Development of a Simple Method for Concentrating Enteroviruses from Oysters

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The development of a simple method for concentrating enteroviruses from oysters is described. In this method viruses in homogenized oyster tissues are efficiently adsorbed to oyster solids at pH 5.5 and low salt concentration. After low-speed centrifugation, the supernatant is discarded and viruses are eluted from the sedimented oyster solids by resuspending them in pH 3.5 glycine-buffered saline. The solids are then removed by low-speed centrifugation, and the virus-containing supernatant is filtered through a 0.2-μm porosity filter to remove bacteria and other small particulates without removing viruses. The virus-containing filtrate is then concentrated to a volume of a few milliliters by ultrafiltration, and the concentrate obtained is inoculated directly into cell cultures for virus assay. When tested with pools of oysters experimentally contaminated with small amounts of different enteroviruses, virus recovery efficiency averaged 63%.

The sanitary quality of edible molluscs such as oysters, clams, and mussels is presently based upon bacterial criteria (1, 13). The lack of simple, reliable, and sensitive methods for quantitating enteric viruses in these shellfish has been a major handicap in evaluating their public health significance as vehicles for enteric virus transmission. A number of studies have been reported on the detection of enteric viruses in oysters, clams, and mussels by methods employing ethyl ether extraction (5, 11, 12), fluorocarbon extraction (6), polyelectrolyte flocculation (8), and acid precipitation (7) to separate the viruses from shellfish tissue components that either interfere with virus recovery or are toxic to the cell cultures used for virus assay.

Previous studies in our laboratory have shown that by controlling pH and ionic conditions, enteroviruses can be efficiently adsorbed to such diverse materials as cellulose ester and fiber glass filters, insoluble organic polymers, activated carbon, and erythrocytes (3, 15–18; unpublished data). The adsorbed viruses can be quantitatively eluted by changing pH and/or ionic conditions. In the present study we describe the development of simple, rapid methodology for quantitatively recovering enteroviruses from experimentally contaminated oysters by adsorption to and subsequent elution from the homogenized oyster tissue, followed by concentration of the viruses with ultrafiltration.

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was done with type PM30 membranes in model 202 stirred cells (Amicon Corp., Lexington, Mass.).

RESULTS

Effects of pH and salt concentration on virus adsorption to homogenized oyster solids. Previous methodology for enteric virus detection in oysters has utilized slow-speed centrifugation or flocculation and filtration (8) of the homogenized oyster tissue to initially remove oyster solids (5-7, 11, 12). Previous studies in our laboratory with a number of different materials used as virus adsorbents showed that enterovirus adsorption was greatly influenced by salt concentration and pH level. Therefore, a series of experiments was conducted to determine if the pH and salt concentration of virus-contaminated oyster homogenate would influence the extent of virus adsorption to the oyster solids.

In the first experiment a poliovirus-contaminated oyster homogenate was prepared and divided into eight equal portions. The portions were adjusted to the pH levels and salt concentrations shown in Table 1 with minimal volume change. The homogenate portions were assayed for virus and centrifuged, and the resulting supernatants were assayed for virus. The results are given in Table 1 as percentage of initial homogenate virus remaining in the supernatant. At salt concentrations of either 1,000 or 10,000 mg of NaCl per liter and pH levels of 3, 7, and 9 and at a salt concentration of 10,000 mg of NaCl per liter and pH 5, considerable percentages of the initial homogenate virus were found in the supernatant. However, at a salt concentration of 1,000 mg of NaCl per liter and a pH of 5, only 1% of the total homogenate virus was detected in the supernatant.

The effect of salt concentration in pH 5.0 oyster homogenates on the degree of virus adsorption to oyster solids was then examined. A virus-contaminated oyster homogenate, adjusted to pH 5.0 and having a low conductivity, was prepared and then divided into nine equal portions. The homogenate portions were adjusted to the NaCl concentrations shown in Table 2 with minimal volume change, assayed for virus, and centrifuged, and the resulting supernatants were assayed for virus. Only a small percentage of the initial homogenate virus was found in the supernatant at salt concentrations of 2,000 mg of NaCl per liter or less, whereas at salt concentrations of 3,000 mg of NaCl per liter or more all or most of the initial homogenate virus was detected in the supernatant (Table 2).

