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Development of a Method for Concentration of Rotavirus and Its Application to Recovery of Rotaviruses from Estuarine Waters

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As part of our studies on the ecology of human enteric viruses, an improved method for detection of rotaviruses in water was developed, and their presence in Galveston Bay was monitored. Samples (378 liters) of estuarine water adjusted to pH 3.5 and a final $AlCl_3$ molarity of 0.001 were filtered through 25-cm pleated cartridge-type filters (Filterite Corp., Timonium, Md.) of 3.0- and 0.45-μm porosity. Adsorbed virus was eluted with 1 liter of 10% tryptose phosphate broth, pH 9.5. Primary eluates were reconcentrated to a final volume of 10 to 20 ml by a simple and rapid magnetic iron oxide adsorption and elution procedure. Two percent casein at pH 8.5 effectively eluted rotavirus from iron oxide. A total of 21 of 72 samples of water, suspended solids, fluffy sediments, and compact sediments collected in different seasons in Galveston Bay yielded rotaviruses. Recovery of rotaviruses varied from 119 to 1,000 PFU/378 liters of water, 1,200 PFU/1,000 g of compact sediment, 800 to 3,800 PFU/378 liters of fluffy sediment, and 1,800 to 4,980 PFU from suspended solids derived from 378 liters of water based on immunofluorescent foci counts on cover slip cultures of fetal monkey kidney cells.

Rotaviruses are now recognized as a major cause of severe infantile diarrhea (1, 7, 9). Rotaviruses may also cause gastroenteritis in adults either by contact with infected infants (17) or through contaminated water (6, 8, 16, 19). Outbreaks of rotavirus illness associated with sewage-polluted waters emphasize the existence of a capability for transmission by water.

As many as 10^6 or more rotavirus particles are often present in 1 g of stool from a child with acute gastroenteritis. Rotavirus has been detected in 25% of raw sewage samples examined in Kiel, Federal Republic of Germany (15). In Houston, a seasonal distribution of rotavirus exists in raw sewage with low levels from May through September, and high levels during winter and spring (5, 13). Using temporally matched 24-h composite samples of raw sewage and effluent, we found that the final chlorinated effluent from an activated sludge plant treating 1.5×10^6 gallons (ca. 5.7×10^6 liters) of domestic sewage per day was discharging 4.8×10^7 total infectious rotaviruses per day. Large volumes of sewage effluents from treatment plants in Houston and neighboring towns flow into Galveston Bay daily. The bay is a popular recreational area and a commercially important source of shellfish.

A high percentage of viruses are associated with solids in sewage effluents (2) and, upon discharge into coastal waters, viruses associated with large particles (>6 μm) leave the water column and settle down in the bottom sediments. Viruses adsorbed on smaller solids, including colloids (<3 μm), tend to stay afloat in the water for a longer duration. Suspended solid-associated virus, when recently settled out of a water column, accumulates in a loose, fluffy layer over the compact bottom sediments (11). Sediments in coastal waters serve as a reservoir from which human viruses can be released when the water is disturbed by storms, dredging, and boating. Viruses from fluffy sediments are more easily resuspended by mild turbulence or water movement. Resuspended viruses from polluted waters can be transported to distant nonpolluted areas used for shellfish production and recreational purposes.

To assess health hazards attributed to rotaviruses in polluted coastal waters and to study virus survival and transport in water, sensitive and reliable methods for detection of rotavirus are needed. This report describes the use of the model rotavirus SA-11 for developing concentration methods which were then used to recover human rotaviruses naturally present in Galveston Bay estuarine water, suspended solids, and fluffy and compact sediments.

MATERIALS AND METHODS

Cell cultures. A continuous line of fetal monkey kidney cells, MA104, was used for culturing. The cell line was grown in Eagle minimal essential medium supplemented with 10% fetal calf serum, 5% tryptose phosphate broth (TPB), 2% basal Eagle medium, vitamins, 0.25% glucose, 0.02% glutamine, 0.075% sodium bicarbonate, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 25 μg of gentamicin per ml. Routine subculturing of cells was completed with 0.2% trypsin in phosphate-buffered saline containing 0.025% EDTA.

