APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1993, p. 631–635
0099-2240/93/020631-05$02.00/0

Vol. 59, No. 2

Detection of Enteric Viruses in Oysters by Using the Polymerase Chain Reaction

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Received 7 July 1992/Accepted 21 November 1992

A procedure for the detection of enteric viral nucleic acid in oysters by the polymerase chain reaction was developed. Known quantities of poliovirus type 1 were seeded into oysters. Virus was extracted and concentrated by using organic flocculation and polyethylene glycol precipitation. Inhibitors of reverse transcription-polymerase chain reaction were present in the oyster extracts, preventing amplification of target viral nucleic acid. The use of cetyltrimethylammonium bromide precipitation sufficiently removed inhibitors to allow detection of as few as 10 PFU of poliovirus. Norwalk virus also could be detected after being seeded into oysters. This methodology may be useful for the detection of these and other shellfish-borne viral pathogens.

Public health concern about the safety of shellfish for human consumption has arisen periodically as outbreaks of shellfish-transmitted viral illness have occurred. The viruses responsible for the majority of nonbacterial, shellfish-transmitted illness are (i) hepatitis A virus (HAV) and (ii) Norwalk and Norwalk-like viruses. In recent years, the latter group of viruses has been responsible for the greatest incidence of shellfish-associated gastroenteritis illness in the United States (10, 22). Although outbreaks of gastrointestinal illness have occurred chiefly among consumers of raw shellfish, the concern over shellfish safety has not been limited to this group. Outbreaks of type A hepatitis and acute nonbacterial gastroenteritis have contributed to a public confidence problem over shellfish safety and also have resulted in serious financial losses by the shellfish industry and related seafood business (2, 20).

Fecal coliforms have long been considered the best, if slightly imperfect, indicator of fecal pollution of water. On the basis of many years of study, the National Shellfish Sanitation Program regards fecal coliforms to be the most practical indicator of shellfish water sanitary quality (17). However, these indicators have come under increasing challenge as acceptable indicators of viral health hazard in marine waters and shellfish (3). The two criticisms best substantiated against the use of fecal coliforms as a standard are the following: (i) shellfish and water samples meeting fecal coliform standards have been positive for enteric viruses on a number of occasions, and (ii) fecal coliforms may have a nonhuman source and, therefore, are unable to distinguish between human and nonhuman fecal pollution. Methods to remove bacterial pathogens from shellfish by depuration have not consistently removed viruses, and depurated shellfish have been the source of outbreaks of gastroenteritis (21, 26).

Until recently, direct tests of shellfish water or shellfish for the detection of viruses have been considered economically, technically, and practically unfeasible for routine monitoring purposes (reviewed in references 19 and 30). However, new biotechnology developments are making available rapid, sensitive, and specific tools for detecting HAV and gastroenteritis viruses in shellfish and shellfish-growing waters. This article describes the first phase of developing methods for detecting HAV and Norwalk viruses in shellfish by using the polymerase chain reaction (PCR). Poliovirus added to oysters was used initially to develop the method and assess its quantitative capability. The method then was applied to the detection of HAV and Norwalk virus in shellfish.

Poliovirus type 1 (strain LSC-1) was propagated and plaqued in Buffalo green monkey kidney cultures as described previously (18). Clarified virus suspensions were passed consecutively through two sterile 0.22-μm-pore-size low-protein-binding filters to give monodispersed preparations which were stored at −70°C until used. Cell culture-adapted HAV (strain HM-175) was propagated in persistently infected African green monkey kidney cultures as described previously (18). Virus was harvested and processed in the same manner described for poliovirus. Norwalk virus was obtained from the stools of human subjects who had been challenged with virus (14).

