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Evaluation of a Method for Recovering Poliovirus 1 from 100-Gram Oyster Samples

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ABSTRACT

A method is described that uses commonly available laboratory equipment and materials to detect low numbers of poliovirus 1 in oysters. Thirty 100-g oyster samples inoculated with poliovirus 1 were processed by blending at pH 4.8 in water, centrifuging, extracting the pellet at pH 9 in a mixture of Eagle's medium, nonfat dry milk, $MgCl_2 \cdot 6H_2O$, and Freon TF, and centrifuging again. The supernatant fluids were diluted in water, precipitated at pH 4.8 and centrifuged. The pellets were resuspended in Na_2HPO_4 and Cat-Floc, and centrifuged. The final supernatant fluids (~10 ml per sample) were assayed for viral plaque-forming units (PFU) in BGM African monkey green kidney cell monolayers. The average inoculum per sample was 95 PFU, and the average recovery from 30 samples was 53 PFU. The percent recovery with 95 percent confidence intervals was 55.4 ± 2.1.

Viruses have been isolated from estuary waters throughout the world, and indigenous filter-feeding shellfish have been shown to bioaccumulate viruses from these waters (*14*). Enteroviruses were the most frequently isolated viruses, with a contamination level ranging from 1 to 224 viral infectious units per 100 g of shellfish tissue (*6*). Viruses have been isolated from shellfish harvested from both approved and unapproved waters. However, the incidence of virus contamination in animals recovered from approved areas was sporadic and of low level (*12*).

Virus diseases in humans have been associated with the consumption of raw shellfish (*15,16*). The reported incidence of disease is usually low, but numerous shellfish-associated outbreaks occurred in 1982 (FDA, unpublished data). When minimal shellfish contamination occurs, susceptible consumers may become infected and spread the disease to others. Thus the transmission route could be construed to be person-to-person contact, and the primary shellfish vehicle might go undetected (*1*). Such infections of unknown frequency would probably not be reported or considered in shellfish-associated disease incidence.

Relationships between human viral diseases and shellfish consumption are unclear because few effective methods exist for recovering low numbers of viruses (*11*). In a previous study, analysis of 20-g samples of oyster homogenate worked well in laboratory studies on virus persistence in refrigerated oysters (*20,21*). When the method was used to study oysters from different sources, cytotoxicity was encountered. This study describes a method that uses commonly available laboratory equipment and materials, has minimum cytotoxicity problems, and detects low numbers of poliovirus 1 in 100-g samples of oysters. The potential for detecting viruses in the sample was enhanced by increasing the sample size.

MATERIALS AND METHODS

Oysters (Crassostrea virginica)

Oysters were harvested from Mobile Bay, AL, and shipped to the Cincinnati Research Laboratory, where they were stored in the shell at 5°C until used. All oysters were processed within 1 wk after harvest, and the shellfish were shucked and assayed on the same day.

Cells

The BGM African green monkey kidney cell line (BGM) was used in passages 158 through 160 (*3*). Cells were incubated at 36°C in planting medium composed of equal volumes of Leibovitz L-15 medium (*13*) and Eagle's minimum essential medium with Hanks' balanced salts (MEMH) (*4,7*), supplemented with 10% fetal bovine serum and antibiotics (100 units of penicillin G, 100 μg of streptomycin sulfate and 50 μg of gentamicin per ml). Double-distilled water was used in the preparation of media and reagents. The cells were treated with 0.02% tetrasodium ethylenediamine tetraacetate (EDTA) in phoshate buffered saline, split 1 to 10, and passaged weekly in 150-cm^2 polystyrene tissue culture flasks (Corning Glass Works, Corning, NY).

Virus

Poliovirus 1 (Chat strain, ATCC VR-192) was propagated in monolayers of BGM cells and harvested after a 4+ cytopathic effect was observed. The culture fluid containing the virus was filtered through a 0.22 μm-porosity membrane. The filtrate was shaken vigorously with an equal volume of Freon TF[1] (DuPont, Inc., Wilmington, DE) and centrifuged at $4000 \times g$ at 10°C for 30 min. The supernatant fluid was then refiltered. A stock solution containing approx. 100 viral plaque-forming units (PFU) per ml was prepared in planting medium and stored at 5°C.