The effect of pH on poliovirus adsorption to oyster solids at a constant salt concentration of 1,000 mg of NaCl per liter was also determined. A virus-contaminated oyster homogenate with a low salt concentration was prepared and then divided into seven equal portions. The portions were adjusted to the pH levels shown in Table 3 and then brought to a constant salt concentration of 1,000 mg of NaCl per liter with minimal volume change. The homogenates were assayed for virus and centrifuged, and the resulting supernatants were assayed for virus. Little of the initial homogenate virus was detected in the supernatants when the pH values were 5.0, 5.5, or 6.0, suggesting that most of the viruses were associated with the sedimeted oyster solids. At either higher or lower pH values, the majority of the initial homogenate virus was found in the supernatants. From the results of these experiments, we decided to use pH 5.5 and a salt concentration of 1,500 mg of NaCl per liter or less to adsorb viruses to oyster solids.

The effects of pH and salt concentration on the ability of sedimeted oyster solids to retain adsorbed viruses were examined. A virus-contaminated oyster homogenate with a pH of 5.5 and a salt concentration of 1,500 mg of NaCl per

| Table 1. Effect of pH and conductivity on poliovirus adsorption to oyster solids |
|--------------------------|--------------------------|--------------------------|
| pH          | Homogenate virus remaining in supernatant (%)* |
|             | 1,000 mg of NaCl/liter | 10,000 mg of NaCl/liter  |
|-------------|-------------------------|-------------------------|
| 3           | 93                      | 90                      |
| 5           | 1                       | 68                      |
| 7           | 28                      | 100                     |
| 9           | 100                     | 93                      |

*Homogenate virus concentration was $5 \times 10^4$ PFU/ml.

| Table 2. Effect of NaCl concentration on poliovirus adsorption to oyster solids at pH 5.0 |
|-----------------------------------------------|-----------------------------------------------|
| Salt concn (mg of NaCl/liter) | Homogenate virus remaining in supernatant (%)* |
|--------------------------------|-----------------------------------------------|
| 500                            | 4                                             |
| 1,000                          | 5                                             |
| 1,500                          | 6                                             |
| 2,000                          | 8                                             |
| 3,000                          | 72                                            |
| 4,000                          | 100                                           |
| 5,000                          | 80                                            |
| 15,000                         | 91                                            |
| 30,000                         | 100                                           |

*Homogenate virus concentration was $5 \times 10^4$ PFU/ml.
The teroviruses subsequently and resulting water, which were centrifuged, and the resulting supernatants were assayed for virus. The results of this experiment (Table 4) showed that poliovirus which was adsorbed to homogenized oyster solids would be retained by the solids when washed at pH 5.5 and low NaCl concentration, but would readily elute from the solids when washed at pH 3.5 and 7.5 with 0.05 M glycine-NaCl solutions of either high or low NaCl concentration. The eluted viruses could be separated from the oyster solids by simply centrifuging out the solids and decanting the virus-containing supernatant. Additional experiments showed that oyster solids containing adsorbed viruses could be repeatedly washed and centrifuged with no appreciable virus loss to the supernatants if the pH was 5.5 and the conductivity was 1,500 mg of NaCl per liter or less.

Based upon these experiments, an adsorption-elution procedure for recovering enteroviruses from oysters consisting of the following steps was developed and tested. (i) A pool of three oyster meats is homogenized in distilled water, and the homogenate is adjusted to pH 5.5 with dilute HCl and to a conductivity of 1,500 mg of NaCl per liter or less. The mixture is centrifuged at 1,900 × g for 10 min, and the supernatant is discarded. (ii) The sedimented oyster solids are washed by resuspending in 150 ml of 0.05 M glycine, and the mixture is adjusted to a pH of 5.5 and a conductivity of 1,500 mg of NaCl per liter or less and then centrifuged. (iii) The supernatant is discarded and the adsorbed viruses are eluted from the sedimented oyster solids by resuspending in 150 ml of pH 3.5, 0.05 M glycine-buffered saline. The mixture is centrifuged at 1,900 × g for 10 min, and the virus-containing supernatant is decanted. When this procedure was tested with poliovirus-contaminated oysters, virus recovery efficiencies in the final supernatant averaged 60%.

