Polyelectrolyte Flocculation as an Aid to Recovery of Enteroviruses from Oysters

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A simple and rapid method for recovering enteroviruses from oysters is described. A polycation sewage flocculant promoted cohesion of oyster solids and thereby aided separation of these from the viruses. Recovery of 80 to 100% of experimentally inoculated virus was achieved, and the suspension or extract obtained could be inoculated directly into tissue cultures or concentrated first for greater sensitivity.

Edible oyster species of northern (Ostrea edulis; 7) and eastern United States (Crassostrea virginica; 10, 11) accumulate human enteroviruses from water under experimental conditions. Enteroviruses and reoviruses have been detected in the latter species in samples taken from a polluted estuary (10, 11). Oysters have also been the vehicles in large common-source outbreaks of infectious hepatitis in Sweden (13) and the United States (9).

Means of detecting oyster-borne hepatitis virus are not yet at hand. The procedures used to detect enteroviruses in oysters have been either very laborious or relatively insensitive. This is true because oyster suspensions are toxic to the tissue cultures used to demonstrate these viruses. One has had either to treat the suspensions intensively to reduce toxicity (6, 8, 10–12) or to dilute them to minimize toxic effects (7).

Studies in our laboratory, unrelated to oysters, led to use of two polyelectrolyte products which had been developed to flocculate solids in water and wastewater (1). Aquafloc 418, “a mixture of high-molecular-weight cationic flocculants” produced by the Dearborn Chemical Division, W. R. Grade and Co., Chicago, Ill., had been tested for recovery of enteroviruses from experimentally contaminated ground beef (D. J. Engeseth, unpublished data). Cat-Floc, a polydimethylallyl ammonium chloride (molecular weight ≈ 500,000) produced by the Calgon Corp., Pittsburgh, Pa., had been used in tests on enterovirus-contaminated water (2). The present report describes tests of these two polyelectrolytes in flocculating shellfish solids for enterovirus recovery from two species of inoculated oysters, and the finding that one of them, Cat-Floc, is well suited to this purpose.

MATERIALS AND METHODS

Viruses and tissue cultures. Poliovirus type 1, strain CHAT, coxsackievirus type A9, strain P.B., and echovirus type 6, strain d’Amori, were obtained from the Virus and Rickettsial Registry, American Type Culture Collection. Coxsackievirus type B2 was a clinical strain obtained from Dorothy Hamre, then of the University of Chicago. All had been standardized by three serial subcultures from isolated plaques and were propagated in primary rhesus (Macaca mulatta) monkey kidney (PMK) cell cultures. Our methods for propagation and assay of enteroviruses for plaque-forming units (PFU) in PMK cultures have been described in detail elsewhere (3).

Oysters. Gulf Coast oysters (C. virginica) had been tonged in Galveston Bay and were shipped to us frozen by K. J. Fugate, U.S. Food and Drug Administration, Dallas, Tex. English oysters (O. edulis) were gathered from the Tamar River, Cornwall, England, and were sent frozen by air by Betty C. Hobbs, Central Public Health Laboratory, London, England. Both lots were tested extensively and judged to be free from enteroviruses as received. The oysters were stored at −16 °C and were quick-thawed at the time of use. Virus was injected into the digestive tract or pipetted onto the oyster’s surface in different experiments: no differences in results, attributable to mode of inoculation, were observed.

Clarification. The “SF, F” method, which served as the basis for comparison in these studies, has been described in detail previously (8). Briefly, the experimentally contaminated oyster (≈20 g) was minced with scissors and shaken with 80 ml of glycine-NaOH (G/N) buffer (pH 8.8), 20 ml of a gamma or fetal calf serum, 20 g of MgCl2-6H2O, and 100 ml of Freon TF (E. I. Du Pont De Nemours & Co. Inc).
The mixture was homogenized in an ice bath and centrifuged for 45 min at 8,000 \( \times g \). The supernatant fluid was homogenized with another 100 ml of Freon TF and centrifuged again. The resulting supernatant fluid was designated SF, F oyster extract. Its pH was \( \sim 7.4 \).

