Improved Method and Test Strategy for Recovery of Enteric Viruses from Shellfish

THEODORE G. METCALF,* ELLEN MOULTON, AND DANIEL ECKERSON

Department of Microbiology, University of New Hampshire, Durham, New Hampshire 03824

An improved recovery method and testing strategy were devised for recovery of low numbers of enteric viruses from each of three commercially important shellfish species. Effective recovery of virus depended as much upon details of the test strategy adopted for use of the improved method with each species as on the method itself. The most important test details involved sample composition, pool size, and method of use of cell cultures. Recovery sensitivity measured permitted detection of 25 to 3 plaque-forming units of enteroviruses and 100 to 27 plaque-forming units of reovirus through their recovery in cell culture, with effectivenesses averaging 64 and 46%, respectively. Test samples prepared by the improved recovery method were virtually cytotoxicity free. Optimal recovery of virus on 45-cm² cell culture monolayers was obtained with 1-ml inocula adsorbed for 2 h. The most effective recovery of virus from shellfish samples was made by a sequential adsorption procedure which allowed equal exposure of an entire sample to each of two or more cell cultures. Removal of nonviral contaminants from test samples by antibiotic treatment was preferable to the use of ether or membrane filtration procedures.

Surveillance of the sanitary quality of shellfish and shellfish-growing waters has been carried out since 1925 in the United States, when a certification program administered by the U.S. Public Health Service was instituted (1). This monitoring program, which developed into the National Shellfish Sanitation Program (NSSP), was instituted after a typhoid epidemic resulting in 1,500 cases with 150 deaths was attributed to sewage-polluted oysters (13). This epidemic, coming after many previously reported outbreaks of enteric disease attributed to shellfish, helped to focus critical attention upon the health hazards associated with the consumption of polluted shellfish. Since then a number of shellfish-associated outbreaks of infectious hepatitis and viral gastroenteritis have been reported. These reports have been reviewed and summarized recently by Gerba and Goyal (5). All of the commercially important shellfish, oysters, hard- and soft-shell clams, and mussels, have been incriminated as vehicles for the transmission of enteric virus diseases to humans.

The NSSP recommends the use of both total coliform and fecal coliform standards for monitoring the microbial quality of shellfish and shellfish-growing waters (21). The relationship of these bacterial standards to the presence of enteric viruses in shellfish is not clear (4, 6, 15, 18). The adequacy of these standards for assessing the safety of shellfish from virus contamination has been challenged, most notably after outbreaks of infectious hepatitis from oysters harvested from NSSP-approved growing areas (16).

Suitable virus monitoring strategies and methods are needed which will make it possible to accurately compare enteric virus and fecal coliform contents of shellfish to determine when a virus carriage state exists and its significance to the process of transmission of virus pathogens to humans.

Monitoring strategies, to be effective, require decisions for each of the commercially important species to determine how many shellfish are needed for a representative sample, whether samples should include the entire shellfish or only selected tissues, what method(s) possesses maximum sensitivity and accuracy for the recovery of small numbers of natural virus, and how many shellfish per pool can be tested without loss of a recovery method's sensitivity and accuracy. Factors important to the effectiveness of a recovery method include not only the cell culture(s) used but also how it is used, test sample adsorption procedures, and whether a test sample is cytotoxic for the cell culture(s).

No serious attempt has been made to develop a single comprehensive monitoring strategy for all commercially important shellfish species in which development and use of a virus recovery method has been coordinated with the other parameters influencing monitoring effectiveness on a species-to-species basis. An improved re-
covery method developed with oysters has not been critically assessed in soft- and hard-shell clams or mussels (19). Coordination and combinations of cell cultures recommended for wastewater and fecal samples and inclusion of procedures to measure cytopathic effect (CPE) along with plaque-forming unit (PFU) formation have not been adequately investigated with shellfish samples (17). This study describes the development of a virus monitoring strategy for recovery of enteric viruses from each of three commercially important shellfish species. The monitoring strategy described includes the development of a single recovery method equally effective with oysters and hard- and soft-shell clams and identification of the test strategy needed for effective use of the improved method for recovery of low numbers of enteric viruses from each of these species.

MATERIALS AND METHODS

Cell cultures. A Buffalo Green monkey kidney continuous cell line (BG(M) obtained in passage 104 from Gerald Berg, Environmental Protection Agency, Cincinnati, Ohio, was used for virus propagation and assay. Cells were grown in a medium consisting of equal parts of Eagle minimum essential medium (MEM) with Hanks balanced salt solution and modified L-15 medium (MEM-L15), supplemented with 1% nonessential amino acids and 10% heat-inactivated fetal calf serum (FCS) and buffered with 0.4% HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer. Antibiotics were added to give final concentrations (per milliliter) of: 100 U of penicillin G, 100 µg of streptomycin sulfate, and 2 µg of amphotericin B (Fungizone). Cultures were maintained in MEM-L15 medium containing 2% heat-inactivated FCS.

Primary African Green monkey kidney cultures (PMK) were used in studies of methods of use of cell cultures promoting the most effective virus recovery from shellfish samples. Cultures were prepared from suspensions purchased from Microbiological Associates, Walkersville, Md. Melnick A medium with 10% FCS was used for growth, and Melnick B medium with 2% FCS was used for maintenance of cultures. Antibiotics as described for BG(M) cultures were used with PMK cultures.

Human epidermoid carcinoma culture, ATCC-CCL23 (HBep-2), was also used in cell culture system methods of use studies. Eagle MEM with Hanks balanced salt solution and 5% FCS was used for growth, and the same basal medium with 2% FCS was used for maintenance.

