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Concentration of Seeded Simian Rotavirus SA-11 from Potable Waters by Using Talc-Celite Layers and Hydroextraction

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There is mounting evidence for the waterborne transmission of diarrhea caused by rotaviruses. As a result, proper techniques are required for their recovery from samples of incriminated water. The combined efficiency of the talc-Celite technique and polyethylene glycol 6000 hydroextraction was, therefore, tested for this purpose, using Simian rotavirus SA-11 and MA-104 cells. Conditioning of the dechlorinated tap water samples was carried out by pH adjustment to 6.0 and the addition of Earle balanced salt solution to a final concentration of 1:100. Passage of a 1-liter volume of such a conditioned sample through a layer containing a mixture of talc (300 mg) and Celite 503 (100 mg) led to the adsorption of nearly 93% of the added SA-11 plaque-forming units. For the recovery of the layer-adsorbed virus, 3% beef extract and 1× tryptose phosphate broth were found to be superior to a variety of other eluents tested. When we tested 100-liter sample volumes, layers containing 1.2 g of talc and 0.4 g of Celite were employed. Virus elution was carried out with 100 ml of tryptose phosphate broth. The eluate was concentrated 10-fold by overnight (4°C) hydroextraction with polyethylene glycol. With a total input virus of 7.0×10^5 and 1.4×10^2 plaque-forming units, the recoveries were about 71 and 59%, respectively.

In the past few years, a number of hitherto unknown types of viruses have been identified as causative agents of acute diarrhea (18, 30, 32). Rotaviruses appear to represent a major proportion of these newly discovered agents (1).

Rotaviruses were recognized as agents of acute diarrhea in human infants only about 6 years ago (2, 12, 23). That rotaviruses can cause diarrhea in human adults as well has now been shown (15, 22, 33-35, 38).

In the United States during 1971 through 1977, acute diarrhea was involved in nearly 57% of the waterborne disease ("sewage poisoning") outbreaks recorded (6). Many of these outbreaks now appear to have a viral etiology (5). Rotaviruses have recently been implicated as etiological agents in such outbreaks (13, 20, 24).

In spite of the mounting evidence for the potential of human rotaviruses to be transmitted through sewage-polluted waters, no techniques are as yet available for their efficient concentration and recovery from the water environment. This is in part due to the difficulties in the in vitro cultivation of human rotaviruses. It is, however, well established that the Simian rotavirus SA-11 (21) not only closely resembles (17, 29, 37) human rotaviruses but can also be readily grown (21) and quantitated (26, 31) in cell cultures. In view of this, it was decided to use rotavirus SA-11 as a model for human rotaviruses in the following study.

MATERIALS AND METHODS

Cells. MA-104 cells, an established line derived from rhesus monkey kidneys, were used throughout this study. A seed culture of these cells was originally received by us through the courtesy of H. Malherbe of the University of Texas at San Antonio. As stock cultures, the cells were routinely cultivated as monolayers in 75-cm^2 plastic tissue culture flasks (Flow Laboratories), using Eagle minimum essential medium in Earle base (Autopow; Flow Laboratories, Inc.). Each 450 ml of the medium was supplemented with 25 mg of gentamicin (Schering Corp.), 13.5 ml of a 5.6% solution of sodium bicarbonate, 5.0 ml of a 200 mM solution of L-glutamine (Flow Laboratories) and 50 ml of virus- and mycoplasma-tested fetal bovine serum (Microbiological Associates).

Each monolayer was trypsinized, using 2.0 ml of a mixture of trypsin (0.25%) and ethylenediaminetetraacetic acid (0.05%) in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline. A split ratio of 1:4 was generally used for the passage of the cells, and cultures for plaque tests were put up in 25-cm^2 plastic flasks (Flow Laboratories).

Virus. Simian rotavirus SA-11 (strain H96) was also kindly supplied to us by H. Malherbe. The virus was first plaque purified in MA-104 cells, and the same cells were used for the preparation of virus pools to be used here.

Because fetal bovine serum was found to be inhibitory to rotaviruses by us and other investigators (4), it was necessary to wash the monolayers at least twice with Earle balanced salt solution (EBSS) before virus inoculation. After allowing the virus to adsorb for 1 h at 37°C, maintenance medium (minimum essential medium without serum and trypsin) was introduced into the cultures and they were placed back at 37°C. When nearly 75% of the monolayer was affected by virus cytopathic effects, the cultures were frozen (−20°C) and thawed three times. After centrifugation at 1,000 × *g* for 15 min, the supernatant was dispensed in 0.5-ml portions and kept frozen at −80°C.