Adsorption of poliovirus in oyster homogenate eluates to filter surfaces. Because the virus-containing eluates from three oysters had volumes of about 150 ml, they could not be conveniently assayed for viruses in a small number of cell cultures without somehow concentrating the viruses to a volume of a few milliliters. Previous studies in our laboratory showed that viruses in aqueous fluids such as water, sewage, and infected cell culture harvest could be efficiently concentrated by adsorbing the viruses to a filter surface and then eluting them off in a small volume. Therefore, experiments were conducted to determine if poliovirus in oyster homogenate eluates would adsorb to filter surfaces.

The supernatant of a pH 3.5 oyster homogenate eluate obtained from six oysters and containing 5 × 10⁴ PFU per ml was divided into eight portions of 40 ml each. The portions were adjusted to the pH and ionic conditions shown in Table 5. In previous studies it was shown that, under these pH and ionic conditions, viruses in aqueous fluids could be concentrated by adsorbing them to filter surfaces and then eluting them with a small volume. Some of the portions were treated with 20-g amounts of various ion exchange or adsorbent resins at neutral pH prior to adjustment to the pH and ionic conditions shown in Table 5. The purpose of the resin treatments was to determine if possible interfering materials pres-

| pH | Homogenate virus remaining in supernatant (%) |
|----|---------------------------------------------|
| 3.5| 100                                        |
| 4.0| 72                                         |
| 4.5| 82                                         |
| 5.0| 2                                          |
| 5.5| 3                                          |
| 6.0| 2                                          |
| 6.5| 80                                         |

* Homogenate virus concentration was 5 × 10⁴ PFU/ml.

| pH | NaCl concn of wash solution (mg/liter) | Homogenate virus remaining in supernatant (%) |
|----|---------------------------------------|---------------------------------------------|
| 3.5| 1,000                                 | 86                                         |
| 3.5| 5,000                                 | 92                                         |
| 5.5| 1,000                                 | 92                                         |
| 5.5| 5,000                                 | 62                                         |
| 7.5| 1,000                                 | 90                                         |
| 7.5| 5,000                                 | 90                                         |

* Homogenate virus concentration was 5 × 10⁴ PFU/ml.
ent in the sample might be removed with a specific resin. The use of resins to remove materials from aqueous fluids that interfere with virus adsorption to filter surfaces has been previously reported (15, 17). Each sample was then filtered through a 25-mm diameter, 5-, 1-, and 0.45-μm porosity Cox filter series, and the filtrate was assayed for virus. Little or no virus in the oyster sample adsorbed to the filters, regardless of the resin treatments or ionic conditions employed (Table 5). Apparently, the samples contained a considerable amount of material that interfered with virus adsorption to the filters.

To determine if these interfering substances were membrane-coating components (17), the supernatant of a pH 3.5 oyster homogenate eluate containing no added poliovirus was treated with AlCl₃ to a final 0.0005 M concentration, and then serially diluted by half-log dilutions in pH 3.5, 0.05 M glycine-0.0005 M AlCl₃. A 10-ml volume of each dilution was filtered through a set of 25-mm diameter, 5-, 1-, and 0.45-μm porosity Cox filters in series and the filtrate was discarded. Each set of filters was then challenged with 5 ml of pH 3.5, 0.05 M glycine-0.0005 M AlCl₃ containing 5 × 10⁴ PFU of type 1 poliovirus per ml, and the filtrate was assayed for virus. One set of filters which received only the challenge virus served as a positive control. The percentages of total initial challenge virus present in the filtrates of the filter sets receiving undiluted and 1:3, 1:10, 1:33, and 1:100 dilutions of oyster sample were 100, 100, 84, 1, and 0.2%, respectively. The virus challenge filtrate of the control filter set contained only 0.2% of the total initial challenge virus. The results of these experiments indicate that the supernatant of an oyster homogenate eluate contains relatively large amounts of material that coats a filter surface and thereby prevents virus adsorption. For this reason we abandoned our efforts to concentrate viruses in supernatants of oyster homogenate eluates by adsorption to and elution from filter surfaces. However, these experiments did indicate that, due to the presence of membrane-coating components, the supernatants of oyster homogenate eluates could be filtered through submicron filters without appreciable virus losses due to adsorption.