Viruses and virus assays. Test enteric viruses included poliovirus 2 (W-2), coxsackievirus B1 (natural virus isolate), coxsackievirus B3 (Nancy), coxsackievirus B5 (natural virus isolate), echovirus 7 (Wallace), and reovirus 1 (Lang). All viruses were plaque purified, and their identities were confirmed in serum neutralization tests. Crude stocks were prepared by infection of BG(M) monolayers on maintenance medium in 670-cm² roller bottles. Virus harvests were made after generalized CPEs occurred by freezing and thawing infected cultures three times. Harvests were clarified at 12,000 × g for 15 min at 5°C, passed through serum-treated membrane filter assemblies to monodisperse virus (22), and frozen and stored at -76°C. Virus titers of 10⁷ to 10⁹ PFU/ml were obtained routinely.

Virus assays performed in duplicate were made by plaque counts (PFU) on either 9- or 45-cm² monolayers in drained 1-ounce (30-ml) or 6-ounce (180-ml) bottles, respectively. Inoculum volumes were 0.1 ml for 9-cm² monolayers and 1 ml for 45-cm² monolayers. Adsorption was carried out at 37°C for 1.5 h with 9-cm² monolayers (1-ounce bottles) and 2 h with 45-cm² monolayers (6-ounce bottles). Assays for poliovirus, coxsackievirus, and echovirus were made with an agar overlay medium containing (per 100 ml): 100 U of penicillin; 100 µg of streptomycin; 1.5% agar (Difco Laboratories, Detroit, Mich.); 40 ml of 2X MEM; 1 ml each of 100X L-glutamine, 100X nonessential amino acids, 1% MgCl₂, and undilute sterile liquid milk (Real Fresh Inc., Visalia, Calif.); 2 ml of heat-inactivated FCS; 3 ml of 7.5% NaHCO₃; and 0.6 ml of 1-300 neutral red (2). Reovirus assay overlays substituted pancreatic (Oxoid Ltd., London, England) in a final concentration of 1/70 for MgCl₂. Five-milliliter agar overlay volumes were used with 1-ounce bottles, and 20-ml volumes were used with 6-ounce bottles. After solidification of the agar, assay bottles were inverted, covered with aluminum foil, and incubated in the dark at 37°C.

Shellfish and shellfish preparations. Oysters (Crassostrea virginica), hard-shell clams (Mercenaria mercenaria), and soft-shell clams (Mya arenaria) were used. Both hard- and soft-shell clams were obtained as shell stock from approved commercial sources. The soft-shell clams had been depurated at the time of purchase. Oysters (from New Hampshire estuary waters) and hard-shell clams were retained in running clean seawater tanks for 1 to 1.5 months before use.

The procedure for induction of virus carriage status in the shellfish depended upon the purpose to be served. Shellfish used in virus recovery method development studies received a known PFU input injected directly into hepatopancreatic tissue. Studies of the tissue distribution of virus were carried out with shellfish allowed to bioaccumulate solid-associated virus from environmental seawater. Solid-associated virus was either feces-associated natural virus (stools from infants receiving Sabin-type polyvalent poliovirus vaccine, obtained from M. Michael Sigel) or kaolinite-associated stock enterovirus.

Test samples were prepared from freshly shucked shellfish. Regular samples included meats and shell liquor. Special samples identified as siphon (afferent and efferent), hepatopancreatic (stomach, digestive diverticula and tubules, midgut), or "remaining" (mantle, muscle, gill structures) tissues were prepared by dissection of test samples.

Salt concentration adjustments were made with a Yellow Springs Instrument conductivity meter, model 31, and pH adjustments were made with a Sargent-Welch model PBX meter.

RESULTS

Virus recovery method development. (i) New procedure for release and separation

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of virus from shellfish tissues. The first step in developing a method equally effective in oysters and hard- and soft-shell clams was to examine new ways for releasing and separating virus from homogenized tissues. Homogenates were adjusted to pH 9 and a salt concentration of $\geq 10,000$ mg of NaCl per liter. Beef extract (Inolex Corp., Glenwood, Ill.) was added at the same time in anticipation of an ability to induce secondary separation of released virus through its adsorption to an organic floc formed at acid pH (10). The effectiveness of the new procedure for release and separation of virus was compared with oyster-developed procedures used for the same purpose in studies carried out in hard- and soft-shell clams. The results are given in Table 1.

The new procedure was more effective for recovery of test viruses in each species but the degree of effectiveness varied between both species and test viruses. Recovery rates in soft-shell clams were considerably less than the rates obtained in hard-shell clams, and poliovirus was more easily recovered than coxsackievirus and echovirus in both species. The results indicated the extent to which differences of species and virus identity could influence the success of procedures for the release and separation of enteric viruses. The results also raised a question about the influence that the method used for tissue disaggregation might have upon release and separation of virus.

Three ways of preparing homogenates were tested for their effectiveness in disaggregating oyster and hard- and soft-shell clam tissues preparatory to the release and separation of virus. Disaggregation by kneading scissors-fragmented tissues in plastic bags (20) was ineffective. Tissues could not be reduced to homogeneous cell suspensions, and virus remained occluded within intact fragments. Manually operated food grinders also were unsatisfactory for reduction of tissue masses to homogeneous cell suspensions. High-speed homogenization (Omnimix homogenizer at 16,000 rpm) was the only satisfactory way found to disaggregate tissue masses. Microscopic examinations showed that the disaggregation resulted in homogeneous suspensions of well-dispersed individual cells. The most effective practice consisted of four 30-s homogenizations of phosphate-buffered saline-suspended tissues.