Plaque assay. The quantitation of infectious virus was carried out by using the plaque assay technique described in detail elsewhere (26). It consisted of the following major steps. After washing the cell monolayers twice with about 5 ml of EBSS, each culture received 0.5 ml of appropriately diluted virus inoculum. For virus adsorption, inoculated cultures were kept at 37°C for 1 h. At the end of the adsorption period, excess inoculum was removed, and when necessary, each monolayer was washed twice with 5.0 ml of EBSS. Each culture was then overlaid with 5 ml of a medium containing minimum essential medium, 0.7% Ionagar no. 2 (Oxoid Ltd.), and 5 μg of trypsin per ml. The cultures were left to incubate in an inverted position for 5 days at 37°C. When the plaques were ready to be examined, the monolayers were fixed and stained as described before (26).

Virus eluting agents. Powdered beef extract (Oxoid Ltd.), casein hydrolysate (GIBCO Laboratories), and lactalbumin hydrolysate (GIBCO) were prepared as 3% solutions in deionized water. Nutrient broth (Difco Laboratories) and tryptose phosphate broth (Difco) were rehydrated in deionized water by the instructions of the manufacturer. Separate 3% aqueous solutions of the amino acids arginine (Nutrional Biochemicals Corp.), asparagine (Nutritional Biochemicals Corp.), glutamine (GIBCO), and glycine (Eastman Organic Chemicals) were also tested as virus eluents in this study. All the eluent solutions were autoclave sterilized, and their pH was adjusted to 9.0, using 5 N NaOH.

Talc-Celite layers. Talc-Celite layers were prepared by using an autoclave-sterilized stock suspension of a mixture of 10 g of talc (J. T. Baker) and 3.3 g of Celite 503 (J. T. Baker) in 1 liter of distilled water. The layers for 1-liter sample volumes were made with 30 ml of the suspension and held in a 47-mm diameter glass filter holder (Millipore Corp.). For sample volumes of 20 and 100 liters, 120 ml of the suspension was used, and the layers were prepared in either a specially designed Plexiglas (27) or a 142-mm diameter stainless-steel membrane filter holder (Sartorius).

Sample conditioning and concentration. Water samples used here represented a municipal supply of treated water (Ottawa River). The basic physical, chemical, and biological characteristics of raw and treated water from this source have already been described (28). The details of sample conditioning and concentration of viruses from water, using the talc-Celite technique, have also been previously outlined (28). In brief, conditioning of dechlorinated water samples was carried out by pH adjustment to 6.0 and the addition of EBSS (as a source of divalent cations) to give a final concentration of 1:100. A measured amount of the conditioned sample was contaminated with a known amount of SA-11 virus. After removing portions of the virus-contaminated sample to act as control, the remaining sample volume was passed through a talc-Celite layer of the appropriate diameter.

Virus elution. Elution of the layer-adsorbed virus was carried out in situ by passing through the layers the eluent under test; for the small (47-mm) layers, 10 ml, and for the large (142 mm) layers 100 ml of the eluent were required.

Hydroextraction. Eluates (100 ml) from the large layers were subjected to second-step concentration with polyethylene glycol (PEG) 6000 as has been described earlier (25). Briefly, a dialysis sac containing the eluate was placed in a glass beaker and surrounded with PEG powder. The beaker was then placed at 4°C overnight. The material remaining in the sac was suspended in 10 ml of EBSS and passed through a 0.2-μm membrane filter (Nalge/Sybron Corp.) before plaque assay.

RESULTS

Virus adsorption to talc-Celite layers. A 1-liter volume of conditioned water was contaminated with a known number of SA-11 plaque-forming units (PFU). It was then passed through a 47-mm diameter talc-Celite layer. The filtrate was collected and plaque assayed. The results of these experiments are presented in Table 1.

As can be seen from the data, the filtrate contained less than 7% of the input PFU. This clearly indicated that, at the sample pH of 6.0 and in the presence of EBSS at a final concentration of 1:100, talc-Celite layers could efficiently adsorb the virus. In this respect, the behavior of SA-11 is, therefore, very similar to that of other enteric viruses (28, 28a).

Elution of layer-adsorbed virus. A 1-liter volume of conditioned and experimentally contaminated sample was passed through a 47-mm diameter talc-Celite layer. A 10-ml amount of eluent under test was then passed through the layer in an attempt to elute and recover the layer-adsorbed virus. The eluate was plaque assayed to determine the amount of input virus recovered. The results of these experiments are summarized in Table 2. In the first two experiments, lactalbumin hydrolysate gave virus recoveries of 62.5 and 66.0%. Because these recoveries were consistently lower than those obtained with the other eluents, it was eliminated from subsequent experimentation. The mean recoveries with beef extract, casein hydrolysate, nutrient broth, and tryptose phosphate broth were 90, 79, 82, and 93%, respectively.