Viruses and viral assays. Rotavirus SA-11 was grown in MA104 cells as previously described (12). Virus samples were diluted in Tris-buffered saline containing 20 mM Tris, 140 mM NaCl, 5 mM KCl, 0.5 mM Na_2HPO_4, 6 mM glucose, 0.5 mM $MgCl_2$, 0.7 mM $CaCl_2$, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 50 μg of gentamicin per ml, pH 7.2

Virus was plaque assayed on 10-cm^2 MA104 monolayers as previously described (12). Natural rotaviruses were recovered by modification of a previously reported indirect immunofluorescence method (13). Cover slip monolayers of MA104 cells were inoculated with 0.25 ml of inoculum adsorbed at 37°C for 1.5 h. Centrifugation of cover slips and treatment of inocula with pancreatin were not found to be essential. Cover slips were alcohol fixed and were stained with fluorescent antibody after 24 h of incubation at 37°C in a CO_2 incubator. An entire cover slip was examined for fluorescent foci (FF) with a microscope (Carl Zeiss, Inc.,

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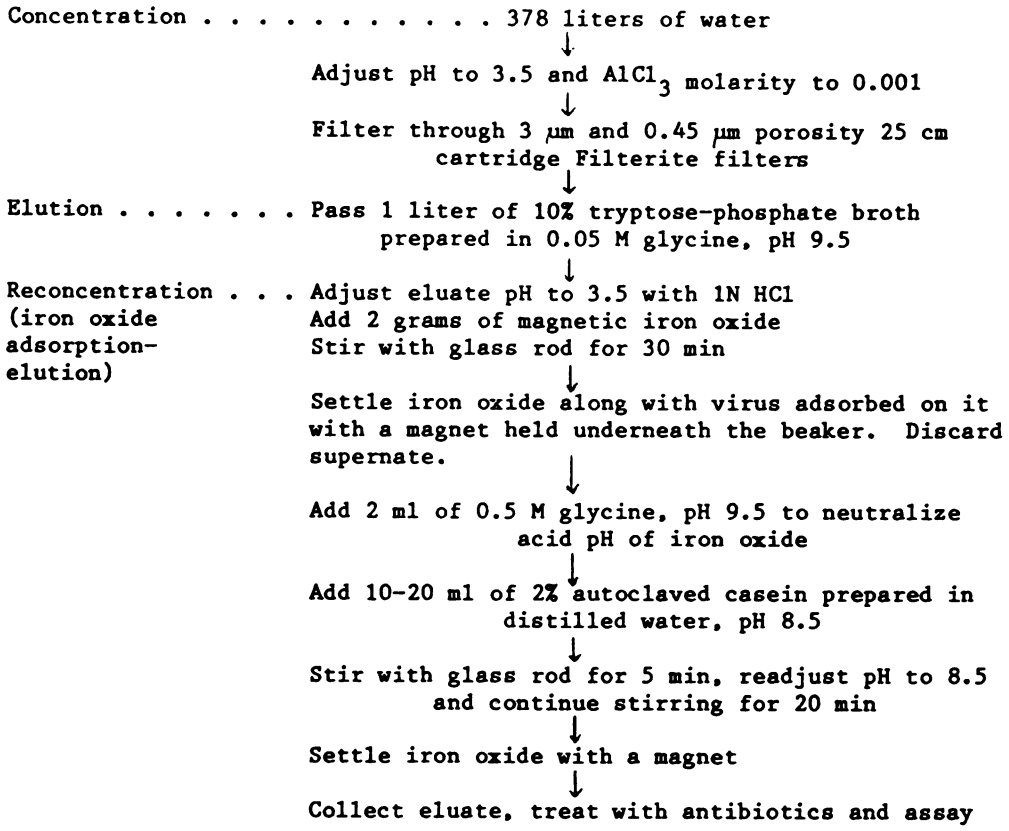

FIG. 1. Scheme for concentration of rotavirus from estuarine water.

Thornwood, N.Y.) fitted for epifluorescence, using a filter and beam-splitter combination for blue-green excitation (filter pack no. 487709; wavelength, 450 to 490 nm).

Sampling sites. Water samples were collected from sites at Kemah and Seabrook in Galveston Bay, located within 80 km of our laboratory on the Gulf of Mexico. The two areas were receiving secondarily treated, chlorinated sewage effluent. Water depth at the sampling sites ranged from 1 to 2 m at low tide. Sampling at Kemah was conducted 30 m offshore from a partially sunken barge, whereas samples at Seabrook were collected within 1 m of the shore. From July 1981 through February 1982 a total of 72 samples were collected and processed. Water characteristics at the collection sites varied as follows: temperature, 14 to 32°C; pH, 7.6 to 8.3; turbidity, 10 to 115 nephelometric turbidity units; salinity, 2 to 20‰.