The lower recovery rates from soft-shell clams indicated that virus was not being as effectively released and separated from this species as from hard-shell clams. Sonication after homogenization was investigated for its usefulness as an adjunct treatment for release of tissue-bound virus. Sonication (model W-375 sonicator; Heat Systems-Ultrasonics Inc., Plainview, N.Y.) was carried out at 100 W for 10 min in a rosette cooling cell at ice bath temperature. Virus released was recovered in supernatant after centrifugation at 10,000 $\times g$. Variables of sonication time, diluent, and pH were investigated to determine conditions optimal for release of virus. Test results are shown in Table 2. Virtually all virus was recovered after 10 min of sonication of phosphate-buffered saline-prepared and beef extract-fortified (3% beef extract) homogenate adjusted to pH 8 or 9. Virus survival was not influenced by a 15-min sonication time at pH 9 to 9.5. Good results were obtained also with a diluent of equal volumes of 3% beef extract and phosphate-buffered saline.

The ability of the new procedure featuring the use of sonication to release and separate virus from beef extract-fortified homogenates at pH 9 was tested with oysters and hard- and soft-shell clams. Parallel tests in the same species were made with the same procedures minus sonication. Results of these tests are given in Table 3.

The new procedure with sonication improved release of virus 23% in oysters, 72% in soft-shell

<table>
<thead>
<tr>
<th>Table 1. Test virus released and separated from hard- and soft-shell clam homogenized tissues by oyster-developed and new procedures</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Percent test virus released from:</strong></td>
</tr>
<tr>
<td><strong>Hard-shell clams</strong></td>
</tr>
<tr>
<td>Oyster procedure</td>
</tr>
<tr>
<td>P2</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td><strong>Avg</strong></td>
</tr>
<tr>
<td><strong>Soft-shell clams</strong></td>
</tr>
<tr>
<td>Oyster procedure</td>
</tr>
<tr>
<td>P2</td>
</tr>
<tr>
<td>47</td>
</tr>
<tr>
<td>49</td>
</tr>
<tr>
<td>41</td>
</tr>
<tr>
<td><strong>Avg</strong></td>
</tr>
</tbody>
</table>

* Sobsey et al. (19).
* Poliovirus 2 (P2), coxsackievirus (CB5), and echovirus 7 (E7).
* Virus inputs for hard-shell clams: P2, $2.3 \times 10^5$ PFU; CB5, $4.8 \times 10^5$; E7, $1.9 \times 10^5$.
* Virus inputs for soft-shell clams: P2, $3.2 \times 10^5$; CB5, $1.9 \times 10^5$; E7, $2.7 \times 10^5$.
clams, and 4% in hard-shell clams. The new procedure with sonication was judged necessary for effective release of virus from oysters and soft-shell clams. Effective release of virus from hard-shell clams could be obtained without sonication, but since no adverse effects resulted from sonication, the new procedure with sonication was used with this species also.

(ii) Recovery of separated virus. Virus separated by the new procedure was recovered by resuspension of a postsonication organic flocculation precipitate. The precipitate was formed by adjustment of a clarified beef extract-fortified supernatant to pH 4 and a salt concentration of ≤4,000 mg of NaCl per liter. The precipitate was suspended in a volume of 0.15 M Na₂HPO₄ solution, pH 9.5, equal to 7 times the precipitate mass (optimal results were obtained with volumes 7 to 10 times the precipitate mass). Precipitates were suspended with the help of a mortar and pestle, Cat-Floc (Calgon Corp., Pittsburgh, Pa.) was added at a rate of 0.5 ml per 100 ml of suspension volume, and a supernatant was collected after centrifugation at 10,000 × g.

The effectiveness of the organic flocculation procedure for recovery of virus released and separated from shellfish homogenates was assessed in a series of tests and then compared with virus recoveries accomplished by the oyster-developed method. In the case of the latter, virus recovered represented the virus content of the eluate obtained through elution of precipitated shellfish tissues with a glycine-saline eluent at pH 7.5 and a salt concentration of 8,000 mg of NaCl per liter. Results of the comparison are given in Table 4.

Recovery of separated virus was improved by organic flocculation in all species, slightly in oysters and markedly in both clam species. Recoveries in clams were improved ca. threefold. Toxicity for cell cultures of three soft-shell and one hard-shell clam sample prepared by the oyster-developed method recapitulated earlier toxicity experiences encountered with these species during new-recovery-method development studies. It was discovered that addition of Cat-Floc during the organic flocculation step virtually eliminated the cytotoxicity of soft- and hard-shell clam samples for most cell cultures investigated. Freon treatment of shellfish samples to eliminate cytotoxicity was tried, but it was less effective than Cat-Floc and necessitated an additional processing step. Consequently, the routine use of Cat-Floc for removal of cytotoxicity was retained as an integral part of the organic flocculation procedure.

### Table 2. Influence of sonication conditions upon release of test virus* from homogenates of soft-shell clam tissues

<table>
<thead>
<tr>
<th>Diluent*</th>
<th>Virus assay (PFU recovered)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10-min sonication at pH:</td>
</tr>
<tr>
<td></td>
<td>7  8  9  9.5</td>
</tr>
<tr>
<td>3% BE</td>
<td>—  110 114 112</td>
</tr>
<tr>
<td>PBS</td>
<td>—  104 107 106</td>
</tr>
<tr>
<td>Equal volumes of 3% BE and PBS</td>
<td>— 105 110 107</td>
</tr>
<tr>
<td>Distilled water</td>
<td>— 100 96 93</td>
</tr>
<tr>
<td>0.15 M Na₂HPO₄</td>
<td>92  —  —  —</td>
</tr>
<tr>
<td>1.0 M glycine</td>
<td>—  —  —  —</td>
</tr>
</tbody>
</table>

* Poliovirus 2 used at a constant input of 112 PFU/100 g of homogenate.
*— None detectable (<2 PFU of virus).