It was reported (9) that certain solutions containing single amino acids could be used in the elution of poliovirus adsorbed to membrane fil-

ters. There are obvious advantages involved in the use of single amino acids compared with the use of more complex protein solutions. Therefore, in this study, the efficiency of a number of single amino acid solutions in the elution of the rotavirus was also tested.

The basic amino acids arginine (83%) and glycine (80%) were superior to the acidic amino acids glutamine (40%) and asparagine (29%) in their rotavirus eluting efficiency (Table 3).

That the low virus recovery with an acidic amino acid was not due to virus inactivation was ruled out in the following experiment: SA-11 virus adsorbed to a talc-Celite layer was first eluted with a solution of glutamine, and then beef extract solution was passed through it. Amino acid solution was able to recover not more than 40% of the input PFU, whereas the subsequent treatment of the same layers with beef extract eluted an additional 54% of the added virus (Table 4). This indicated that the amino acid was not inactivating the virus but leaving most of it still adsorbed to the layers.

TABLE 1. *Efficiency of rotavirus SA-11 adsorption to talc-Celite layers*[a]

Expt no.	Input virus (PFU × 10^4/liter)	Total PFU (×10^4) in filtrate	% PFU lost in filtrate
1	8.0	0.60	7.5
2	7.8	0.60	7.7
3	3.8	0.25	6.6
4	3.8	0.20	5.3
Mean	5.9	0.4	6.8 ± 1.1[b]

[a] After the adjustment of pH to 6.0 and the addition of EBSS, 1-liter volumes of experimentally contaminated samples of potable water were passed through talc (300 mg)–Celite (100 mg) layers. Plaque assays were performed in monolayers of MA-104 cells to determine the amount of virus unadsorbed to the layers. Five cultures were used for each sample dilution tested.

[b] Value represents mean ± standard deviation.

Hydroextraction. For processing sample volumes of greater than 1 liter, larger (142-mm diameter) talc-Celite layers are required. This also makes it necessary to use at least 100 ml of a suitable eluent for the efficient recovery of the layer-adsorbed virus. A subsequent reduction in the volume of the eluate, therefore, becomes essential before its inoculation into cell cultures. Hydroextraction with PEG 6000 was shown to be highly suitable for such a second-step concentration of eluates containing a variety of enteric viruses (25). In this study, the suitability of this method for working with rotaviruses was evaluated.

A 100-ml volume of either tryptose phosphate broth or 3% beef extract was contaminated with a known amount of SA-11 virus. It was then subjected to overnight hydroextraction. A 10-fold reduction in the volume of the suspension lead to virtually no loss of the input virus (Table 5). This clearly indicated that second step concentration by PEG hydroextraction could be extended to working with rotaviruses.

Effect of sample volume and SA-11 input dose on the virus recovering efficiency of the layers. In the foregoing experiments, which were conducted with 1-liter sample volumes, it was demonstrated that SA-11 could be readily concentrated by the talc-Celite technique. In the actual field studies, however, much larger sample volumes need to be examined. Therefore, it was considered essential to test the system not

TABLE 2. *Comparison of different eluates in the recovery of rotavirus SA-11 absorbed to talc-Celite layers*[a]

Expt no.	Input virus (PFU × 10^4/liter)	3% Beef extract		3% Casein hydrolysate		3% Lactalbumin hydrolysate		Nutrient broth		Tryptose phosphate broth	
		Total PFU (×10^4) in eluate	% Recovery	Total PFU (×10^4) in eluate	% Recovery	Total PFU (×10^4) in eluate	% Recovery	Total PFU (×10^4) in eluate	% Recovery	Total PFU (×10^4) in eluate	% Recovery
1	8.0	7.4	92.5	6.0	75.0	5.0	62.5	7.0	87.5	7.4	92.5
2	7.6	7.2	95.0	6.6	87.0	5.0	66.0	7.0	79.0	7.3	96.0
3	3.8	3.2	84.0	3.0	79.0	ND	—	3.0	79.0	3.4	89.0
4	3.8	3.4	89.0	2.8	74.0	ND	—	3.2	84.0	3.6	95.0
Mean	5.8	5.3	90.0 ± 4.76[b]	4.6	79.0 ± 5.91[b]	5	64.3 ± 2.47[b]	5.05	82.3 ± 4.15[b]	5.40	93.0 ± 3.12[b]

[a] The pH of a 1-liter experimentally contaminated potable water sample was adjusted to 6.0, and EBSS was added to a final concentration of 1:100. It was passed through a layer of talc (300 mg)–Celite (100 mg). For elution of adsorbed virus, a 10-ml volume of eluate under test was then passed through the layer. All eluates were prepared in deionized water, and their pH was adjusted to 9.0. Virus plaque assays were performed in monolayers of MA-104 cells using five cultures for each sample dilution tested. ND, Not determined.