Sample preparation

Each 100-g sample consisted of approx. 10 standard size oysters. The shellfish were shucked, and the liquor and meat were poured into a sterile,

[1] *Use of trade names is for identification purposes only and does not constitute endorsement by the U. S. Department of Health and Human Services.*

disposable 220-ml polyethylene specimen container (Beckton, Dickinson Labware, Oxnard, CA).

Viral stock solution (1 ml) was inoculated into the sample, and 1 ml from this solution was analyzed concurrently with each final oyster eluate. The eluates of thirty 100-g oyster samples and thirty 1-ml portions of the stock solution were assayed. This procedure provided a good estimate of the number of PFU injected and the number of PFU recovered, in that all of the final eluate was assayed. Five inoculated 100-g oyster samples, five 1-ml stock virus controls, one 100-g sample of negative oysters, and one uninoculated BGM cell monolayer (negative cells) were processed on each of the 6 assay days. Three batches of oysters that were harvested weekly from the same area were used in the study.

Adsorption of viruses onto oyster tissues

The 100-g oyster sample was poured into a 1-L blender, and 1 ml of Antifoam C (Dow Corning, Midland, MI) was pipetted onto the oysters. The oyster specimen container was rinsed with 150 ml of 37°C-distilled water, which was then poured into the blender. Into the mixture was pipetted 2.5 ml of 1 N HCl. The container contents were then blended for 20 s at 18,500 rpm (Waring Blender Products, New Hartford, CT). The homogenate was adjusted (using 1-s blending mixes) to pH 4.8 ± 0.1, with addition of 1 N HCl or 1 N NaOH as needed. The blend was poured through a PF 100 polypropylene funnel (Nalge Co., Rochester, NY) into a 250-ml linear polyethylene centrifuge bottle (Nalge). The centrifuge bottles were tightly capped to prevent leakage during the various assay procedures. The blend was centrifuged at $5000 \times g$ (5500 rpm) for 10 min without refrigeration in a GSA rotor using a Sorvall RC-5B refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, CT). The supernatant fluid was poured off and discarded.

Elution of viruses from oyster tissues

The following cold reagents (~5°C) were added to the pellet (~40 g): 50 ml of MEMH without phenol red or $NaHCO_3$, 10 ml of 10% wt/vol nonfat dry milk (Carnation Co., Los Angeles, CA), 2 ml of 50% $MgCl_2 \cdot 6H_2O$, 50 ml of Freon TF, and 3 ml of 1 N NaOH. The mixture was vigorously shaken horizontally for 5 min on a wrist action shaker (Burrell Corp., Pittsburgh, PA), the pH was adjusted to 9.1 ± 0.1 with 1 N NaOH as required, and the mixture was centrifuged at $5000 \times g$ for 20 min at 10°C.

Concentration of viruses by precipitation

The supernatant fluid (~80 ml) was pipetted into a 250-ml centrifuge bottle, taking care not to harvest the Freon TF which settled below the supernatant fluid. The sediment and Freon TF were discarded. Enough 37°C-distilled water (~170 ml) was added to the supernatant fluid to bring the liquid level to the shoulder of the top of the bottle. A floc was formed (15 min) by adjusting the pH of the liquid to pH 4.8 ± 0.1 by adding 1 N HCl (~1 ml). The sample was centrifuged at $1500 \times g$ (3000 rpm) without refrigeration for 10 min. The supernatant fluid was poured off and discarded.

Elution of viruses from precipitate

Four ml of 0.2 M Na_2HPO_4 (~23°C) and 1 ml of freshly prepared 10% Cat-Floc (Calgon Corp., Ellwood, PA) were added to the pellet (~10 g), and the pellet was suspended by vortex mixing for 30 s. The suspension (pH 7.5 ± 0.2) was centrifuged at $10,000 \times g$ (8000 rpm) for 20 min at 10°C.

Plaque assay of eluate

The supernatant fluid (~10 ml) was pipetted onto two 150-cm^2 BGM cell monolayers that had been previously rinsed with 50 ml of MEMH adjusted with 7.5% $NaHCO_3$ to pH 7.0. The pellet was discarded. The inoculated cell monolayers were incubated at 36°C for 2 h. The culture flasks were rocked at least twice during incubation to redistribute the eluate over the cell monolayers. The monolayers were then overlaid.