In the polyelectrolyte method, the experimentally inoculated oyster was minced as described above and stirred for 1 min with 100 ml of GN buffer. A 2-ml amount of stock solution (usually 1%, w/v) of the polyelectrolyte being tested was added, and stirring was continued for 15 min. The flocc which had formed was allowed 5 min to settle. The entire mixture was poured through Miracloth (Chicopee Mills), reinforced with cheesecloth, in a potato ricer (R. Sullivan, personal communication). The cloth was folded over the retained flocc, and residual fluid was expressed by pressure from the plunger. The filtrate was filtered again, in an 11-cm Büchner funnel with vacuum, through successive layers of Miracloth, cheesecloth, glass-fiber prefilter, and a 0.45-\( \mu \)m porosity GA-6 membrane filter (Gelman Instrument Co.).

**Concentration.** Extracts prepared by either the SF, F or Cat-Floc method could be concentrated by ultracentrifugation. An extract was divided among two or three tubes for the no. 50.1 rotor of a Spinco model L ultracentrifuge and run at 200,000 \( \times g \) for 2 hr. The supernatant fluids were discarded; the pellet in each tube was collected with 0.5 ml of phosphate-buffered saline (pH 7.4) plus 2% gamma calf serum and inoculated directly into a PMK culture.

Cat-Floc oyster extracts containing low levels of virus were more frequently concentrated by ultrafiltration. An entire extract could be concentrated in one step in a model 202 Diaflo filter holder (Amicon Corp.) with nitrogen gas pressure (25 psi) and an XM100A filter which had been pretreated with gamma calf serum. Stirred at a rate just less than that which would cause foaming, the extract was reduced to \( \sim 5 \) ml and inoculated directly into PMK cultures at 0.5 ml per culture. This degree of concentration was attained within 1 hr.

**Coproantibody.** For reasons discussed elsewhere (8), we were concerned that virus neutralized with coproantibody might sometimes occur in oysters. A modification of Urbano's method (14) was used to prepare coproantibody to poliovirus type 1 (8). The liquid preparation comprised the supernatant fluid (after 9,000 \( \times g \) for 20 min) from a 20% (w/v) fecal suspension, heated at 56 C for 30 min to inactivate any residual virus. Equal volumes of this fluid and of virus suspension were incubated at 37 C for 6 hr and stored until use at \( \sim 16 \) C.

**RESULTS**

**Aquafloc 418.** The manufacturer's recommendations did not indicate the proper Aquafloc concentration for our purpose. Suspensions of *C. virginica* were treated with 2 ml each of 0.1, 1.0, and 10% (w/v) Aquafloc solutions. Each of the extracts obtained was turbid, but that treated with the 1% solution was clearer than the others. This concentration was used in clarifying suspensions of *C. virginica* which had been inoculated with poliovirus type 1. Recovery of virus was poorer than in extracts obtained by the SF, F method (Table 1). Adjusting the pH of the oyster suspension (in either direction) after adding the polycation did not help (Table 1). When oysters were inoculated with 100 PFU or less of virus and the extracts were concentrated by ultracentrifugation, no virus was recovered. This must have been at least partly a result of the large quantity of sediment formed in the ultracentrifuge tubes. No further experiments were done with Aquafloc.

**Cat-Floc.** The solutions added, at 2 ml per oyster suspension, contained 15 or 1% (w/v) Cat-Floc. The oysters (*C. virginica*) had been inoculated with \( \sim 3,000 \) PFU of poliovirus type 1 each. The suspension treated with the 15% solution yielded 71 ml of extract containing 21% of the inoculated virus. The yield with the 1% solution was 98 ml with 89% of the inoculated virus. An oyster treated by the SF, F method, for comparison, yielded 94 ml with 96% of the inoculated virus.

