

## Glass Wool-Hydroextraction Method for Recovery of Human Enteroviruses from Shellfish

J. T. TIERNEY, A. FASSOLITIS, D. VAN DONSEL, V. C. RAO, R. SULLIVAN and E. P. LARKIN\*

*Virology Branch, Division of Microbiology, Food and Drug Administration, Cincinnati, Ohio 45226*

(Received for publication July 16, 1979)

### ABSTRACT

The Glass Wool-Hydroextraction Method was developed to analyze a number of foods for the presence of contaminating human enteroviruses. This method was modified to examine a variety of shellfish, including oysters and hard- and soft-shell clams. The method consistently recovered ca. 50% of viruses inoculated into shellfish at levels of ca. 10 virus units/100 g. In a multilaboratory study, the method successfully detected all but one of the eight test viruses, and the quantitative recoveries compared favorably with the control laboratory data.

For a number of years, we have been developing methods in this laboratory for recovery of viruses from foods (6,8,9). The number and complexity of foods in the marketplace presented a formidable challenge to development of a method that could be used to analyze a variety of different foodstuffs. Because of the limited virus susceptibility of cell culture systems, the methods were restricted to detection of human enteroviruses. This decision was made because of the known public health significance of these human viruses and because of past experience with outbreaks shown to be associated with foods contaminated by the food handlers or by human wastes.

In 1975, a collaborative study was made of the glass wool filtration method, and six investigators demonstrated the effectiveness of the method for detection of virus levels of > 10 units/g of ground beef (6). However, data from this laboratory and others in the United States and Europe demonstrated that virus levels in contaminated foods were of a low order of magnitude (2,3,4,7,8). Therefore the method was modified to detect virus levels of ca. 10 units/100 g of food. This procedure was used to analyze a number of shellfish samples known to be contaminated with human enteroviruses.

### METHOD

(a) Blend at low speed in a Waring blender two shellfish for 20 sec at 1:10 dilution of 0.01 M Tricine containing 5 ml of  $MgCl_2 \cdot 6H_2O$  (475 g/L) and 5 ml of DEAE-dextran (10 g/L), pH 9.0. Add 1 ml of antifoam emulsion (Dow-Corning, Midland, MI) to each sample before blending. Blend sufficient shellfish to obtain at least 100 g. The pH of the homogenate must be readjusted periodically to ca. 9.0 with 1 N NaOH. A pH test paper (Micro Essential Lab., Brooklyn, N.Y.; VWR Scientific Box 855, Columbus, OH) was used to prevent contamination that might occur with the use of a pH meter.

(b) Stir on a magnetic stirrer in an incubator at 37 C for 1 h.

(c) Pour the contents into a sterile 150-mm funnel containing 5 g of glass wool that has been pretreated in situ with 100 ml of Tricine, pH 9.0. Attach 8 to 10 ft of sterilized dialysis tubing, 1-1/8 in. in diameter to the base of the funnel.

(d) After 0.5 h, or when filtration is complete, rinse with 100 ml of Tricine and depress the glass wool with a tongue depressor to remove excess fluid.

(e) Remove the dialysis bag and seal with special closures (Spectrum Medical Industries, Inc.; Cole-Palmer Instrument Co., Chicago, IL). Wet the outside of the dialysis bag with tap water and place it in a large beaker or other container. Add ca. 70 g of polyethylene glycol (20,000 mw) plus 10 ml of water to the container. Place the bag and container in the refrigerator to hydroextract overnight.

(f) The following morning, remove the dialysis bag and wash the outside thoroughly with tap water. Add 30 ml of Tricine ( $MgCl_2$  and DEAE), pH 9.0. Knead the bag thoroughly by hand; then place it on a flat surface and, using a large (rubber) spatula, squeeze the contents slowly out into a 50-ml (screw capped) centrifuge tube, moving from the lower to the upper portion of the dialysis tubing.

(g) Add 1 g of Celite, mix, adjust to ca. pH. 8.5, and centrifuge at 2000 RPM for 0.5 h. (If the sample is expected to be toxic, add 1 part Freon to 5 parts concentrate before centrifugation.)

(h) Decant the supernatant fluid into an 8-oz. specimen cup, add antibiotics, and bring the volume to 100 ml with Tricine (6). Place it in the refrigerator overnight.

(i) The following morning, add 5 ml of inoculum to each of 20 Buffalo green monkey kidney (BGM) cell cultures for the plaque assay; incubate at 37 C. Approximately 4.5 to 5 h later, pour off the inoculum into an additional 20 bottles of BGM cultures (1). To the first set of bottles, add 18 ml of the overlay medium, and to the second group, add 15 ml of growth medium (6).

(j) Incubate, observe cytopathic effect (CPE) and count plaques daily for a period of 14 days.