### Table 3. Release of virus* from oyster and clam homogenates with and without sonication

<table>
<thead>
<tr>
<th>Trial</th>
<th>Without sonication</th>
<th>With sonication</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oysters  Soft-shell clams  Hard-shell clams</td>
<td>Oysters  Soft-shell clams  Hard-shell clams</td>
</tr>
<tr>
<td>1</td>
<td>42/103  9/91 93/100</td>
<td>66/120 160/135 606/506</td>
</tr>
<tr>
<td>2</td>
<td>39/92  &gt;100/100</td>
<td>67/100 67/135 440/506</td>
</tr>
<tr>
<td>3</td>
<td>10/107 76/100</td>
<td>59/88 112/120 98/112</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Known numbers of coxsackievirus B3 injected into hepatopancreatic tissue.
(iii) Concentration of recovered virus. Recovery of separated virus by the organic flocculation procedure resulted in a resuspension volume which needed to be reduced before tests in cell culture could be made. Ultrafiltration (Amicon pressurized stirred cell with PM-30 membranes) and hydroextraction techniques were studied for their usefulness for virus concentration. Reduction of the resuspension volume to 15 to 30 ml, with good retention of virus, could be achieved by both procedures, but a minimum of 16 h usually proved necessary.

An alternative method of concentration was sought which would be at least equally effective but completed in a shorter period of time. A shorter processing interval was sought in order to carry out the entire preparation of a sample in 1 working day. This was considered desirable for routine laboratory practice purposes as well as for increasing the chances for recovery of virus.

A modification of the organic flocculation procedure adopted for recovery of separated virus was used for sample reconcentration. The small precipitate mass formed at this stage of the recovery method made it possible to reduce the volume of 0.15 M Na₂HPO₄ solution needed for resuspension to ca. 15 to 20 ml. The rest of the procedure remained unchanged. The greater convenience of ultrafiltration over hydroextraction plus its equal effectiveness led to its selection for comparison with the modified flocculation procedure to determine which was more effective for reduction of sample volume with minimal loss of virus. The results of the comparison are given in Table 5.

Both procedures were effective for reduction of sample volume, but consistently greater virus recovery rates and noncytotoxic samples were obtained with organic flocculation. In addition, samples prepared by ultrafiltration required a minimum of 16 h for completion, whereas organic flocculation-prepared samples were completed within 3 to 4 h.

(iv) Elimination of nonviral contaminants. Three ways of treating test samples were compared for their effectiveness in eliminating nonviral contaminants without simultaneous loss of virus. Filtration with membrane filters was compared with ether and antibiotic treatments. Membrane filter systems used included Gelman TCM-200 (0.2 μm) and Nucleopore (0.2 μm) filters, 47-mm size. Filters were pretreated with sterile phosphate-buffered saline, pH 9.5,

### Table 4. Recovery of virus separated from shellfish tissues by organic flocculation and oyster-developed precipitation-elution procedures

<table>
<thead>
<tr>
<th>Test virus</th>
<th>Organic flocculation</th>
<th>Oyster-developed precipitation-elution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oysters</td>
<td>Soft-shell clams</td>
</tr>
<tr>
<td>Poliovirus 2</td>
<td>82</td>
<td>79</td>
</tr>
<tr>
<td>Coxsackievirus B5</td>
<td>77</td>
<td>74</td>
</tr>
<tr>
<td>Echovirus 7</td>
<td>81</td>
<td>68</td>
</tr>
<tr>
<td>Avg</td>
<td>80</td>
<td>74</td>
</tr>
</tbody>
</table>

* Organic flocculation was carried out by adjustment of beef extract-fortified viral supernatant to pH 4 and salt concentrations of ≤4,000 mg of NaCl per liter. Cat-Floc was added at rate of 0.5 ml/100 ml of supernatant. Recoveries are reported as average percent recovery [(PFU recovered/PFU input) × 100] for three trials per test virus per shellfish species per method.

* Sobsey et al. (19).

* One of three samples was toxic.

* Two of three samples were toxic.

### Table 5. Effectiveness of ultrafiltration and organic flocculation procedures for reduction of virus sample volume

<table>
<thead>
<tr>
<th>Trial</th>
<th>Ultrafiltration</th>
<th>Organic flocculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oysters</td>
<td>Soft-shell clams</td>
</tr>
<tr>
<td>1</td>
<td>37*</td>
<td>53</td>
</tr>
<tr>
<td>2</td>
<td>83</td>
<td>76</td>
</tr>
<tr>
<td>3</td>
<td>72</td>
<td>T*</td>
</tr>
<tr>
<td>Avg</td>
<td>64</td>
<td>64</td>
</tr>
</tbody>
</table>

* Coxsackievirus B5 input values varied from 212 to 334 PFU per pool of five shellfish. Ultrafiltration was carried out in a pressurized Amicon stirred cell with PM 30 membranes.

* Percent recovery of virus [(PFU recovered/PFU input) × 100].

* T, Cytotoxic for cell cultures.
containing 1 to 5% FCS, and test samples to be filtered were adjusted to pH 9.0.