Oligonucleotide primers were selected from published sequences of the viruses being amplified: for poliovirus, the primers amplified the 5′ noncoding region (7, 28); for HAV, the primers amplified the VP3 region (11); for Norwalk virus, the primers amplified the polymerase region (14, 15). The sequence of the downstream primer for poliovirus was 5′-AC GGACACCCAAAGTA-3′, and for the upstream primer it was 5′-AGCACTTCTGTTTCCC-3′. The sequence of the downstream primer for HAV was 5′-CTCCAGAATCATC TCC-3′, and for the upstream primer it was 5′-ACAGGTAT ACAAAGTCAG-3′. The sequence for the downstream primer for Norwalk virus was 5′-CTTGTTGGTTTGAGGC CATAT-3′, and for the upstream primer it was 5′-ATAAAA GTTGGCATGAACA-3′. Complementary DNA was synthesized from RNAs recovered from ethanol precipitation. The RNA pellet was suspended in 30 μl of reverse transcription (RT) reaction mix containing 10 mM Tris hydrochloride (pH 8.3), 50 mM potassium chloride, 1.5 mM magnesium chloride, 3.3 μM downstream primer or 3.3 μM random hexamers (Promega Corp., Madison, Wis.), 667 μM deoxynucleoside triphosphates, 20 U of RNasin (Promega), and 5 U of avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.). In one experiment, several concentrations of type II oyster glycogen (Sigma, St. Louis,

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TABLE 1. Conditions for PCR amplification of viral nucleic acids

Virus	Conditions (temp and time) of:				
	Initial denaturation	Template denaturation[a]	Primer annealing[a]	Primer extension[a]	Final extension
Polio	94°C, 4 min	94°C, 1 min	49°C, 1 min 30 s	72°C, 1 min	72°C, 5 min
HAV	94°C, 3 min	94°C, 1 min	49°C, 1 min 20 s	72°C, 40 s	72°C, 15 min
Norwalk	94°C, 4 min	94°C, 1 min	55°C, 1 min 30 s	72°C, 1 min	72°C, 15 min

[a] Forty cycles of denaturation, annealing, and extension.

Mo.) were added to the RT mix. The RT mix was incubated for 1 h at 43°C and then placed on ice. Seventy microliters of PCR mix was added to the RT mix to yield a mixture containing 10 mM Tris hydrochloride (pH 8.3), 50 mM potassium chloride, 1.5 mM magnesium chloride, 1 μM of each primer, 200 μM deoxynucleoside triphosphates, and 5 U of *Taq* polymerase (Perkin-Elmer Cetus Corp., Norwalk, Conn.). The mixture was overlaid with mineral oil. In some PCR reactions, the plasmid pGHAV 1307A, which contains the VP3 region of the HAV genome, was used as the positive control or template DNA (12). All cDNA was amplified by using a PTC-100 thermal cycler (MJ Research, Inc., Cambridge, Mass.). Cycling conditions are shown in Table 1. Amplified products were 394, 207, and 470 bp in length for poliovirus, HAV, and Norwalk virus, respectively, and they were detected by agarose gel electrophoresis and ethidium bromide staining.

Bioaccumulation of poliovirus was performed in either 2 or 3 liters of Galveston Bay seawater or distilled water with Instant Ocean salts added to a 9-liter tray, and the salinity was adjusted to 16 ppt. From 0.5 to 1 g of soluble starch was added and distributed throughout the water. A virus stock suspension was added and distributed throughout the water. Two to three oysters were positioned centrally in the tray, and two aeration stones were placed on either side of the oysters. The tray was covered with aluminum foil, aeration was introduced, and bioaccumulation timing was started. Bioaccumulation periods of 15 to 16 h were used. At the end of the bioaccumulation period, the oysters were removed, rinsed with tap water and 95% ethanol, and then dried.

The final method developed involves two main steps. First, the virus is extracted and concentrated from oyster homogenates. Second, the viral nucleic acid is extracted from the oyster extract by using a method to remove inhibitors, and the nucleic acid is detected by RT-PCR (Fig. 1).