Virus concentration procedures. (i) Suspended solids. About 378 liters of seawater at ambient pH (7.6 to 8.3) was filtered through a combination of 10-in. (ca. 25.4 cm) pleated cartridge filters of 3.0- and 0.45-μm nominal porosity (Filterite Corp., Timonium, Md.) at a flow rate of 19 liters/min with a 1.9-kW (2.5 horsepower) gasoline-engine water pump. The intake water hose was submerged only 1 to 2 cm below the water surface to prevent agitation of sediments. Suspended solids trapped on the filters were backwashed with 1 liter of 10% TPB prepared in 0.05 M glycine (GLY) (pH 9.5). Eluates were neutralized to pH 7.0, carried to the laboratory on ice, stored in a cold room overnight, and the next day were reconcentrated by the iron oxide method (Fig. 1).

(ii) Fluffy sediment. Samples were collected on 3.0- and 0.45-μm-pore cartridge filters by a sediment-associated virus sampler (10). The fluffy sediment, separated from collecting filters by backwash with sterile 0.01 M phosphate-buffered saline, was returned to the laboratory on ice and stored overnight in the cold room. The sample was centrifuged at 1,500 × *g* for 10 min. The deposit was shaken for 15 min with 3 volumes of 10% TPB-GLY (pH 9.5) to extract the virus. The sample was centrifuged at 1,500 × *g* for 10 min. The sediment was discarded. The supernatant containing the virus was reconcentrated by the iron oxide method (Fig. 1).

(iii) Sediment. Samples of compact sediment were collected with an Ekman dredge from the same site and were transferred into polyethylene bags. After overnight storage in the cold room, processing began on the following morning. A 300-g portion of the sample was shaken for 15 min with 3 volumes of 10% TPB-GLY (pH 9.5) and was clarified by centrifugation at 1,500 × *g* for 10 min. The supernatant was concentrated by the iron oxide method (Fig. 1).

(iv) Water. A 378-liter water sample from which suspended solids had been removed by filtration through 3.0- and 0.45-μm-pore cartridge filters was collected in a tank (Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y.). Viruses from this sample were concentrated by using a single 0.45-μm-pore cartridge filter as described previously (3). Viruses in the primary eluate were reconcentrated by the iron oxide method (Fig. 1). Final eluate volumes of 10 ml each were obtained from water, suspended solids, and fluffy sediments, and 20-ml volumes were obtained from compact sediments.

Virus survival. Solid-associated virus survival was studied by addition of aliquots of SA-11 to 100 ml of estuarine water

(salinity, 15‰) and suspensions of compact sediment, suspended solids, and fluffy sediments, respectively. Samples of 1 ml each were withdrawn for assay at regular intervals during a 19-day period.

RESULTS

Factors affecting adsorption and recovery of rotavirus on Filterite filters. (i) pH. The ability of Filterite filters to concentrate rotavirus SA-11 from estuarine water was tested at different pH levels. Virus (2.3×10^5 PFU) was seeded into 100-ml volumes of estuarine water with a salinity of 13‰ (pH 7.8), the pH was adjusted as indicated, and the samples were filtered through assemblies of 47-mm, 3.0- and 0.45-μm-pore Filterite filters. Virus in the filtrates was assayed in triplicate experiments. Percentages of virus adsorbed (± standard deviation) were 72.3 ± 7.5 at pH 3, 59.3 ± 9.0 at pH 4, 31.3 ± 7.6 at pH 5, 16.6 ± 2.4 at pH 6, and 11.0 ± 3.6 at pH 7.

(ii) Addition of $AlCl_3$. Virus-seeded estuarine water samples adjusted to a pH of 3.5 and supplemented with different concentrations of $AlCl_3$ (0.0001 to 0.005 M) were filtered through separate sets of Filterite filters. The filtrates were assayed for their virus content. Virtually all virus adsorbed to filters at $AlCl_3$ concentrations of >0.001 M.