Diethyl, anesthetic-grade ether (final concentration, 25%) was mixed with a test sample, allowed to remain in contact for 10 to 16 h at 5°C, and then removed through centrifugation followed by evaporation at 26°C.

Antibiotics were added to give the following final concentrations (per ml): 200 U of penicillin, 200 μg of streptomycin, 50 μg of gentamicin, and 2.5 μg of Fungizone. A 2-h interaction at 26°C was allowed for inactivation of contaminants. Twenty-milliliter test samples prepared from each of the three shellfish species and containing known numbers of test enteroviruses were treated by each procedure. The results of several trials are given in Table 6.

The most effective procedure for eliminating nonviral contaminants (bacteria, yeasts, fungi, protozoa) was membrane filtration, but virus losses averaging greater than 30% occurred. Ether treatment of test samples occasionally failed to remove contaminants, but the average virus loss dropped to about 12%. In the four trials shown, antibiotic treatments were as effective as ether in the elimination of contaminants and resulted in the lowest loss of virus. The greater loss of virus in the ether and membrane filter methods, which ranged from two- to five-fold, plus the chance for loss of ether-susceptible viruses, was considered sufficient justification for selection of antibiotic treatment as the preferred procedure.

(v) Outline of improved virus recovery method. The improved method developed is outlined in Fig. 1. It consists of five steps for preparation of a sample to be tested for virus. It differs principally from the oyster-developed method in the procedures used for release and separation of virus, recovery of separated virus, and concentration of recovered virus. It resulted in final samples possessing virtually no cytotoxicity for cell cultures. Its effectiveness was assessed in a series of trials in oysters and hard- and soft-shell clams, in which its ability to recover low numbers of test viruses was determined. The results of these trials are given in Table 7.

An average recovery effectiveness of 64% was obtained in 32 trials with members of the three enterovirus groups where inputs were less than 100 PFU. A similar effectiveness was found in 17 of these trials where inputs were from 25 to 3 PFU. Recoveries of 8, 4, and 1 PFU where inputs were 9, 7, and 3 PFU, respectively, were

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**Table 6. Effectiveness of membrane filtration and ether and antibiotic treatments for elimination of nonviral contaminants from test samples**

<table>
<thead>
<tr>
<th>Trial</th>
<th>Samples remaining contaminated after treatment</th>
<th>% Virus loss</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ether</td>
<td>Antibiotics</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Avg</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Oysters and hard- and soft-shell clam samples containing coxsackievirus B3 (1.4 × 10⁵ PFU) or poliovirus 2 (2.2 × 10⁵ PFU) were tested in each treatment procedure.

† Adjust pH to 9.0, salt concentration to 2,000 mg of NaCl per liter; mix 10 min; centrifuge at 10,000 × g, collect supernatant.

‡ Add 10% FCS, pH 7.2, at rate of 100 ml/100 g of sample.
considered indicative of method sensitivity. Reo-
virus recovery was less effective. An average
recovery of ca. 2 per 4 PFU input of virus was
obtained, compared with enterovirus recoveries
of ca. 2 per 3 PFU input of virus. This indicated
ca. 3 or 4 PFU of reovirus would be the lowest
number possible to detect through recovery of
infectious virus in cell culture, compared with
about 2 PFU for enteroviruses.

Monitoring strategies influencing virus
recovery effectiveness. (i) Tissue composition
of sample being tested. Decision on what shellfish tissues should be included in a sample
tested for enteric viruses was based upon com-
parisons of virus numbers recovered from pools
of entire shellfish (tissues and shell liquor) ver-
sus numbers recovered from pools of selected
tissues. Separate pools of selected tissues and
whole shellfish samples were prepared from lots
of shellfish exposed to virus. All samples were
prepared by means of the improved recovery
method. The results of the comparisons are
given in Table 8.

The data indicated that use of the whole shell-
fish resulted in recovery of greater numbers of
virus than a selected tissue sample in all but one
instance. The proportion recovered in a selected
tissue sample was similar for each virus in each
species. The results for soft-shell clams sug-
gested that use of samples representing whole
shellfish could be crucial to monitoring effective-
ness in the presence of low numbers of virus.
The results in general showed that use of a
whole shellfish sample was preferable because
chances for recovery of virus were increased ca.
50% in all species.

(ii) Effect of pool size upon recovery ef-
fectiveness. A series of trials were carried out
with oysters and soft-shell clams in which recov-
ery effectiveness was determined for pools con-
taining the same virus input but different num-
bers of shellfish. The results of these trials are
given in Table 9.

Within the numbers used, limitation of oyster
but not soft-shell clam pool size was indicated to
be advisable in order not to adversely affect
recovery effectiveness. Approximately 20 oysters
or a pool mass preferably not greater than 200
to 250 g or both was found to be the maximum
pool size to be used for this species. Little dif-
ference in recovery effectiveness was found for soft-
shell clam pools with masses varying from less