We first developed the method to extract and recover virus from oysters. This method required optimization to permit the subsequent detection of viral nucleic acid by RT-PCR. Previously, we had shown that virus could be effectively extracted and concentrated from oysters by using an extraction procedure coupled with concentration by polyethylene glycol (PEG) precipitation (18). Initially, the use of this method resulted in the formation of a large, dark viscous aqueous phase when the PEG pellet was treated with phenol-chloroform for nucleic acid extraction. This product made the separation of nucleic acid from oyster sample concentrates difficult. Trials of a virus concentration method reported by Bemiss et al. (1) resulted in the effective recovery of virus but also yielded a final sample which contained nonsuspendable particulate matter. However, by combining the Bemiss et al. extraction and PEG concentration procedures, a PEG pellet could be easily processed further for extraction of nucleic acid. The final method used in the study was as follows. Virus was extracted from shucked gulf oysters (*Crassostrea virginica*; The Dutchman's Seafoods, Houston, Tex.) which had been seeded with virus or which had bioaccumulated virus. Fifteen to twenty grams of oyster meats and fluids was added to 160 ml of 0.2 M glycine sodium chloride buffer (pH 9.5) in a Waring blender (Waring Products Division, Dynamics Corp. of America, New Hartford, Conn.). Four milliliters of antifoam B (J. T. Baker, Inc., Phillipsburg, N.J.) was added, and the mixture was blended at low speed for four 30-s intervals. Sixty milliliters of ice-cold trichlorotrifluoroethane (Freon 113; E. I. du Pont de Nemours & Co., Wilmington, Del.) was added, and four more cycles of homogenization were performed. After removal of the homogenate, the blender was washed with 50 ml of glycine sodium chloride buffer, and this wash was added to the homogenate. After the pH of the solution was adjusted to 9.5, 1.5 ml of Cat-Floc T (Calgon

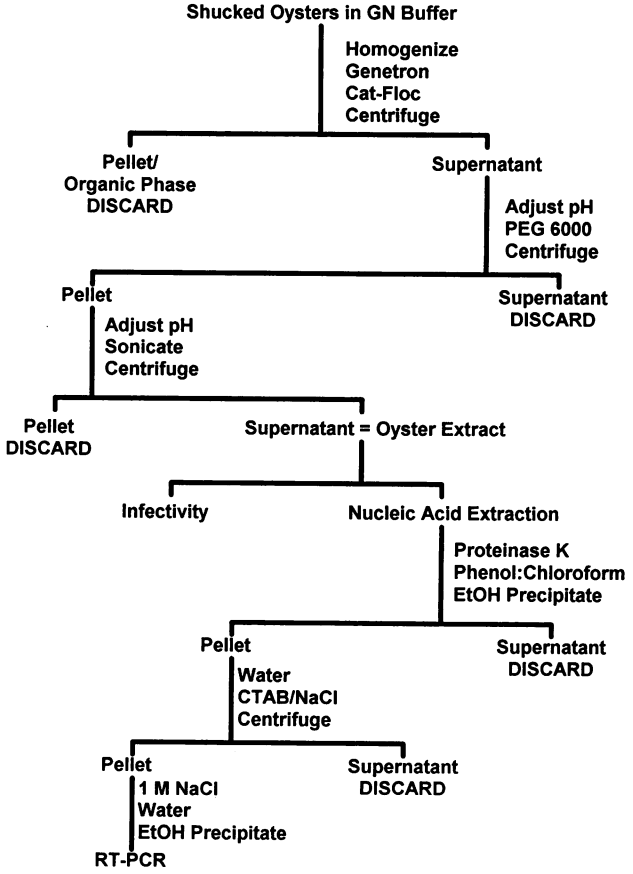

FIG. 1. Outline of method for concentration of virus and extraction of nucleic acid from oysters. CTAB, cetyltrimethylammonium bromide; EtOH, ethanol.

TABLE 2. Extraction of poliovirus from seeded oysters by using an organic flocculation-PEG method

Oyster	Input virus[a] (PFU)	Recovery[a] (PFU)	% Recovery
1	2.2×10^5	1.7×10^5	77
2	7.5×10^5	6.8×10^5	91
3	6.0×10^5	9.3×10^5	155
4	6.0×10^5	1.0×10^6	167
5	1.3×10^7	7.3×10^6	56
6	1.3×10^7	7.4×10^6	57
7	9.7×10^5	1.1×10^6	113
8	9.7×10^5	1.2×10^6	124
Mean (SD)			105 (42)

[a] Virus quantitated by plaque assay in Buffalo green monkey kidney cells.