(iii) Eluent efficacy. Three different eluents, 3% beef extract (pH 9.5) plus GLY (BE-GLY), 0.05 M GLY, pH 10.5, and 10% TPB (pH 9.5) plus GLY (TPB-GLY), were tested for their efficiency in recovering rotavirus adsorbed to Filterite filters. Volumes (100 ml) of estuarine water (salinity, 15‰; pH 7.9) were seeded with 1.4×10^5 PFU of SA-11 virus and passed through assemblies of 47-mm, 3.0-, and 0.45-μm-pore Filterite filters after adjustment of pH to 3.5 and $AlCl_3$ molarity to 0.001. Filters were eluted with 10 ml of each eluent. GLY recovered 38 ± 19.5% (± standard deviation) of the virus. BE-GLY recovered 54 ± 15.6%, and TPB-GLY recovered 68 ± 14.2%.

The efficiency of 10% TPB for recovering SA-11 from filters was tested with 68 to 110 liters of estuarine water seeded with virus. Results of four trials are given in Table 1. The data suggest that recoveries of about 50% of input virus could be expected.

Reconcentration of rotavirus. Pleated cartridge filters with a large surface area are needed for recovery of small numbers of virus from large volumes of seawater. Elution of virus from these filters requires 1 to 2 liters of eluent. Reconcentration of a primary eluate is necessary to achieve a further reduction of sample volume. Organic flocculation and magnetic iron oxide adsorption-elution were compared to assess their efficiency for this purpose. In three trials, 19% of seeded rotavirus could be recovered by the organic flocculation method, while 66% virus recovery was obtained by the iron oxide method. Subsequently, a study of the various factors affecting rotavirus reconcentration by iron oxide adsorption-elution was done. In the first set of experiments rotavirus was suspended in fresh BE, GLY, and TPB-GLY and the optimum pH for adsorption on iron oxide was determined. Maximum adsorption of virus occurred at pH 3 in all three media tested, the relative percent adsorption being 70, 28, and 99% for BE, GLY, and TPB-GLY, respectively. A decrease in acidity of the virus-suspending media was accompanied by a gradual reduction in the amount of virus adsorbed to iron oxide. Only results obtained with 10% TPB are presented in Table 2.

TABLE 1. Recovery of rotavirus SA-11 by elution with TPB-GLY after filtration of large volumes of estuarine water[a]

Trial	Vol of water (liters)	Mo	Virus input (PFU)	% Virus recovered in primary eluate
1	68	Aug.	1.0×10^7	42
2	100	Oct.	6.2×10^6	58
3	110	Jan.	7.2×10^6	50
4	85	Apr.	7.8×10^6	50

[a] Samples (68 to 110 liters) of seasonally collected estuarine water were passed through 3.0- and 0.45-μm-pore pleated cartridge filters at ambient pH, and suspended solids were removed. SA-11 was seeded into filtered water, the pH was adjusted to 3.5, and $AlCl_3$ was added to 0.001 final molarity. A sample was filtered through a single cartridge filter of 0.45-μm porosity. Virus was eluted from the filter with 1 liter of 10% TPB-GLY, pH 9.5.

TABLE 2. Effect of pH upon iron oxide adsorption of rotavirus SA-11 from TPB-GLY[a]

pH	% Adsorption of virus	
	With $AlCl_3$ (0.001 M)	Without $AlCl_3$
3	98.7	99.0
4	97.0	97.6
5	78.1	79.0
6	71.5	72.1
7	57.5	55.3
8	39.4	56.7

[a] 100-ml volumes of 10% TPB-GLY were seeded with 2.4×10^4 PFU of SA-11 and their pHs were adjusted to indicated levels. $AlCl_3$ was added to a final molarity of 0.001 to one set of samples; 0.2 g of magnetic iron oxide was introduced, and each sample was stirred with a glass rod intermittently for 30 min. Iron oxide was settled by a magnet, and supernatants were adjusted to pH 7 and assayed.