Three oysters (*C. virginica*) were inoculated with \( \sim 10^9 \) PFU of poliovirus type 1 each. They were homogenized with GN buffer, and 2 ml of 1% Cat-Floc was added to each suspension. One suspension was adjusted to pH 8.5 with 1 n NaOH. The second was left unmodified (pH 7.5), and the third was adjusted to pH 6.8 with 1 n HCl. Each yielded 100 ml of very clear extract, and virus recoveries ranged from 90 to 100%.

In a first attempt at concentrating oyster extracts prepared by the Cat-Floc method, two oysters (*C. virginica*) were inoculated with \( \sim 20 \) PFU of poliovirus type 1 each. After clarification-

**Table 1. Recoveries of inoculated poliovirus type from Crassostrea virginica oysters by the Aquafloc and SF, F methods**

| Virus input (PFU/oyster) | Aquafloc extracts | SF, F extracts |
|-------------------------|-------------------|---------------|
|                         | Titer (PFU/ml)    | Vol (ml)      | Recovery (%) | Titer (PFU/ml) | Vol (ml) | Recovery (%) |
| 2.3 \( \times 10^9 \)   | 1.1 \( \times 10^8 \) | 82            | 39           | 2.0 \( \times 10^4 \) | 98       | 87           |
| 5.0 \( \times 10^9 \)   | 1.3 \( \times 10^8 \) | 85            | 22           | ND*          | ---      | ---          |
| 3.5 \( \times 10^9 \)   | 25                | 76            | 5            | ND*          | ---      | ---          |
| 2.0 \( \times 10^9 \)   | 4.2 \( \times 10^8 \) | 90            | 18           | 2.7 \( \times 10^3 \) | 90       | 122         |

* Oyster suspension adjusted to pH 8.4 with 3 n NaOH.
* Not done.
* Oyster suspension adjusted to pH 6.0 with 6 n HCl.
tion, one extract was concentrated by ultracentrifugation and the other by ultrafiltration. The plaque counts for the virus controls and for the concentrated extracts ranged from 90 to 100% of the estimated number of PFU inoculated.

**Oyster species.** Eight oysters (four *C. virginica* and four *O. edulis*) were inoculated with ~70 PFU of poliovirus type 1 each. Two of each species were processed at a time, one by the SF, F method and one by the polycation method. The SF, F extracts were concentrated by ultracentrifugation, and the Cat-Floc extracts were concentrated by ultrafiltration. The results of this and of a second, identical run showed no effect of oyster species upon the efficiency with which the virus was recovered (Table 2).

**Enterovirus type.** Three other enterovirus serotypes were tested because their plaquing efficiency had been affected by other aqueous polymer solutions (4, 5). The oysters used in these experiments were *C. virginica*. Two were inoculated with ~73 PFU of coxsackievirus type A9 each, extracted with the aid of Cat-Floc, pooled, and concentrated by ultrafiltration: 97% of the inoculated virus was recovered. Two were inoculated with 89 PFU of coxsackievirus type B2 each; after the same treatment, the yield was an apparent 108% of the inoculated virus. The last two oysters received 58 PFU of echovirus type 6 each, and yielded 88% of the inoculated virus. There were no significant differences in recovery of different virus types; neither did these three in particular appear to be inhibited by the polycation.

**Coproantibody.** A suspension of poliovirus type 1 virus, ~99% neutralized by coproantibody, and a control suspension lacking coproantibody were sampled and inoculated into two oysters (*C. virginica*) each. One oyster of each pair was extracted by the SF, F method and concentrated by ultracentrifugation. The other two oysters were extracted by the Cat-Floc method and concentrated by ultrafiltration. At the end of this time (~4 hr), the original virus suspensions, which had been held at room temperature during the oyster manipulations, were sampled again. Reactivation of coproantibody-neutralized virus by the SF, F method was close to 50%, whereas that by the Cat-Floc method was essentially nil (Table 3).