[b] Value represents mean ± standard deviation.

TABLE 3. *Comparison of different single amino acids in the recovery of rotavirus (SA-11) adsorbed to talc-Celite layers*[a]

Expt no.	Input virus (PFU × 10^4/liter)	3% Arginine		3% Glycine		3% Glutamine		3% Asparagine	
		Total PFU (×10^4) in eluate	% Recovery	Total PFU (×10^4) in eluate	% Recovery	Total PFU (×10^4) in eluate	% Recovery	Total PFU (×10^4) in eluate	% Recovery
1	8.0	6.6	82.5	6.6	82.5	3.2	40.0	2.2	27.5
2	7.8	6.6	85.0	6.2	79.0	3.2	41.0	2.4	31.0
3	7.8	6.4	82.0	6.2	79.0	3.0	38.0	2.2	28.0
Mean	7.9	6.5	83.0 ± 1.61[b]	6.3	80.0 ± 2.02[b]	3.1	40.0 ± 1.53[b]	2.3	29.0 ± 1.9[b]

[a] The pH of a 1-liter experimentally contaminated potable water sample was adjusted to 6.0, and EBSS was added to a final concentration of 1:100. It was passed through a layer of talc (300 mg)-Celite (100 mg). For elution of adsorbed virus, 10 ml of eluate under test was then passed through the layer. All eluates were prepared in deionized water, and their pH was adjusted to 9.0. Virus plaque assays were performed in monolayers of MA-104 cells, using five cultures for each sample dilution tested.

[b] Value represents mean ± standard deviation.

TABLE 4. *Sequential elution of rotavirus SA-11 adsorbed to talc-Celite by acidic amino acids followed by a protein solution*[a]

Expt no.	Input virus (PFU × 10^4/liter)	First eluate: 3% glutamine		Second eluate: 3% beef extract	
		Total PFU (×10^4) in eluate	% Recovery	Total PFU (×10^4) in eluate	% Recovery
1	7.8	3.2	41.0	4.0	51.0
2	7.8	3.0	38.0	4.2	54.0
3	4.0	1.6	40.0	2.3	57.5
Mean	6.5	2.6	40.0 ± 1.53[b]	3.5	54.0 ± 3.25[b]

[a] The pH of a 1-liter, experimentally contaminated potable water sample was adjusted to 6.0, and EBSS was added to a final concentration of 1:100. It was passed through a layer of talc (300 mg)-Celite (100 mg). A 10-ml amount of 3% glutamine in deionized water (pH 9.0) was first used as an eluate. Then the layer was again eluted with 10 ml of 3% beef extract in deionized water (pH 9.0). Virus plaque assays were performed in monolayers of MA-104 cells, using five cultures for each sample dilution tested.

[b] Value represents mean ± standard deviation.

only with larger sample volumes but also using lower virus input doses.

Either 20- or 100-liter volumes of conditioned and experimentally contaminated water samples were passed through the larger layers. Virus elution was carried out by the subsequent passage of 100 ml of tryptose phosphate broth through the layers. A 10-fold reduction in the volume of the eluate was achieved by overnight PEG hydroextraction. The data obtained in these experiments are presented in Table 6.

When a 20-liter sample with a total of either 1.4×10^5 or 5.6×10^2 SA-11 PFU was concentrated, between 81 and 84% of the input virus could be recovered. Concentration of a 100-liter sample containing a total of about 1.4×10^2 PFU gave a virus recovery of 59%. With the same sample volume but containing approximately 7.0×10^5 PFU, the virus recovery was nearly 12% higher.