(i) Effect of organic and other components of estuarine water on adsorption and recovery of rotavirus. Primary eluates derived from filters through which large volumes of estuarine water were filtered would contain organic and other components of water initially adsorbed to the filters. To determine whether these components exert any influence on the adsorption and recovery of rotavirus from iron oxide, we conducted the following experiment. A volume of 378 liters of estuarine water adjusted to pH 3.5 and on $AlCl_3$ molarity of 0.001 was filtered through 25-cm pleated cartridge Filterite filter combinations of 3.0- and 0.45-μm porosity. The filter was eluted with 1 liter of 3% BE-GLY, pH 9.5, and 10% TPB-GLY, pH 9.5, in separate experiments. Volumes (100 ml) of these primary eluates were seeded with 3,660 PFU of SA-11, pH adjusted to 3.5 and were concentrated by the iron oxide method (Fig. 1). Effectiveness of 2-ml volumes each of 3% BE-GLY (pH 9.5), 10% TPB-GLY (pH 8.5), and 2% casein (pH 8.5), for elution of rotavirus adsorbed on iron oxide was evaluated. Data indicate a median casein recovery of 88 ± 14.2% compared with 66 ± 2.84% for the second-best BE-GLY eluent (Table 3).

(ii) Effect of primary eluate volume on rotavirus concentration and recovery. A primary eluate volume from cartridge filters is usually between 1 and 2 liters. The effect of volume on iron oxide recovery of rotavirus from TPB eluates of 100 to 1,600 ml was tested to see if volume was a critical factor. The eluates were seeded with 600 to 5,400 PFU of the model rotavirus. Data suggest that recovery of virus is more apt to be favored when an eluate volume is ≤800 ml, but do not indicate a significant loss of recovery effectiveness with a 1,600-ml volume.

Recovery of rotavirus from estuarine water of Galveston Bay. The distribution of rotaviruses in the water column at two study sites was investigated seasonally during an 8-month period. Specimens were suspended solids retained on filters, water from which suspended solids had been re-

TABLE 3. Iron oxide concentration of rotavirus SA-11 from BE-GLY and TPB-GLY eluates[a]

Virus-suspending medium (pH 3.5)	Eluent	Virus input (PFU)	% Virus recovery
3% BE	10% TPB-GLY, pH 8.5	3,660	34
	3% BE-GLY, pH 9.5	3,660	64
	2% Casein, pH 8.5	3,660	78
10% TPB-GLY	10% TPB-GLY, pH 8.5	3,660	86
	3% BE-GLY, pH 9.5	3,660	68
	2% Casein, pH 8.5	3,660	98

[a] 100-ml volumes of 3% BE-GLY and 10% TPB-GLY primary eluates from Filterite filters were seeded with 3,660 PFU of SA-11, adjusted to pH 3.5, and concentrated by iron oxide. Virus adsorbed to iron oxide was eluted with 2 ml of the indicated eluents.

moved, fluffy sediments, and compact bottom sediments. Results show that virus was associated with suspended solids in 9 of 18 (50%) samples (Table 4). Six of fifteen (40%) fluffy-sediment samples contained rotavirus, but recovery from compact sediments was made on only one occasion. Rotavirus recoveries from water were made in only 5 of 31 (16%) samples tested. A total of 29% of the samples were positive for rotaviruses.

Numbers of rotaviruses recovered from the different samples tested (Table 4) varied from 119 to 4,980 FF. The greatest number of recoveries was made from suspended solids, 1,800 to 4,980 FF in solids per 378 liters followed by fluffy sediment, 800 to 3,800 FF in sediment per 378 liters. Recoveries from compact sediments were calculated as 1,200 FF/1,000 g, and 119 to 1,000 FF/378 liters were recovered from water.

Survival of rotavirus. Survival data for rotavirus in water and in association with suspended solids and fluffy and compact sediments in estuarine water are summarized in Table 5. Test suspensions of the model rotavirus were kept at 22°C with assays at 3-day intervals. The influence of association with solids in prolonging virus survival is evident. Considerable numbers of the original SA-11 virus remained at day 19 in all solids samples, an indication that rotavirus persistence could allow transmission of infectious virus from polluted to nonpolluted water areas.

DISCUSSION

Pleated cartridge-type Filterite filters are known to adsorb rotaviruses from sewage (13) and estuarine water (3). An advantage of these negatively charged filters is their ability to allow filtration of >378 liters of moderately turbid estuarine water in about 30 to 40 min. Filterite filters were used in the present investigation in view of their rapid filtration capability (14, 18). The use of positively charged filters for recovery of rotavirus seeded into tap water was recently reported (4). Water pH had to be adjusted to 6.5 and it took 2 h to filter 64.5 liters through a 142-mm-diameter 60S Zeta-plus filter (Filterite). An average virus recovery effectiveness of 16% was reported for these filters in three trials. We recovered an average of 50% of the rotavirus added to 68 to 110 liters of estuarine water with Filterite filters. These higher recoveries of the virus coupled with the advantage of filtering large volumes of water in a short time make the Filterite filters very useful in estuarine studies.