Agar-medium overlay

The plaque assay agar was prepared by adding 12 g of purified agar (Oxoid) to 500 ml of distilled water, autoclaving at 121°C for 15 min, and tempering at 47°C for 30 min. The plaque assay medium was prepared by adding 10 ml of 50% $MgCl_2 \cdot 6H_2O$, 10 ml of 1% diethylaminoethyl (DEAE) dextran ($MW = 5 \times 10^5$), 15 ml of 7.5% $NaHCO_3$, 15 ml of 0.1% neutral red, 30 ml of 10% (wt/vol) autoclaved (10 min) nonfat dry milk and 1 ml (50 mg) of gentamicin sulfate to 420 ml of double-strength MEMH. The medium was brought to 36°C in a waterbath and placed in a 36°C-incubator until use.

Overlay procedure

The tempered agar was poured into the 36°C-meidum, and the two were mixed by inverting the flask a few times. The mixture (60 ml) was poured into the cell monolayer flask onto the side opposite the cell sheet, and the flask was rotated so that the agar flowed over the monolayer once and then covered the cells. The overlay was allowed to solidify at room temperature (~23°C). The flasks were covered with cloth to exclude light during solidification (~15 min) and then incubated at 36°C, agar side up, covered with cloth in a dark incubator. The monolayers were incubated for 7 d, and the plaques (which appeared as expanding clear foci of cellular necrosis during days 2 through 5), were marked and counted as they appeared.

RESULTS AND DISCUSSION

An overall mean of 52.9 PFU were recovered from thirty 100-g samples of oysters that had been inoculated with an average of 95.5 PFU per sample. No PFU were detected in the negative controls. The estimated 100% input inoculum for each of five oyster samples assayed on a given day was determined by averaging the PFU counted in the five virus stock solution assays of that day (Table 1). The virus recovery over days did not differ significantly ($\alpha = 0.05$). The percent coefficients of variation for the assays on different days ranged from 4.1 to 25.3. The 95% confidence interval for the mean percent recovery of viruses from the oyster samples was 55.4 ± 2.1 (*5*).

The method is a composite of published techniques and innovative modifications. Enhancing the adsorption of viruses to cells was shown to depend on the temperature of the suspending media. More efficient adsorption occurred at 37°C, and more efficient elution occurred at temperatures approaching 0°C (*17*). Viruses adsorbed to precipitates more efficiently in solutions of low salt concentration and acid pH (*18*). These data were used to develop the first step of the method in that 37°C-distilled water was used to dilute the 100-g sample to 250 ml and permitted initial use of a single centrifuge bottle for each sample (*9,22*). Six samples could be processed within 6 h.

Salt concentrations of approx. 1% and an alkaline pH have been used to elute viruses from food (*19,21*), and nonfat dry milk was used to elute viruses from filters (*2*). Freon TF, in

TABLE 1. *Means and coefficients of variation for recovery of poliovirus 1 from 100-g samples of oysters* (Crassostrea virginica).

Assay day	Average PFU/100 g recovered		Average PFU/ml of inoculum	
	Mean	% CV[a]	Mean	% CV
1	47.2[b]	22.3	89.0	10.3
2	44.8	16.7	105.2	4.1
3	64.0	16.9	91.2	11.2
4	53.4	13.3	104.4	10.9
5	52.0	25.3	93.4	7.0
6	56.0	10.0	89.8	13.5
Overall mean	52.9	----	95.5	----

[a]Percent coefficient of variation (% CV) = (standard deviation/mean) × 100.

[b]Five observations per mean.

addition to being a lipid solvent, has been shown to disrupt coproantibody-viral complexes, thereby enhancing the probability of detecting these viruses (*8*). These procedures were incorporated into the second step of the method. The $MgCl_2 \cdot 6H_2O$ solution was used to enhance separation of the sediment during centrifugation.

The third step of the method was similar to the first step. The centrifuged sediment was more homogeneous in this low-salt concentration, acid-pH precipitation, and it was lower in bulk than the sediment recovered in the first step.

The final elution of the viruses from the sediment required a nontoxic, isotonic solution for assay on the BGM cell monolayers. The Cat-Floc removed toxicity (*10*), and the 0.2 M Na_2HPO_4, pH 9.1, solution was diluted by residual sample liquid to a concentration of ~0.8% salts (pH~7.5) in the final 10-ml eluate. Assay of the eluate in two 150-cm^2 monolayers was convenient and reduced initial and subsequent handling associated with the use of many smaller monolayers.

The method is uncomplicated and could be finished in 1 d with a consistent recovery of low numbers of viruses from six large oyster samples.

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