### RESULTS

In early developmental studies, problems were encountered when lipids present in the shellfish solidified at refrigeration temperatures. This problem was especially troublesome during the filtration and hydroextraction procedures. Mixing the homogenate at 37 C for 1 h liquefied the fat and appeared to monodisperse the lipid globules. When the homogenates were cooled, no further globule aggregations were encountered. Occasionally, a fat layer was detected after low-speed centrifugation (step g).

Initially, 30 ml of Tricine (pH 9.0) was used to recover the virus concentrate from the dialysis tubing. This volume was increased to 70 ml to attempt to enhance virus recovery efficiency, but no difference was noted. About 50% of the input viruses were detected with either procedure. Whether the pH is maintained at 8.5 or 9.0 is not critical, since virus recovery was the same when the

pH ranged from 8.0 to 9.0. Therefore pH indicator paper could be used successfully to monitor the sample with a minimum loss of sample volume.

Because of cell culture toxicity associated with some shellfish tissues, the concentrate was diluted to 100 ml, and 5 ml was inoculated onto the cell cultures. It was anticipated that the toxic substance thus diluted would be less likely to produce nonspecific cytopathologic changes on the cell sheet. An additional safeguard was taken by pouring the inoculum into a second culture; we anticipated that any toxicity associated with the inoculum would be removed by the cells in the first bottle. On several occasions the cells in the first bottle were destroyed, but those in the second remained intact during the incubation period.

The addition of 5 ml of concentrate to the cell culture was a change in our normal procedure of adding 1-ml portions to the cell culture. To determine what effect this increase in volume would have on virus adsorption, a study was initiated to monitor the differences in concentrate volume and adsorption times (Table 1). To obtain virus titers comparable to the 1-ml/1-h adsorption procedure, the 5 ml concentrate adsorption time was increased to 5 h. Little if any increase in total virus

recovery occurred when the 5-ml concentrate was transferred to a second cell sheet. If cells with a different virus susceptibility were used in the second cultures, different viruses might be detected (4).

In sample F, Freon (1 part) was added to the concentrate (5 parts) and removed from the dialysis tubing. This step was taken because two investigators examining the samples in this laboratory had encountered toxicity problems with this sample. Some of the samples were found to be toxic after addition of the concentrates, but sufficient cultures remained unaffected to process the sample.

The effectiveness of the Glass Wool-Hydroextraction Method was demonstrated in a multilaboratory study of methods for recovery of viruses from shellfish meats contaminated with a variety of enteroviruses (5). The recovery data obtained using the Glass Wool-Hydroextraction method from this study are shown in Table 2. All but one of the eight test viruses were detected, and the quantitative recoveries compared favorably with the control laboratory data. In all the samples containing Echovirus, the CPE was more sensitive than the plaque technique (PFU) in detecting the presence of virus.

TABLE 1. Effect of volume of inoculum and adsorption time on virus titer.

Procedure	No. of tests	Virus titer after 5 h adsorption volume added			Virus titer after 1 h adsorption volume added		
		10 ml	5 ml	1 ml	10 ml	5 ml	1 ml
1. Inoculate-overlay	11 <sup>a</sup>	10 <sup>b</sup>	10.7	14.2	7.3	10.3	11.3
2. Inoculate, pour off, overlay; 2nd bottles CPE	10	10.5	10.6	11.3	7.5	10.8	10.4
3. Inoculate, pour off, overlay; 2nd bottles CPE, overlay negative CPE	16	11.0	10.8	10.8	5.3	10.3	9.5
4. Inoculate, pour off, overlay; 2nd bottles overlay after 1 h	3	10.3	8.7	15.3	9.0	13.4	13.7

<sup>a</sup>5 bottle cultures used in each test plus an additional set of 5 cultures when inocula were poured off into the 2nd bottle cultures.

<sup>b</sup>Total of plaque numbers plus CPE. Each culture showing CPE was counted as 1 PFU.

TABLE 2. Recovery of viruses from seeded shellfish.<sup>a</sup>

Virus numbers reported as	Cell cultures used		A	B	C	D	E	F
	PFU	BGM	Oysters	Clams, hardshell	Clams, hardshell	Oysters	Clams, softshell	Clams, softshell
Glass Wool-Hydroextraction Method	PFU	BGM	113	11	4	17	1	2
	CPE <sup>b</sup>	BGM	18	14	9	ND <sup>c</sup>	8	8
	Total		131	25	13	17	9	10
Types			P1	CB3	E7	P1	E17	E7,P2
Control laboratory recoveries:								
Initial <sup>e</sup> samples	PFU	BGM	9	6	6	4	6	8
	CPE	BGM	22	3	7	17	0 <sup>d</sup>	30
Final samples	PFU	BGM	ND	ND	20	ND	ND	25
	CPE	BGM						
Total			22	3	27	17	0	55
Types			P1	CB3	E7	P1,P2	E17	E7,P2

<sup>a</sup>P1, Poliovirus 1; P2, Poliovirus 2; B3, Coxsackievirus B-3; E7, Echovirus, 7; E17 Echovirus 17.

<sup>b</sup>Monolayers showing CPE expressed as 1 PFU.