**DISCUSSION**

The polyelectrolyte method of preparing oyster samples for virus recovery is considerably simpler and quicker than comparable procedures (6-8, 10-12). Studies by others have not indicated that enteroviruses are held tenaciously in or on tissues of oysters of these species (6, 7, 10-12). This and our experience in developing method SF, F, which has now been used successfully to detect viruses in field samples of oysters (K. J. Fugate, personal communication), indicate that results with "naturally" contaminated oysters can be expected to be similar to those with oysters inoculated with virus in the laboratory.

The extract obtained contains essentially all of the inoculated enterovirus and is ready for further concentration, if desired, in an ultracentrifuge or ultrafilter. It can also be filtered rapidly at 0.2-μm porosity to eliminate bacteria, if necessary. The species of oysters, the pH of the prepared suspension, and the type of enterovirus had no effect upon the efficiency of virus recovery, within the range of variables in these experiments. Virus was as efficiently recovered at low as at high initial levels of inoculation. The time elapsed in preparing a sample to inoculate into tissue cultures was less than 2 hr. Since several could be processed concurrently, the labor requirement per sample was well under 2 man-hr. The costs of consumed supplies and of equipment for the extraction process were very low.

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**Table 2. Percentage of inoculated poliovirus type 1 recovered from two species of oysters by two extraction methods**

| Expt | Crassostrea virginica | Ostrea edulis |
|------|-----------------------|---------------|
|      | SF, F*                | Cat-Floc*     | SF, F | Cat-Floc |
| 1    | 94                    | 96            | 87    | 97       |
| 2    | 85                    | 94            | 86    | 98       |
| Mean | 90                    | 95            | 86    | 98       |

* SF, F extracts concentrated by ultracentrifugation.
* Cat-Floc extracts concentrated by ultrafiltration.

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**Table 3. Recovery of neutralized poliovirus type 1 from Crassostrea virginica oysters by two extraction methods**

| Treatment | Virus inoculum (PFU) | Recovery (total PFU) |
|-----------|----------------------|----------------------|
|           | 0 hr                 | 4 hr                 |
| Control   | 3.7 x 10^4           | 3.4 x 10^4           |
| Coproantibody | 20                  | 15                   |

* SF, F extracts concentrated by ultracentrifugation.
* Cat-Floc extracts concentrated by ultrafiltration.
The costs for concentration would depend upon the method selected. There may well be other polyelectrolyte flocculants which would serve as well as Cat-Floc, but some, such as Aquafloc 418, would not. The three virus types other than poliovirus type 1 were selected because each had been shown to be affected, with regard to efficiency of plaque initiation, by other aqueous polymer solutions (4, 5). No such effect was seen with Cat-Floc.

An apparent disadvantage of the polyelectrolyte method, when compared with the “SFF” procedure, is inability to recover virus neutralized by coproantibody (8). The significance of this deficiency depends upon the proportion of virus that an infected host sheds in coproantibody-neutralized form, the stability of such virus outside the host, and the capability of such virus to infect by the oral route. The literature seems not to contain answers to these questions.

We expect this method to be applicable to other species of shellfish, but certainly not to all other foods. Extremely variable results were obtained in limited tests of the method with cheddar cheese (R. M. Herrmann, unpublished data). However, given the apparent significance of virus transmission through shellfish, a better method to apply to this problem alone would seem worth having.

ACKNOWLEDGMENTS

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ERRATA

Activation of a Toxic Component of Clostridium botulinum Types C and D by Trypsin

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Volume 24, no. 1, p. 108, abstract; p. 110, column 2, paragraph 3; and p. 110, column 2, last paragraph to end of text, including Tables 3 and 4: Wherever C1 toxin is mentioned, add “and possibly D toxin.”

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Volume 24, no. 4, p. 541, column 1, lines 11-13: Change “...stirring was continued for 15 min. The floc which had formed was allowed 5 min to settle.” to “...stirring was continued for 5 min. The floc which had formed was allowed 15 min to settle.”