Corp., Elwood City, Pa.) was added, and the mixture was stirred for 5 min. After 15 min of incubation of the mixture at room temperature, the sample was centrifuged for 20 min at 11,000 × *g* at 4°C. The aqueous phase was harvested, and the pH was adjusted to 7.4; PEG-6000 (BDH Ltd., Poole, England; as a 75% [wt/vol] solution in phosphate-buffered saline) was added to a final concentration of 8%. The mixture was stirred for 2 h at 4°C and centrifuged for 20 min at 11,000 × *g* at 4°C. The PEG pellet was suspended in 0.15 M disodium hydrogen phosphate (pH 9.3). The pH of the suspension was adjusted to 9.0 to 9.5, and the sample was sonicated for 30 s, shaken for 10 min at 250 rpm, and sonicated again for 30 s. The sample then was centrifuged for 10 min at 15,000 × *g* at 4°C, the supernatant was collected, and the pH was adjusted to 7.5. This supernatant was designated the oyster extract and used for enumeration of virus by plaque assay or processed further to extract nucleic acids. Good (105% ± 42%) recoveries of virus from seeded oysters were obtained by using this method (Table 2).

We then optimized the method to extract viral nucleic acid from oyster extracts to permit RT-PCR. The simple extraction of nucleic acid from oyster extracts was not sufficient for the recovery of nucleic acid which could be amplified in RT-PCR assays. For example, when oyster extracts were treated with sodium dodecyl sulfate-proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation in initial studies, no amplified products were seen after RT-PCR. When these concentrates were seeded with pGHAV-1307A plasmid DNA (12), no PCR products were seen unless the seeded sample was diluted more than 1,000-fold. An adsorption of the extracted nucleic acid to CF-11 cellulose powder (Whatman BioSystems, Ltd., Maidstone, England) was then tried, but no PCR products were seen. These results suggest that inhibitors that prevented amplification of nucleic acid by PCR were present in the oyster extracts. Hill et al. (9) reported a similar interference in attempts to amplify DNA from *Vibrio vulnificus*. Zhou et al. (30) suggested that polysaccharides present in the oyster and virus concentrates might be responsible for problems with nucleic acid detection by dot blot hybridization and that the use of the cationic detergent cetyltrimethylammonium bromide could remove these inhibitors (16). To determine if polysaccharides might also inhibit RT-PCR, several dilutions of type II oyster glycogen were added to reaction mixtures containing a constant amount of poliovirus RNA. Low concentrations of glycogen (≤3.13% [wt/vol]) did not inhibit enzymatic amplification of poliovirus RNA, but higher concentrations partially (6.25% glycogen; decreased band intensity) or completely (12.5% and 25% glycogen; no visible band) inhibited the RT-PCR. When precipitation with cetyltrimethylammonium bromide was used to remove glycogen or other inhibitors after a phenol-chloroform extraction step, viral nucleic acids were amplified successfully from oyster extracts.

The final method used for the extraction of viral nucleic acids from the virus in the oyster extracts was as follows. Nucleic acids were obtained from oyster extracts by an initial digestion with proteinase K (50 μg/ml) in 10 mM Tris hydrochloride (pH 7.5), 5 mM EDTA, and 0.5% sodium dodecyl sulfate at 56°C for 30 min. Subsequently, the digest was extracted twice with an equal volume of phenol-water-chloroform (68:18:14) (Applied Biosystems, Foster City, Calif.), and the aqueous phase was precipitated in ethanol. The resulting pellet was suspended in water, and cetyltrimethylammonium bromide (Sigma) and sodium chloride were added to final concentrations of 1.4% and 0.11 M, respectively. This mixture was incubated at room temperature for 30 min, and the nucleic acids were pelleted by centrifugation for 30 min at 12,100 × *g*. The pellet was suspended in 1 M sodium chloride. The sodium chloride was diluted with water and ethanol (final concentrations, 0.32 M NaCl and 35% ethanol). In some instances, the nucleic acids were further purified by adsorption onto 30 mg of CF-11 cellulose powder by shaking for 60 min at 250 rpm at 4°C (6, 29). After centrifugation for 6 min at 12,000 × *g*, the pellet was washed three times in 65% STE buffer (0.01 M sodium chloride, 0.05 M Tris hydrochloride [pH 7.0], 1 mM EDTA) and 35% ethanol. After the final ethanol wash, the nucleic acid was eluted from the cellulose in 100% STE buffer and precipitated in ethanol. Early experiments suggested that the use of a CF-11 cellulose adsorption step increased the amount of amplified products detected, but subsequent studies showed that the use of CF-11 cellulose did not increase the level of virus detection. The nucleic acid pellet was washed in 70% ethanol and suspended in RT mix for further use in RT-PCR assays. Viral nucleic acids were extracted from cell lysates in equal volumes of phenol-water-chloroform (68:18:14). After an additional chloroform extraction, the nucleic acids were precipitated in ethanol.