Recovery of small quantities of rotavirus from natural waters is dependent not only on the efficacy of primary concentration on microporous filters, but also on the reconcentration of the primary eluate to a small volume. The iron oxide adsorption-elution procedure used in this study recovered 50 to 61% of virus seeded into 100 to 1,600 ml of TPB primary eluates. Another reconcentration method, organic flocculation with beef extract primary eluates, was reported to recover 68 (3) and 61% (4) of rotavirus. In our study, TPB-GLY was found superior to BE-GLY for recovery of rotavirus from Filterite filters. Since reconcentration of TPB by organic flocculation resulted in only 14 to 28% recovery of seeded virus, we chose the iron oxide procedure for reconcentration of primary eluates to assess naturally occurring rotavirus in polluted estuarine waters. Reconcentration by the iron oxide procedure, using a casein eluent, was effective not only for recovery of rotavirus from BE-GLY eluates, but also for recovery of poliovirus (V. C. Rao and J. L. Melnick, Abstr. Annu. Meet. Am. Soc. Microbiol., 1983, Q8, p. 261) and hepatitis A virus (V. C. Rao, V. Tara Rao, T. G. Metcalf, D. R. Dahling, and J. L. Melnick, Abstr. Am. Water Works Assoc., Water Qual. Conf., 1985, 2C-3, p. 2C). It would be a definite advantage in field studies if a single eluent like casein could be used in the iron oxide method to recover polio-(entero-), rota-, and hepatitis A viruses.

The distribution and quantification of rotavirus among water, suspended solids, fluffy sediments, and compact sediments reported in this paper support the view that solid-associated virus should be an important consideration when monitoring estuarine waters. Results of this study indicate that solid-associated virus in estuarine waters represents a potential health hazard in recreational and shellfish waters.

Furthermore, a potential public health hazard exists when virus-containing fluffy sediments are resuspended by water turbulence. Our data indicate a potential for transport of solid-associated rotavirus over ≥3 miles (1.852 km = 1 mile) in Galveston Bay. The frequency of finding virus associated with solids and the protective effect of solids on rotavirus survival supports this view. Water circulation patterns plus

TABLE 4. Distribution of virus in Galveston Bay samples

Sample[a]	Ratio of samples tested/positive samples[b]	% Positive samples	Quantity of virus
Water	31/5	16	119–1,000
Suspended solids	18/9	50	1,800–4,980
Fluffy sediment	15/6	40	800–3,800
Compact sediment	8/1	12	1,200

[a] Volumes: water, 378 liters; suspended solids, derived by filtering 378 liters of water; fluffy sediment, separated from 75 to 150 liters of water and quantity of virus calculated for 378 liters; compact sediment, 300-g samples processed and virus numbers calculated for 1,000 g.

[b] Of 21 samples positive for rotavirus, one sample each of water and sediment and three samples each of fluffy sediment and suspended solids were negative for enteroviruses.

TABLE 5. Effect of solid association status on survival of rotavirus[a]

Sample	PFU	
	Initial titer	Final titer (day 19)
Water	7.7×10^7	0
Sediment	5.6×10^7	1.0×10^3
Suspended solids	9.4×10^7	1.1×10^4
Fluffy sediment	4.0×10^7	1.1×10^2

[a] All experiments were performed at room temperature (ca. 22°C), using rotavirus SA-11.

prevailing winds influence both distance and direction of virus transport. Of immediate importance is the location of shellfish beds not far from our sampling area. Our data indicate the presence of solid-associated enteroviruses and rotaviruses in substantial amounts in approved shellfish waters of fecal coliform-indicated acceptable sanitary quality (11).

The sanitary acceptability of shellfish and water quality in shellfish growing areas is presently judged by bacteriological standards. Evidence of shellfish-mediated transmission of human virus diseases such as type A hepatitis, Norwalk, and possibly rotavirus-induced gastroenteritis emphasizes the need to recognize the potential hazard represented by the presence of solid-associated viruses in waters overlying shellfish beds.

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