobe hybridization were positive for the presence of HAV RNA (data not shown).

Comparison of methods. Table 1 summarizes the enteric virus detection data for this study. When all methods were combined, a total of 18 of 31 field oyster samples (58%) harvested from a North Carolina coastal site impacted by chlorinated secondary sewage effluent were positive for human enteric viruses. Conventional cell culture infectivity was CPE positive for 12 of 30 samples (40%), RT-PCR of RNA extracts of these cell cultures or their lysates was positive in 6 of 31 samples (19%), and RT-PCR applied directly to oyster extracts further concentrated by Freon extraction, PEG precipitation, and Pro-Cipitate adsorption-elution-precipitation was positive in 5 of 31 samples (13%). When the data were combined, molecular techniques successfully detected human enteric viruses in 9 of 31 samples (29%). The molecular methods detected an additional five virus-positive samples over the samples that were positive by CPE in cell culture. The concordance

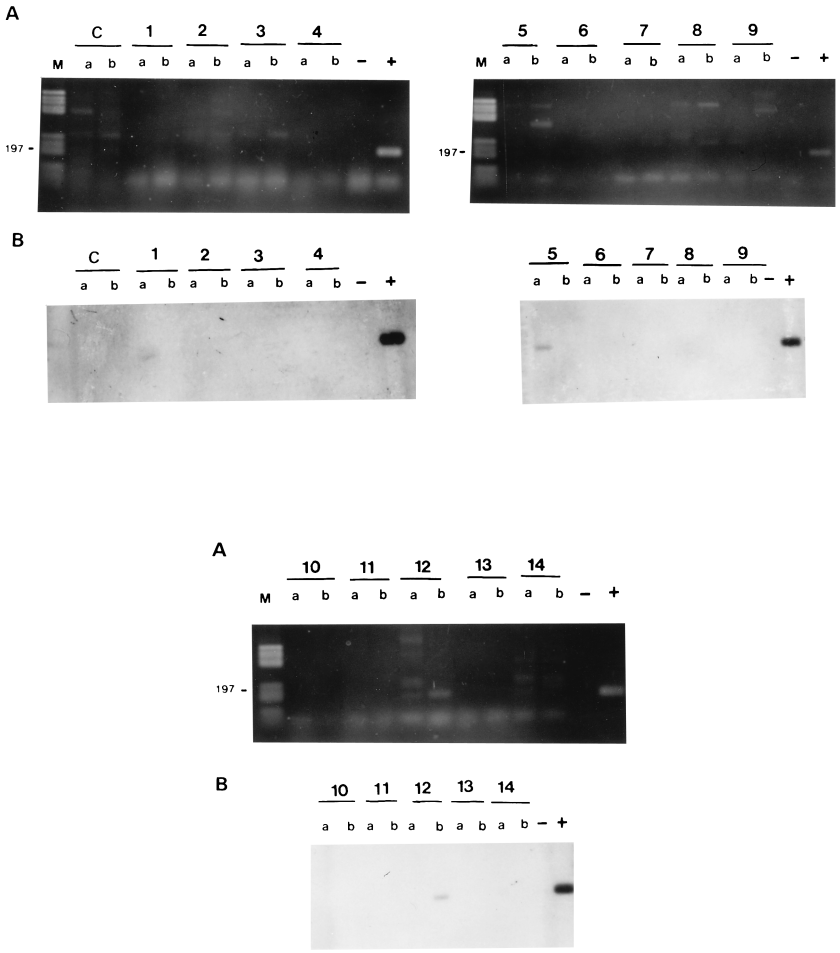

FIG. 5. Detection of enterovirus RNA in representative field oyster samples by direct RT-PCR. Shown are the photographic results from gel electrophoresis (A) and subsequent oligoprobe hybridization (B) after Southern transfer of RT-PCR products. Lane numbers correspond to the harvest date for oyster samples (month-day-year–station number) (see below); small letters below the sample numbers designate $\log_{10}$ dilutions (a and b corresponding to undiluted and 3.16-fold dilutions, respectively) of the final sample concentrates. 1, 9-11-91–2; 2, 11-4-91–2; 3, 7-9-91–2; 4, 6-3-91–2; 5, 8-26-91–2; 6, 7-22-91–2; 7, 6-17-91–2; 8, 5-14-91–2; 9, 9-30-91–2; 10, 3-2-92–2; 11, 11-18-91–2; 12, 6-24-91–2; 13, 5-10-92–2; 14, 4-13-92–2; M, marker; C, negative oyster control; +, positive control reaction containing 500 PFU of poliovirus; −, complete reaction cocktail without virus.

in virus positivity of samples between molecular methods and cell culture infectivity scored by CPE was 57%.

DISCUSSION

Detection of human enteric viruses in oysters and other bivalves has relied primarily on infectivity assays for CPE in primate cell cultures. There is considerable evidence that many naturally occurring enteric viruses may replicate in cell culture but show no apparent CPE (22, 23, 26, 30). Additionally, the development of CPE in cell culture does not ensure that the source of the enteric viruses is human, because some of these viruses, such as reoviruses, may have nonhuman as well as human hosts. Other assay methods such as immunoassays for viral antigens and direct hybridization assays for viral nucleic acids, are unable to detect the small numbers of viruses present in naturally contaminated shellfish. In vitro enzymatic amplification of target nucleic acids provides enrichment that facilitates the detection of low levels of viruses.