We then evaluated the sensitivity of virus detection in oysters. The level of poliovirus detection by RT-PCR was first determined by using serial 10-fold dilutions of stock virus lysate (titer, 3×10^7 PFU/ml). As little as 0.3 PFU of virus was detected (Fig. 2). This represents 9 to 90 virus particles on the basis of an estimated particle/PFU ratio of 30 to 300 (25). Thirty-eight PFU of poliovirus were detected after the addition of virus to oyster extracts (Table 3). Similarly, 10 PFU (an estimated 300 to 3,000 virus particles) of poliovirus were detected in processed samples of whole oysters containing exogenously added virus. For oysters that had bioaccumulated poliovirus, one-half of an extract was used to quantitate bioaccumulated virus; one-half of the remaining extract was used for RT-PCR. As few as 41 PFU of virus per sample tested, representing 164 PFU per whole oyster, were detected by RT-PCR (Table 3).

The same virus extraction methods were applied for the detection of HAV and Norwalk virus. Norwalk virus was detected after it was seeded into whole oysters and processed by the method described above (Fig. 3); Norwalk virus was also detected by using a different primer pair (NV primers 1 and 4 [15]; data not shown). Experiments also were performed to estimate the minimum numbers of Norwalk virus particles able to be detected in seeded oysters. Oyster samples (20 g) were seeded with 100 μl of 10-fold dilutions of a 50% suspension of a stool from a volunteer (no.

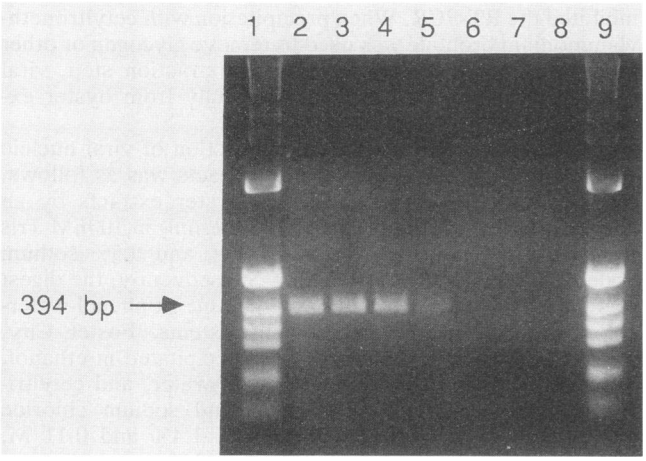

FIG. 2. Limit of detection of poliovirus RNA by using RT-PCR. Lanes 1 and 9 show DNA molecular weight markers (1-kb ladder). Appropriately sized PCR products were detected from the amplification of RNA from 3,000, 300, 30, 3, and 0.3 PFU of poliovirus (lanes 2 to 6, respectively). No PCR products were seen after amplification of RNA from 0.03 PFU of poliovirus or the negative reagent control (lanes 7 and 8, respectively).