### Table 7. Effectiveness of improved method for recovery of test viruses from three shellfish species

<table>
<thead>
<tr>
<th>Shellfish</th>
<th>Test virus*</th>
<th>PFU recovered/PFU input in trial:</th>
<th>Avg (%e)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Oysters</td>
<td>E7</td>
<td>102/98</td>
<td>52/90</td>
</tr>
<tr>
<td></td>
<td>CB1</td>
<td>30/54</td>
<td>16/25</td>
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<tr>
<td></td>
<td>P2</td>
<td>66/120</td>
<td>67/100</td>
</tr>
<tr>
<td></td>
<td>Reo 1</td>
<td>53/110</td>
<td>38/90</td>
</tr>
<tr>
<td>Hard-shell clams</td>
<td>E7</td>
<td>73/100</td>
<td>40/70</td>
</tr>
<tr>
<td></td>
<td>CB1</td>
<td>102/157</td>
<td>110/157</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>586/506</td>
<td>440/506</td>
</tr>
<tr>
<td></td>
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<td>14/30</td>
<td>15/27</td>
</tr>
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<td>Soft-shell clams</td>
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<td>130/170</td>
<td>120/170</td>
</tr>
<tr>
<td></td>
<td>CB1</td>
<td>54/97</td>
<td>58/83</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>67/135</td>
<td>112/120</td>
</tr>
<tr>
<td></td>
<td>Reo 1</td>
<td>32/80</td>
<td>34/74</td>
</tr>
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</table>

* Echovirus 7 (E7), coxsackievirus B1 (CB1), poliovirus 2 (P2), and reovirus 1 (Reo 1).
than 100 up to 231 g and containing from 5 to 30 clams.

Subsequent to the above trials, two tests of recovery effectiveness with 20- and 30-hard-shell-clam pools (183 and 256 g, respectively) were carried out with coxsackievirus B3 inputs of 36 PFU. Recoveries of 71 and 53% for pools containing 20 and 30 specimens, respectively, suggested that hard-shell clam pools probably should be limited to 20 clams or a mass not in excess of ca. 200 g or both for maximum recovery effectiveness.

(iii) Effect of test sample inoculum volume and adsorption time upon virus recovery in cell cultures. Variations of sample volume and adsorption times were studied to determine what combination of inoculum volume and adsorption time resulted in recovery of the greatest numbers of test virus. Inocula of 1 to 5 ml containing the same numbers of virus were adsorbed for 1 to 5 h, after which agar overlay medium was added. The results of three trials in which oysters and soft-shell clams were used are given in Table 10.

The greatest numbers of virus were recovered after 5-h adsorption intervals, but increases over numbers recovered at 2 h were minimal. In view of the minimal increase in numbers recovered after 2 h, an adsorption interval of 2 h was considered adequate for effective recovery of virus. With one exception, recovery effectiveness declined sharply when inoculum volume ex-

| Table 9. Influence of shellfish pool size upon virus recovery effectivenessa |
|---|---|---|
| Oysters | Soft-shell clams |
| Pool size | Pool size |
| TRIAL | No. | Wt (g) | PFU recovered/ PFU input | Recovery (%) | No. | Wt (g) | PFU recovered/ PFU input | Recovery (%) |
| 1 | 12 | 168 | 232/327 | 71 | 30 | 178 | 11/17 | 65 |
| 2 | 6 | 82 | 222/327 | 68 | 15 | 83 | 12/17 | 70 |
| 3 | 24 | 266 | 6/13 | 46 | 15 | 107 | 17/22 | 77 |
| 4 | 18 | 218 | 8/13 | 61 | 10 | 55 | 15/22 | 68 |
| 5 | 12 | 137 | 9/13 | 69 | 5 | 33 | 16/22 | 73 |
| 3 | 24 | 321 | 19/31 | 61 | 30 | 231 | 7/9 | 78 |
| 4 | 20 | 271 | 22/31 | 71 | 20 | 131 | 6/9 | 67 |
| 5 | 12 | 153 | 24/31 | 77 | 15 | 107 | 6/9 | 67 |
| 4 | 30 | 342 | 39/87 | 45 | 30 | 192 | 14/15 | 93 |
| 5 | 24 | 293 | 58/87 | 66 | 15 | 84 | 7/10 | 70 |
| 6 | 18 | 234 | 64/87 | 73 |

a Test pools were prepared by injection of known numbers of coxsackievirus B3 into hepatopancreatic tissue.

| Table 10. Influence upon virus recovery effectiveness of test sample volume and adsorption timea |
|---|---|---|
| Oysters | Soft-shell clams |
| Pool size | Pool size |
| Inoculum vol (ml) | PFU of virus | Inoculum vol (ml) | PFU of virus |
| |输入 | Recovered at: | |输入 | Recovered at: |
| | | 1 h | 2 h | 5 h | | 1 h | 2 h | 5 h |
| 1 | 1 | 74 | 61 | 70 | 76 | 1 | 41 | 14 | — | 39 |
| 2 | 74 | 51 | 54 | 56 | 3 | 41 | — | — | 19 |
| 3 | 74 | 39 | 49 | 51 | 5 | 41 | — | — | T |
| 4 | 24 | 17 | 22 | 23 | 1 | 13 | 8 | 11 | 12 |
| 3 | 24 | 11 | 9 | 11 | 3 | 13 | 3 | 5 | 6 |
| 5 | 24 | 6 | 8 | 7 | 5 | 13 | 4 | 3 | 5 |
| 3 | 96 | 77 | 81 | 89 | 1 | 28 | 19 | 25 | 24 |
| 2 | 96 | 60 | 66 | 70 | 2 | 28 | 11 | 23 | 29 |
| 3 | 96 | 46 | 49 | 53 | 3 | 28 | 13 | 9 | 15 |
| 5 | 96 | 33 | 29 | 28 | 5 | 28 | 15 | 11 | 13 |

a Test virus was added to final samples prepared from non-virus-carrying shellfish. Coxsackievirus B3 was added to oyster samples, and echovirus 7 was added to soft-shell clam samples. Final sample volumes of 30 ml were prepared in all trials. Virus assays were made in 6-ounce bottles containing 45-cm² BGM monolayers.

b Total PFU per sample volume.

c Total PFU recovered per sample volume.

d T, Toxic for cell cultures.
ceeded 1 ml, and even adsorption periods up to 5 h failed to increase the numbers of virus recovered. Recovery results were essentially the same for oysters and clams. One 5-ml soft-shell clam inoculum was cytotoxic after a 5-h adsorption interval.