In this study, RT-PCR was applied to the detection of enteric viruses in naturally contaminated oyster samples and compared with detection by standard cell cultural procedures. Cell culture infectivity successfully detected enteric viruses by CPE in 12 of 30 oyster samples (40%), which is consistent with previous field studies reporting 25 to 40% enteric virus-positive shellfish samples from areas of the United States where harvesting is prohibited (8, 11, 37, 38). Some studies have reported somewhat lower rates of virus contamination (5, 35). The average viral load based on CPE in cell cultures of 7.3 MPN cytopathic units (MPNCU)/100 g of shellfish is within the range of previously reported contamination levels (1 to 200 MPNCU/100 g) (5, 8, 11, 35, 37, 38).

Molecular techniques alone were able to detect human enteric virus RNA in 10 of 31 oyster samples (32%). When the data from all analytical procedures were combined, enteric viruses were detected in 18 of 31 samples (58%). Nucleic acid detection by RT-PCR increased the number of positive samples by 50% over detection by cell culture CPE. This rate of detection (58%) is greater than previously reported in the literature for U.S. samples analyzed by cell culture CPE assays alone (8, 11, 37, 38). Increased virus detection by RT-PCR over cell culture CPE also has been reported for enteric viruses in sewage-contaminated surface waters (29).

HAV RNA was successfully detected by RT-PCR and oligoprobe hybridization in the cell culture lysates of two CPE-positive samples. It is highly likely that the HAV detected was infectious to AGMK cells. Hepatitis A virus is an important cause of shellfish-borne viral disease (1, 25) and yet only fairly recently has the detection of HAV in contaminated shellfish been reported (7, 24). Propagation of HAV in AGMK cells usually occurs without apparent CPE (3, 6, 21, 36). The production of CPE in these samples could have been due to HAV or to the replication of another cytopathic enteric virus that was not otherwise detected by molecular techniques. Nevertheless, RT-PCR amplification and oligoprobe hybridization provided confirmed HAV detection which would have been difficult if not impossible by cell culture methods alone.

The detection of virus-positive oyster samples by the three methods was inconsistent. Of the 12 samples that were CPE positive, only 4 (33%) were confirmed enterovirus positive by molecular techniques. This may be due to the absence of enteroviruses but the presence of other cytopathogenic enteric viruses in these shellfish samples. Grabow et al. (12) reported that the majority of enteric viruses detected on primary vervet kidney cells from polluted seawater and shellfish were reoviruses, and Havelaar et al. (13) detected both reoviruses and enteroviruses by CPE in cell cultures. In a 1-year study of human enteric viruses isolated from sewage effluent, Irving and Smith (15) found that up to one-third of 171 samples were positive for either adenoviruses or reoviruses in the absence of enteroviruses. Alternatively, the lack of concordance between analyses by cell culture infectivity and RT-PCR may be due to genetic differences among enteroviruses. Chapman et al. (4) tested a panenterovirus primer pair similar but not identical to the one used in this study for RT-PCR amplification of 66 known enterovirus serotypes and found that only two-thirds (41 of 66) were detectable. Other possibilities for the lack of enterovirus detection by RT-PCR and oligoprobe hybridization are insufficient RNA target or RT-PCR inhibition in the cell lysates or the final oyster concentrates (14, 17).

Of the samples positive for enteric viruses by molecular techniques, four of nine (44%) could not be confirmed by cell culture CPE. This indicates that a large number of CPE-negative samples did indeed contain enterovirus RNA. The most likely explanation for this observation is that these viruses were either noncytopathic or perhaps even nonculturable in the cell culture systems used (26). Payment and Trudel (22, 23) reported as high as 10-fold-greater detection limits for human enteric viruses in environmental concentrates when using an enzyme-linked immunoassay system in combination with cell culture infectivity. In their study, the absence of visible CPE did not mean the absence of infectious viruses.

A likely explanation for the lack of concordance in virus detection between cell culture CPE and RT-PCR amplification is the sample size analyzed by each method. Ninety percent of the extracts from 50-g oyster samples were inoculated into cell cultures for CPE, of which only 0.04% was actually tested by RT-PCR for enterovirus RNA in either CPE-negative or CPE-positive cultures. For the majority of samples, up to 10% of each 50-g oyster extract sample was processed by Freon extraction, PEG precipitation, and Pro-Cipitate adsorption-elution-precipitation but only 33% of this (3.3% of the original 50-g sample) was tested by direct RT-PCR. The volumes of sample concentrates analyzed by nucleic acid amplification methods were so small that the true extent of virus positivity in these samples was probably underestimated. It is likely that even more viruses would have been detected if a larger proportion of each sample had been tested by molecular techniques.

The significance of virus detection based on RT-PCR amplification of viral genomic RNA and its relationship to virus infectivity is uncertain. The methods used in this study extract, purify, and concentrate infectious particles, thereby allowing for a direct comparison of detection by cell culture infectivity, RT-PCR amplification, and a combination of the two approaches. In some previous applications of RT-PCR to the detection of enteric viruses in environmental samples (17) and shellfish (2, 10), such comparisons were not possible because RNA was extracted from the samples as an early step of processing for later viral RNA detection by RT-PCR alone. Disruption of virion integrity precluded a definitive comparison of virus detection by these nucleic acid-based molecular techniques and cell culture infectivity. The methods used in this study make possible further investigation of the relationships between detection by nucleic acid-based molecular techniques and virus infectivity. They should prove useful in determining virus prevalence in field samples of shellfish and in molecular epidemiological investigation of shellfish-borne disease outbreaks.

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