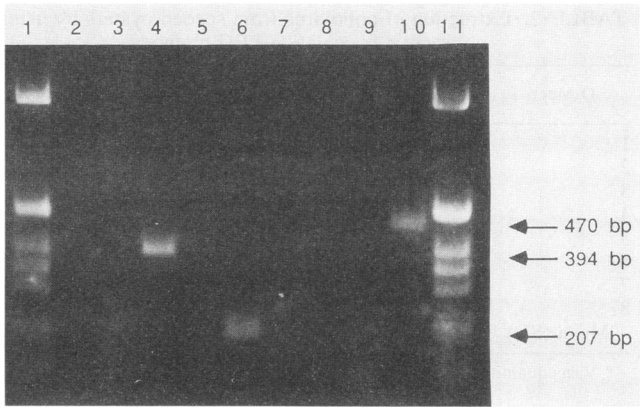

FIG. 3. Detection of viruses in oysters by using RT-PCR. Lanes 1 and 11 show DNA molecular weight markers. Lanes 2 to 4, 5 to 7, and 8 to 10 contain amplified products obtained with poliovirus, HAV, and Norwalk virus, respectively. No PCR products were obtained from reagent negative controls (lanes 2, 5, and 8), negative oyster controls (lanes 3 and 9), and HAV seeded into whole oysters (lane 7). Appropriately sized PCR products were detected after HAV was spiked into an oyster extract (lane 6) and poliovirus and Norwalk virus were spiked into whole oysters (lanes 4 and 10, respectively).

551) who was infected with Norwalk virus. After homogenization and processing of these samples with our protocol, 10 and 90% of the final nucleic acid sample was used for RT-PCR. The limit of virus detection was in the oyster seeded with the 10^{-2} dilution of the stool. This dilution represented a theoretical 0.5 μl of stool sample detected in a whole oyster. If one assumes that this stool contained a maximum of 10^5 to 10^6 particles of Norwalk virus per ml, our method detected 50 to 500 Norwalk virus particles. This estimate is based on the failure to detect virus in the stool by electron microscopy, a method which generally requires a minimum of approximately 10^6 virus particles per ml of feces. Unfortunately, because Norwalk virus cannot be grown or purified in sufficient quantities to obtain accurate particle counts, these numbers are only rough estimates (15). However, this estimate may be correct because we found a 33-fold difference in sensitivity for detection of Norwalk virus in the same stool. This would mean we detected 1.5 to 15 Norwalk virus particles in the stool, close to the theoretical limit of virus detection by using RT-PCR.

The results obtained when using the method in an attempt to detect HAV in seeded oysters were unexpected. HAV was not detected after being added to whole oysters but only after being spiked into oyster extracts. The failure to detect HAV when it was added to whole oysters suggested that the organic flocculation-PEG precipitation method of virus extraction was less effective for this virus. HAV was subsequently shown to be removed from the supernatant after the Cat-Floc step when the quantitative recovery of virus by the plaque assay was monitored for each step of the extraction procedure (data not shown). Other methods of HAV concentration, such as an antigen capture method, will need to be used for RT-PCR detection of HAV in shellfish (5).

TABLE 3. Level of detection of poliovirus by RT-PCR

Virus source	No. of PFU detected
Stock virus	0.3
Seeded oyster extract	38
Seeded whole oyster	10
Bioaccumulated oyster	164[a]

[a] Represents total for whole oyster. Only one quarter of the oyster was assayed by RT-PCR.

The specificity of the amplified products from the RT-PCRs was examined by analyzing the size of the amplicons and their susceptibility to cleavage with appropriate restriction enzymes. The products were of the predicted size, and restriction enzyme (Promega) digestion with *Bam*HI (poliovirus, 61 and 333 bp), *Hin*dIII (HAV, 53 and 154 bp), and *Hin*cII (Norwalk virus, 69 and 401 bp) yielded fragments of the predicted sizes (data not shown). Virus-specific primers did not yield amplified products when nucleic acid from other viruses was used or when virus was not added to oysters. RT-PCR products also were not seen when the Norwalk primers and RNA purified from human calicivirus, rotavirus, and other small round non-Norwalk viruses were used.

The applications of PCR technology are considerable since the usefulness of a thermostable DNA polymerase for the amplication of target nucleic acid has been recognized (24). Previous studies showed the potential of applying hybridization-based tests to the detection of human pathogens in shellfish, such as HAV and Norwalk virus, that are difficult or impossible to detect by other methods (13, 14, 19, 30). PCR technology has improved the level of nucleic acid detection over those of other methods (8). This is illustrated by our studies in which as little as 0.3 PFU of poliovirus was detected in cell lysates; this sensitivity is more than 1,000-fold higher than that of previously reported methods using dot blot hybridization (4, 23). In addition, Jansen et al. (11) have reported the use of RT-PCR in detecting as few as 3 to 30 HAV particles from fecal specimens. Similarly, compared with hybridization methods, RT-PCR increases the level of detection of Norwalk virus in stool samples (15). Because Norwalk virus cannot be cultured, RT-PCR represents a potentially sensitive and specific method of virus detection that can be used in place of cell culture methods and

previously has been estimated to detect as few as 10 to 1,000 virus particles in stool suspensions (15). Our results indicate possibly lower levels of detection in stool samples (1.5 to 15 particles), with the detection of Norwalk virus in oysters being at about 50 to 500 particles. While these numbers are only estimates, they are consistent with our more quantitative determinations for poliovirus.