(iv) Method of exposure of cell cultures to sample inocula. The common practice of splitting samples between two or more different cell culture systems was compared with a strategy that called for sequential addition of inocula representing an entire sample to two or more cell culture systems. The strategy called for collection and pooling of inocula from the first cell culture system after adsorption, followed by redistribution of pooled inocula onto the second cell culture system and observance of a second adsorption interval. The inocula could be recovered from the second cell culture system and adsorbed to a third culture system if desired. The results of a study comparing the two ways of exposing cell cultures to shellfish samples are given in Table 11.

Sequential adsorption of sample inocula to cell cultures was a more effective procedure for recovery of virus in each trial. Taken as a whole, the five trials resulted in a recovery effectiveness of 88% (117/133), compared with 54% (72/133) for the split sample method. The greater recoveries in BGM cultures in sequential adsorption reflected a greater recovery opportunity based upon testing of an entire sample on this cell culture. Inequities in virus distribution between halves in the split sample tests resulted in lower numbers of virus in the halves tested on BGM cultures and a correspondingly lower opportunity to recover virus in this culture. Failure to recover one-half or recovery of more than one-half of a virus input was a further indication of the degree of uneven distribution of virus. The dangers associated with uneven distribution in split sample testing where the numbers of virus in the whole sample are low was illustrated in trial 3, where no recoveries were made from the split sample portion inoculated on BGM. It could not be determined whether the balance of virus inputs not accounted for by BGM and PMK results was represented by HEp-2 results. Greater numbers than the PFU estimated for HEp-2 recoveries could have been present, since it was impossible to tell whether CPE in positive monolayers was caused by one or more infectious virions.

DISCUSSION

The study addressed key questions about the availability and adequacy of methods and strategies for detection of potential virus health hazards in three commercially important shellfish species. It was necessary to devise a monitoring

<table>
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<th>Test virus</th>
<th>Recovered by:</th>
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<tr>
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<td>Sequential adsorption</td>
</tr>
<tr>
<td></td>
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<td>(sample [10 ml] → BGM → PMK → HEp-2)</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>BGM</td>
</tr>
<tr>
<td></td>
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<td>26</td>
</tr>
<tr>
<td>1</td>
<td>Poliovirus 2</td>
<td>33</td>
</tr>
<tr>
<td>2</td>
<td>Echovirus 7</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
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<td>48</td>
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<tr>
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<td>17</td>
</tr>
<tr>
<td>5</td>
<td>Echovirus 1</td>
<td>17</td>
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</table>

* For sequential adsorption, the entire sample sequentially adsorbed in 1-ml inoculum volumes for 1.5 h to 45-cm² monolayers of BGM and PMK (African Green) and 3-cm² HEp-2 cultures, with collection and pooling of inocula before each subsequent adsorption. BGM and PMK monolayers received agar overlay medium. HEp-2 monolayers received 0.5-ml inocula and were overlaid with maintenance medium. For the split sample method, one-half of the sample was adsorbed to BGM only, and the other half was adsorbed to PMK only. Inoculum volumes, adsorption times and temperature of HEp-2 monolayers were the same as described for sequential adsorption. Virus recoveries were calculated from plaques formed on BGM and PMK flask monolayers and CPE produced on HEp-2 test tube monolayers (each monolayer with confirmed virus CPE considered equivalent to 1 PFU).
plan which depended upon both the development of an improved virus recovery method equally satisfactory for all three species and the identification of the test strategies necessary for effective utilization of the recovery method in these species. The improved recovery method represented a synthesis of the features shown to be essential for effective recovery of low numbers of virus in each of the three species. Recovery effectiveness based upon the use of low numbers was considered essential for evaluating the usefulness of a method for virus monitoring in shellfish. The low numbers of natural virus found in shellfish removed from polluted growing areas make it necessary for a recovery method to have the potential for recovering low numbers if it is to be of practical value. The general effectiveness of the method with test enteroviruses and reovirus gave no indication that it would be any less effective with other members of these groups. Prudence dictates further testing of the monitoring plan with other enteric viruses and confirmation of its effectiveness by other laboratories.

The improved recovery method resembled the oyster-developed method of Sobsey et al. (19) in its use of the principle of pH and salt concentration adjustments to separate and recover virus. It differed specifically in the procedures used for release and separation of virus, recovery of separated virus, and recombination of recovered virus. The average recovery effectiveness of 64% in 32 trials with three enteroviruses and that of 46% in 11 trials with reovirus where virus inputs were less than 100 PFU compared favorably with the recoveries reported by Sobsey et al. A recovery effectiveness of 47% for nine trials with poliovirus 1 and one of 41% in seven trials with reovirus 3 were reported. Sobsey et al. also reported a recovery effectiveness of 32% for three trials with a simian adenovirus with inputs less than 100 PFU.

Sonication-induced release of virus from shellfish tissues was considered the counterpart of release of virus from solid-associated states in wastewater and sludge after sonication (23, 24). The effectiveness of sonication as a general treatment for release of virus from the three shellfish species was presumed to be the result of vibrational forces which were responsible for breaking down tissue and cell structures, liberating virus adsorbed to or entrapped within cells.