<sup>c</sup>ND, not done.

<sup>d</sup>No isolates recovered by PFU assay in final test.

<sup>e</sup>Samples were mailed from the control laboratory. After all the investigators had received their samples, the control laboratory analyzed the initial samples; after all investigators had reported their recovery data, the control laboratory analyzed a second set of samples that had been stored at -70 C (final samples).

### DISCUSSION

Methods have been developed for recovery of viruses from complex foods with the objectives of quantitative recovery of low levels of virus contaminants and minimal use of costly equipment and apparatus. Another important objective was to develop methods that could be used to analyze a number of food samples during a normal working period. The Glass Wool-Hydroextraction Method utilizes equipment and supplies that would normally be found in a virus laboratory or that could be purchased at a nominal cost. With this method, one investigator can process 10 to 15 shellfish samples a week with a recovery efficiency of ca. 50% when virus levels are ca. 10 units/100 g of shellfish meat.

### ACKNOWLEDGMENT

The excellent technical assistance of Virgil I. Jones is gratefully acknowledged.

### REFERENCES

1. Dahling, D. R., G. Berg, and D. Berman. 1974. BGM, A continuous cell line more sensitive than primary Rhesus and

- African green kidney cells for the recovery of viruses from water. *Health Lab. Sci.* 11:275-281.
2. Denis, F., and J. F. Brisou. 1976. Contamination virale des fruits de mer: Etude portant sur l'analyse de 15,000 coquillages. *Bull. Acad. Med.* 160:18-22.
3. Fugate, K. J., D. O. Cliver, and M. T. Hatch. 1975. Enteroviruses and potential bacterial indicators in Gulf Coast oysters. *J. Milk Food Technol.* 38:100-104.
4. Kostenbader, K. D., Jr., and D. O. Cliver. 1977. Quest for viruses associated with our food supply. *J. Food Sci.* 42:1253-1258.
5. Larkin, E. P., and T. G. Metcalf. 1980. Cooperative study of methods for the recovery of enteric viruses from shellfish. *J. Food Prot.* In press.
6. Larkin, E. P., J. T. Tierney, R. Sullivan, and J. T. Peeler. 1975. Collaborative study of the glass wool filtration method for the recovery of virus inoculated into ground beef. *J. Assoc. Offic. Anal. Chem.* 58:576-578.
7. Metcalf, T. G., R. Comeau, R. Mooney, and J. H. Ryther. 1977. Opportunities for virus transport within aquatic and terrestrial environments. pp. 77-101. In B. P. Sagik and C. A. Sorber (eds.), *Risk assessment and health effects of land application of municipal wastewater and sludges*. Univ. of Texas at San Antonio.
8. Sullivan, R., A. C. Fassolitis, and R. B. Read, Jr. 1970. Method for isolating viruses from ground beef. *J. Food Sci.* 35:624-626.
9. Tierney, J. T., R. Sullivan, E. P. Larkin, and J. T. Peeler. 1973. Comparison of methods for the recovery of viruses inoculated into ground beef. *Appl. Microbiol.* 26:497-501.

### Pflug, Smith, Holcomb, and Blanchett, *con't from p. 123*

to traverse one log cycle; it is the time required for the temperature difference between product and heating or cooling medium to decrease by 90%;  $f_h$  identifies the heating parameter.

F,  $F_0$ ,  $F_0(\text{BIO})$ ,  $F_0(\text{PHY})$

The F-value is the equivalent time at temperature T of a process delivered to a container or unit of product for the purpose of sterilization; it is the common measure of the level of the sterilization process and is calculated using a specific value of z.  $F_0$  indicates that the temperature was 250 F and the z-value was 18 F.  $F_0(\text{BIO})$  indicates that the  $F_0$ -value was measured biologically;  $F_0(\text{PHY})$  that it was determined from data measured physically.

$j$

Lag factor of the semilogarithmic heating curve for a specific location in a product in a container.

$$j = \frac{(\text{heating medium temperature}) - (Y\text{-intercept temperature})}{(\text{heating medium temperature}) - (\text{initial product temperature})}$$

$r^2$

Statistical correlation coefficient.

$z$

Measure of the direction of the thermal death time curve, the number of degrees of temperature change necessary to cause the F-value to change by a factor of 10.

### REFERENCES

1. Butterfield, C. T. 1933. The selection of a dilution water for bacteriological examinations. *Public Health Rep.* 48:681-691.
2. Conover, W. J. 1971. *Practical nonparametric statistics*. Wiley, New York.
3. Pflug, I. J. 1976. Method and apparatus for sterility monitoring. U.S. Patent 3,960,670.
4. Pflug, I. J. 1980. Converting an F-value determined on the basis of one z-value to an F-value determined on the basis of a second z-value. *J. Food Sci.* 45:35-40.
5. Saseen, D. M. 1969. Computer program for process calculation by the simplified Ball formula method. Pamphlet D-2247, National Canners' Association, Berkeley, Calif.