The ability to detect very low levels of virus in cell lysate shows the potential of RT-PCR, but the results presented in this paper on virus detection in oysters illustrate the realities and difficulties of amplifying virus from samples other than cell culture lysates. Specifically, useful methods must both quantitatively concentrate low levels of virus and effectively remove inhibitors. The use of poliovirus as a model for quantitation in our shellfish studies was accompanied by direct application of the developed method to the successful detection of Norwalk virus in oysters. The results indicate that the RT-PCR technique can be directly applied to the detection of these or other gastroenteritis viruses in shellfish and shellfish waters. Such methods should be useful in the primary prevention of and evaluation of virus-related outbreaks of shellfish-transmitted disease (27).

This work was supported by grants from the National Shellfish Indicator Study funded by the National Oceanic and Atmospheric Administration and the Texas A&M University Sea Grant College Program. R.L.A. is a General Clinical Research Center Clinical Associate Physician and is supported in part by the Division of Research Resources of the National Institutes of Health under grant RR-00350.

We thank Xi Jiang and Cheryl Woodley-Miller for helpful discussions.

REFERENCES

1. **Bemiss, J. A., M. M. Logan, J. D. Sample, and G. P. Richards.** 1989. A method for the enumeration of poliovirus in selected molluscan shellfish. J. Virol. Methods **26**:209–218.
2. **Brown, J. W., and W. D. Folsom.** 1983. Economic impact of hard clam associated outbreaks of gastroenteritis in New York state. NOAA technical memorandum NMFS-SEFC-121. U.S. Department of Commerce, Charleston, South Carolina.
3. **Centers for Disease Control.** 1991. Gastroenteritis associated with consumption of raw shellfish—Hawaii, 1991. Morbid. Mortal. Weekly Rep. **40**:303–305.
4. **Cova, L., H. Kopecka, M. Aymard, and M. Girard.** 1988. Use of cRNA probes for the detection of enteroviruses by molecular hybridization. J. Med. Virol. **24**:11–18.
5. **Desenclos, J.-C. A., K. C. Klontz, M. H. Wilder, O. V. Nainan, H. S. Margolis, and R. A. Gunn.** 1991. A multistate outbreak of hepatitis A caused by the consumption of raw oysters. Am. J. Public Health **81**:1268–1272.
6. **Franklin, R. M.** 1966. Purification and properties of the replicative intermediate of the RNA bacteriophage R17. Proc. Natl. Acad. Sci. USA **55**:1504–1511.
7. **Gama, R. E., P. R. Horsnell, P. J. Hughes, C. North, C. B. Bruce, W. Al-Nakib, and G. Stanway.** 1989. Amplification of rhinovirus specific nucleic acids from clinical samples using the polymerase chain reaction. J. Med. Virol. **28**:73–77.
8. **Guatelli, J. C., T. R. Gingeras, and D. D. Richman.** 1989. Nucleic acid amplification in vitro: detection of sequences with low copy numbers and application to diagnosis of human immunodeficiency virus type 1 infection. Clin. Microbiol. Rev. **2**:217–226.
9. **Hill, W. E., S. P. Keasler, M. W. Trucksess, P. Feng, C. A. Kaysner, and K. A. Lampel.** 1991. Polymerase chain reaction identification of *Vibrio vulnificus* in artificially contaminated oysters. Appl. Environ. Microbiol. **57**:707–711.
10. **Institute of Medicine.** 1991. Microbiological and parasitic exposure and health effects, p. 30–53. *In* F. E. Ahmed (ed.), Seafood safety. National Academy Press, Washington, D.C.
11. **Jansen, R. W., G. Siegl, and S. M. Lemon.** 1990. Molecular epidemiology of human hepatitis A virus defined by an antigen-capture polymerase chain reaction method. Proc. Natl. Acad. Sci. USA **87**:2867–2871.