According to this viewpoint, release of virus would be independent of species differences in binding affinities attributable to anatomical and biochemical factors (3). If this viewpoint was correct, virus should be released with equal effectiveness from a number of different species without regard to the nature or degree of virus binding affinity in existence. Study data on release of virus from three different species supported this viewpoint.

Separation of released virus followed by its recovery from tissue-free supernatant through organic flocculation resulted in virus suspensions considerably less turbid than those prepared by the oyster-developed method. Effective recovery of virus required the use of large suspension volumes during sample preparation. Organic flocculation not only made it possible to selectively recover separated virus from large volumes of aqueous suspension, but it also provided an efficient way for reconcentrating recovered virus through resuspension of precipitated virus in small volumes of fluid.

Organic flocculation in combination with the use of the polyelectrolyte Cat-Floc was considered responsible for the virtually noncytotoxic samples prepared by the improved method. Cat-Floc has been used previously for clarification of shellfish homogenates and as an aid in the separation of enteric viruses (11). The degree of sample clarification obtained was considered an important factor in the virtual elimination of cytotoxicity from samples of each of the three shellfish species studied. This opinion was in agreement with a previous observation that reduction or elimination of the cytotoxicity of Cat-Floc-clarified food samples was dependent upon the degree of clarification achieved (8).

Selection of antibiotic treatment to remove nonviral contaminants ran a risk of occasional failure, but the relative freedom of this treatment from serious loss of virus to membrane filters or destruction of ether-susceptible virus justified this risk.

It was found advisable to use all shellfish tissues plus shell liquor for detection of small numbers of virus. The discrepancy in virus numbers recovered from whole versus selected tissue samples was attributed to translocation or deposition of virus in non-alimentary-tract tissues. Non-alimentary-tract-located virus has been found in mantle, gill, and muscle and may appear in any structure bathed by hemolymph (12, 14). Inclusion of all meats and shell liquor in a sample offered a better chance of recovering both alimentary- and non-alimentary-tract-located virus, thus increasing the chance for detection of low numbers of virus in shellfish.

The number of shellfish to be tested for virus and the number to be included in a test pool represented two different monitoring needs. The number tested should be adequate to insure a representative sample, whereas numbers tested per pool could not exceed a maximum beyond which recovery effectiveness was adversely affected. A more or less fixed relationship between
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The tissue mass or the number of specimens in a pool and the number of viruses possible to recover was postulated. According to this point of view, should too many shellfish be included in a pool, the resulting tissue mass in effect could dilute a low number of viruses to a point which would be beyond the capability of the method to recover. Since the maximum number of shellfish which could be included in a pool would be a function of the tissue mass produced, pool size would be species dependent, to the extent of size and weight differences between species. Study results showing that 18 oysters with a mass of 200 to 250 g could be tested with approximately equal effectiveness as 30 soft-shell clams with a roughly equivalent mass supported a recovery effectiveness-tissue mass relationship. Extrapolating from these data, it was estimated that ca. 20 hard-shell clams could be included in a pool without undue sacrifice of recovery effectiveness.

Sample inoculum volumes added to cell culture monolayers and adsorption times allowed became increasingly critical to recovery effectiveness as test virus numbers to be recovered were lowered. Little attention has been directed to the importance of these procedural details to recovery effectiveness in virus examinations in shellfish. Addition of 0.1- to 0.2-ml inocula to a 10-cm² or greater monolayer surface area followed by a 1- to 1.5-h adsorption period has been a widely accepted practice for plaque-based assays (9). Inoculum volumes varying between 0.1 to 0.2 ml per 10 cm² and as much as 5 ml per 50 to 75 cm², followed by a 1- to 2-h adsorption period, have been used for recovery of natural viruses. Recovery effectiveness in the study depended primarily upon inoculum volume. Optimum recovery with 45-cm² monolayers called for an inoculum volume no greater than 1 ml and an adsorption time of 2 h. Increasing the adsorption time to 5 h led to only minimal increases in virus numbers recovered. Increasing adsorption times could not be used to offset a loss of recovery effectiveness resulting from the use of larger inoculum volumes.

Exposure of an entire sample to each of two or more cell culture systems by means of a sequential adsorption technique allowed each culture to "see" an entire test sample and resulted in an increased recovery effectiveness. Study data showed the dangers inherent in splitting samples between two or more cell cultures where one was more susceptible than another and where unequal distribution of virus between the two split portions occurred. This was shown to be especially critical when a test sample contained low numbers of virus. Unlike the split sample procedure, the outcome of sequential adsorption would not be influenced by which of two or more cultures was more sensitive, because all cultures would have equal opportunity to "see" an entire sample. Study results indicate that further trial and assessment of the merits of sequential adsorption and split sample methods for recovery of virus should be made in field trials where natural virus recoveries from shellfish collected from feces-polluted growing waters are sought.

Comparison of PFU versus CPE techniques for recovery of virus was not made, because experiments were not designed to provide equal opportunities for plaque formation and development of cytopathology. Unlike other studies, in which provision of equal opportunity resulted in recovery of both plaque-forming and non-plaque-forming natural viruses (7), development of CPE on Hep-2 cultures did not reflect non-plaque-forming virus. It showed only that some virus either escaped adsorption or was reversibly adsorbed to the cultures used for recovery of plaque-forming virus.

The Hep-2 results did show that a third culture could be used either for recovery of non-plaque-forming virus, for recovery of a broader spectrum of viruses, or as a backup culture to recover viruses reversibly adsorbed to cultures used for recovery of plaque-forming virus.

The monitoring strategy developed in the study is being tried with mussels (Mytilus edulis). Early results indicate that enteroviruses can be recovered as effectively from this species as from the three species used in the study.

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