DISCUSSION

In earlier investigations we had demonstrated the suitability of the talc-Celite technique (28, 28a) and PEG hydroextraction (25) in the concentration of a variety of enteric viruses expected to be present in sewage-polluted waters. Now there is mounting epidemiological evidence that rotaviruses can also be transmitted through the consumption of sewage-polluted waters (13, 20, 24). However, because of the lack of suitable methodology, rotaviruses could not be demonstrated in the incriminated water samples. It was, therefore, considered important to test the possible extension of the above mentioned techniques to working with rotaviruses. Using SA-11 as a model for human rotaviruses, the present study has shown that these techniques are highly efficient in their concentration of SA-11 and could be equally efficient in the concentra-

TABLE 5. *Overnight PEG 6000 hydroextraction in the concentration of rotavirus SA-11*[a]

Expt no.	Input virus (PFU × 10^3/100 ml)	3% Beef extract		Tryptose phosphate broth	
		Total PFU (×10^3) in concentrate (10 ml)	% Recovery	Total PFU (×10^3) in concentrate (10 ml)	% Recovery
1	3.8	3.6	95.0	3.8	100.0
2	3.8	3.6	95.0	3.6	95.0
3	0.68	0.62	91.0	0.64	94.0
4	0.66	0.64	94.0	0.64	94.0
Mean	2.2	2.1	94.0 ± 1.9[b]	2.2	96.0 ± 2.9[b]

[a] A 100-ml volume of either 3% beef extract (pH 9.0) or tryptose phosphate broth (pH 9.0) was experimentally contaminated with the virus. Hydroextraction was carried out overnight at 4°C. The material remaining in the dialysis sac was suspended in 10 ml of EBSS and plaque assayed in MA-105 monolayers.

[b] Value represents mean ± standard deviation.

tion of other rotaviruses from samples of potable water.

In the talc-Celite process, virus adsorption is carried out at pH 6.0 and recovery of the layer-adsorbed virus is achieved by using an eluent at pH 9.0. This is in contrast to a number of other procedures (14, 15) where pH extremes of 3.5 and 11.5 are necessary for virus recovery from the water environment. In view of the relatively pH-labile nature of rotaviruses (7, 11), such procedures become potentially unsuitable for working with this virus group.

It has been shown that basic differences exist in the adsorptive behavior of entero- and rotaviruses to aluminum hydroxide and activated sludge flocs (10). However, under the experimental conditions used here, the adsorption of SA-11 to talc-Celite layers was as efficient as has been previously reported for other enteric viruses (28, 28a).

Fetal calf serum (10%) had been found to be the best eluent for the recovery of entero- and reoviruses adsorbed to talc-Celite layers (28, 28a). Because of the rotavirus-inhibiting activity of animal sera (4), the use of this eluent could not be extended to working with these viruses. Testing of a number of other eluents showed 3% beef extract and tryptose phosphate broth to be highly efficient for this purpose. Although beef extract is also a good eluent for other enteric viruses (8, 19), in practical terms the use of tryptose phosphate broth offered the following advantage: when tryptose phosphate broth was used as an eluent, the final concentrates obtained after overnight hydroextraction were relatively easy to pass through sterilizing membranes when compared with those obtained with beef extract.

Solutions of individual basic amino acids such as arginine and glycine could also elute the rotavirus, but their efficiency was slightly lower in comparison with beef extract or tryptose phosphate broth. Because the use of single amino acid solutions provides an inhibitor-free and readily standardizable virus eluent, more work is required to bring about a further improvement in their eluting efficiency.

TABLE 6. *Relationship of potable water sample size and rotavirus SA-11 input dose to the virus recovery efficiency of talc-Celite layers*[a]

Sample size (liters)	Virus input (PFU/sample)	Total PFU in final concentrate (10 ml)	% Recovery[b]
20	5.6×10^5	4.7×10^5	85.0 ± 2.31
	1.36×10^2	1.1×10^2	81.0 ± 4.58
100	6.8×10^5	4.7×10^5	71.0 ± 5.13
	1.36×10^2	0.68×10^2	59.0 ± 3.0

[a] Results represent the mean values from three experiments at each sample volume and virus input dose. After adjustment of the sample pH to 6.0 and the addition of EBSS, an appropriate volume of the experimentally contaminated sample was passed through a 142-mm diameter talc (1.2 g)–Celite (0.4 g) layer. Virus was eluted with 100 ml of tryptose phosphate broth (pH 9.0). The eluate was hydroextracted, and the final concentration was plaque assayed in monolayers of MA-104 cells.

[b] Each value represents mean ± standard deviation.

Experiments with sample volumes of greater than 100 liters were not conducted here. But in earlier studies (28), it has been shown that a further increase in the sample volume did not adversely affect the virus-recovering capacity of the technique. It has also been demonstrated before (28a) that the presence of raw sewage in potable waters does not interfere in any way with the performance of this technique.

Experiments are presently underway to see if this technique could also be applied to working with other members of the rotavirus group.

Rotavirus gastroenteritis, which can some-

times be fatal (3), is now well recognized as a public health problem of world-wide significance (36). Any concerted efforts planned for its control would require, among other things, proper methods for the monitoring of the responsible agents in the water environment. The technique reported here may prove to be of use in this regard.

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