Concentration of viruses in supernatants of oyster homogenate eluates by UF. Because attempts to concentrate viruses in supernatants of oyster homogenate eluates by adsorption to and elution from filter surfaces were unsuccessful, virus concentration by UF was tried. The use of UF to concentrate viruses from cell culture harvests (4), water (2, 14), and shellfish extracts (8) has been previously reported. Indeed, one of us (J.L.M.) had used pervaporation through cellulose membranes for this purpose over 30 years ago (9). Supernatants of oyster homogenate eluates, 150 ml in volume, were adjusted to pH 7.5 and contaminated with poliovirus to give a concentration of 5 × 10⁴ PFU/ml. The samples were first filtered through a 90-mm diameter, 0.2-μm porosity Cox filter to remove particulate material, including bacteria and molds. No appreciable virus losses on the filters were observed. Viruses in the clarified filtrates were then concentrated to volumes of about 4 ml by ultrafiltration at a pressure of 30 lb/in² in a time period of about 1 h. After concentrating, heat-inactivated fetal calf serum was added to the concentrates to a final 20% concentration, and the mixture was stirred in the UF cell for a period of 15 min to elute any viruses that may have adsorbed to the surface of the UF membrane. Both the serum-treated concentrates and the UF filtrates were assayed for viruses. In eight replicate experiments, poliovirus recoveries in the concentrates ranged from 49 to 100% and averaged 90% of the total initial virus. Less than 1% of the total initial virus was detected in the UF filtrates.

Concentration of enteroviruses from oysters by adsorption to and elution from oyster solids followed by UF. A series of experiments was done in which poliovirus in pools of three oysters was concentrated by a

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**Table 5. Effects of resin pretreatments and ionic conditions on virus adsorption to Cox filters from the supernatant of an oyster homogenate eluate**

| Adsorption conditions | Resin pretreatment | Initial virus in filtrate (%) |
|-----------------------|--------------------|-----------------------------|
| 0.05 M MgCl₂, pH 5.5  | None               | 94                          |
| 0.0005 M AlCl₃, pH 3.5| None               | 98                          |
| 0.0005 M AlCl₃, pH 3.5| Dowex 1-X8         | 97                          |
| 0.0005 M AlCl₃, pH 3.5| Ionac A540         | 87                          |
| 0.0005 M AlCl₃, pH 3.5| Ionac C-249        | 93                          |
| 0.0005 M AlCl₃, pH 3.5| Duolite S-30       | 83                          |
| 0.0005 M AlCl₃, pH 3.5| Duolite S-37       | 50                          |
| 0.0005 M AlCl₃, pH 3.5| IRA 938           | 60                          |
| Control glycine-saline with 0.0005 M AlCl₃, pH 3.5 | None | <1 |
| Control glycine-saline with 0.05 M MgCl₂, pH 5.5 | None | <1 |

*Each 40-ml sample was treated with 20 g of resin in a column. Dowex 1-X8, strong anion, chloride form, Biorad Laboratories, Richmond, Calif.; Ionac A540, strong anion, mixed chloride and sulfate forms, and Ionac C-249, strong cation, sodium form, both from Ionac Chemical, Birmingham, N.J.; Duolite S-30 and S-37, organic adsorbents, Diamond Shamrock, Redwood City, Calif.; IRA 938, macroreticular, strong anion, chloride form, Rohm and Haas, Philadelphia, Pa.*
two-step procedure consisting of adsorption to and elution from homogenized oyster tissue followed by UF. Both steps of the procedure were conducted as described above. The average, stepwise results for a series of three replicate experiments are shown in Table 6. Poliovirus recovery efficiency in the UF concentrate of 4 ml averaged 57%.

This methodology was then tested for its ability to concentrate relatively small quantities of four different enteroviruses from pools of three experimentally contaminated oysters. In a total of 15 experiments with poliovirus type 1, echovirus type 7, and coxsackieviruses A9 and B3 at input virus levels ranging from 4,790 to 34 PFU, virus recovery efficiency in the UF concentrate ranged from 24 to 97% and averaged 63% (Table 7).