12. **Jiang, X., M. K. Estes, and T. G. Metcalf.** 1987. Detection of hepatitis A virus by hybridization with single-stranded RNA probes. Appl. Environ. Microbiol. **53**:2487–2495.
13. **Jiang, X., M. K. Estes, T. G. Metcalf, and J. L. Melnick.** 1986. Detection of hepatitis A virus in seeded estuarine samples by hybridization with cDNA probes. Appl. Environ. Microbiol. **52**:711–717.
14. **Jiang, X., D. Y. Graham, K. Wang, and M. K. Estes.** 1990. Norwalk virus genome cloning and characterization. Science **250**:1580–1583.
15. **Jiang, X., J. Wang, D. Y. Graham, and M. K. Estes.** 1992. Detection of Norwalk virus in stools using the polymerase chain reaction. J. Clin. Microbiol. **30**:2529–2534.
16. **Jones, A. S.** 1963. Use of alkyltrimethylammonium bromides for the isolation of ribo- and deoxyribo-nucleic acids. Nature (London) **199**:280–282.
17. **Larkin, E. P., and D. A. Hunt.** 1982. Bivalve mollusks: control of microbiological contaminants. Bioscience **32**:193–197.
18. **Lewis, G. D., and T. G. Metcalf.** 1988. Polyethylene glycol precipitation for recovery of pathogenic viruses, including hepatitis A virus and human rotavirus, from oyster, water, and sediment samples. Appl. Environ. Microbiol. **54**:1983–1988.
19. **Metcalf, T. G., X. Jiang, M. K. Estes, and J. L. Melnick.** 1988. Nucleic acid probes and molecular hybridization for detection of viruses in environmental samples. Prog. Med. Virol. **35**:186–214.
20. **Morse, D. L., J. J. Guzewich, J. P. Hanrahan, R. Stricof, M. Shayegani, R. Deibel, J. C. Grabau, N. A. Nowak, J. E. Herrmann, G. Cukor, and N. R. Blacklow.** 1986. Widespread outbreaks of clam- and oyster-associated gastroenteritis. Role of Norwalk virus. N. Engl. J. Med. **314**:678–681.
21. **Richards, G. P.** 1985. Outbreaks of shellfish-associated enteric virus illness in the United States: requisite for development of viral guidelines. J. Food Protect. **48**:815–823.
22. **Richards, G. P.** 1987. Shellfish-associated enteric virus illness in the United States, 1934–1984. Estuaries **10**:84–85.
23. **Rotbart, H. A.** 1990. Enzymatic RNA amplification of the enteroviruses. J. Clin. Microbiol. **28**:438–442.
24. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**:487–494.
25. **Schwerdt, C. E., and J. Fogh.** 1957. The ratio of physical particles per infectious unit observed for poliomyelitis viruses. Virology **4**:41–52.
26. **Sobsey, M. D., A. L. Davis, and V. A. Rullman.** 1987. Persistence of hepatitis A virus and other viruses in depurated eastern oysters, p. 1740–1745. *In* Proceedings Oceans 87, Halifax, Nova Scotia, vol. 5. Marine Technology Society, Washington, D.C.
27. **Todd, E.** 1990. Foodborne illness. Epidemiology of foodborne illness: North America. Lancet **2**:788–790.
28. **Toyoda, H., M. Kohara, Y. Kataoka, T. Suganuma, T. Omata, N. Imura, and A. Nomoto.** 1984. Complete nucleotide sequences of all three poliovirus serotype genomes. Implication for genetic relationship, gene function and antigenic determinants. J. Mol. Biol. **174**:561–585.
29. **Wilde, J., J. Eiden, and R. Yolken.** 1990. Removal of inhibitory substances from human fecal specimens for detection of group A rotaviruses by reverse transcriptase and polymerase chain reactions. J. Clin. Microbiol. **28**:1300–1307.
30. **Zhou, Y.-Z., M. K. Estes, X. Jiang, and T. Metcalf.** 1991. Concentration and detection of hepatitis A virus and rotavirus nucleic acids in shellfish. Appl. Environ. Microbiol. **57**:2963–2968.