**DISCUSSION**

In most of the previously reported methods for enteric virus detection in oysters, the homogenized oyster tissue was initially centrifuged at low speed to remove much of the oyster solids and the resulting supernatant was further processed for virus detection. This supernatant is invariably viscous, turbid, high in dissolved and colloidal organic matter, difficult to filter, and often toxic to the cell cultures used for virus assay. In short, it is a difficult sample to process in attempting to recover viruses, and it has led previous investigators to apply a variety of treatments in an effort to eliminate these difficulties and thereby improve virus recovery. In this present study we found that by controlling the pH and salt concentration of the initial oyster homogenate, viruses could be easily and rapidly separated from the homogenized oyster tissues by a procedure involving two basic steps. In the first step of the procedure, the viruses are adsorbed to the oyster solids at pH 5.5 and a low salt concentration. When this homogenate is centrifuged at low speed, nearly all of the viruses sediment with the oyster solids so that the first supernatant can be discarded. The adsorbed viruses are then eluted from the sedimented oyster solids by resuspending the solids in pH 3.5 glycine-buffered saline, and the virus-free solids are then removed by low-speed centrifugation. The virus-containing supernatant is

| Sample | Vol (ml) | Total input virus detected (%) |
|--------|----------|-------------------------------|
| Input virus* | 1 | (100) |
| Oyster homogenate, pH 6.6 | 250 | 83 |
| Oyster homogenate, pH 5.5 | 254 | 69 |
| First supernatant | 230 | <1 |
| pH 5.5 glycine wash supernatant | 150 | <1 |
| Eluate supernatant, pH 3.5 | 150 | 64 |
| Eluate supernatant, pH 7.5 | 155 | 62 |
| 0.2-μm filtrate | 155 | 60 |
| UF filtrate | 150 | <1 |
| UF concentrate | 4 | 57 |

* 10^3 PFU as determined by titration of stock virus.
* Pools of three oysters, 20 to 65 g/pool.
* Conductivity ≤1,500 mg of NaCl per liter.

**Table 7. Enterovirus recovery from oysters by solids adsorption-elution and UF**

| Virus | Concentrate vol (ml)* | Inoculated virus (PFU) | Virus recovered in concentrate (PFU) | Inoculated virus recovered in concentrate (%) |
|-------|----------------------|------------------------|-------------------------------------|---------------------------------------------|
| Poliovirus type 1 | 11 | 800 | 385 | 48 |
| Echovirus type 7 | 4 | 186 | 49 | 26 |
| | 5 | 119 | 78 | 65 |
| | 5 | 39 | 29 | 74 |
| Coxsackievirus B3 | 4 | 2,070 | 1,984 | 96 |
| | 4 | 80 | 19 | 24 |
| | 4 | 34 | 33 | 97 |
| | 5 | 786 | 684 | 87 |
| | 4 | 590 | 483 | 82 |
| | 4 | 58 | 37 | 64 |
| | 4 | 42 | 28 | 67 |
| Coxsackievirus A9 | 4 | 4,790 | 2,307 | 48 |
| | 4 | 4,790 | 2,516 | 52 |
| | 4 | 37 | 33 | 89 |
| | 15 | 37 | 10 | 27 |

* Final concentrate was sterile filtered through a 25-mm diameter, 0.22-μm porosity cellulose ester membrane prior to inoculation into cell cultures.
relatively low in turbidity and dissolved and colloidal organic matter, nontoxic to cell cultures, and, after adjustment to pH 7.5, easily filtered through a 0.2-μm porosity filter to remove bacteria, molds, and other particulate matter without removing viruses.

The viruses in the resulting filtrate are efficiently concentrated to a volume of a few milliliters by UF, and the concentrate obtained is nontoxic to cell cultures, at a physiological pH, isotonic, and, therefore, ready for virus assay. Because the final concentrate is of small volume, the viruses obtained from the entire initial oyster pool can be inoculated into a small number of cell cultures.

When this procedure was tested with relatively small amounts of four different enteroviruses in experimentally contaminated pools of three oysters (20 to 65 g/pool), virus recovery efficiency averaged about 63%. This methodology is now being used to detect enteroviruses in Texas Gulf Coast oysters (M. D. Sobsey, C. P. Gerba, C. Wallis, and J. L. Melnick, manuscript in preparation).

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