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Concentration of Simian Rotavirus SA-11 from Tap Water by Membrane Filtration and Organic Flocculation

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Simian rotavirus SA-11 was concentrated from tap water by adsorption to and elution from microporous filters, followed by organic flocculation. Two types of filters were compared for their ability to concentrate the virus. Both Zeta Plus 60S and Cox AA type M-780 filters were efficient for virus adsorption, but the efficiency of virus elution was higher with Zeta Plus than with Cox filters. Optimum conditions for virus recovery from Zeta Plus filters included an input water pH of 6.5 to 7.5 and the use of 3% beef extract (pH 9.0) for elution. Under these conditions, an average of 62 to 100% of the virus was recovered in the concentrate. Organic flocculation was used as a second-step concentration method, with average recoveries of 47 to 69%. When the two methods were used to concentrate small numbers (7 to 75 PFU/liter) of input rotavirus, an average of 75 ± 40% recovery was achieved. With large volumes of input water, however, recovery was reduced to 16 ± 7%.

Rotaviruses are viral agents of gastroenteritis in animals and humans and are distributed worldwide (5, 19). They are fecally excreted (5) and thus enter the water environment as potential agents of waterborne epidemics of diarrhea. They have been detected in sewage (13, 17), but at present there is little firm evidence for their role as waterborne disease agents. For determination of the extent of contamination of the environment with these viruses it is essential to have a reliable and sensitive technique for their detection. Furthermore, these viruses may be present in water in low concentrations, and an efficient concentration technique is required for their detection by standard virological procedures.

Most methods of virus concentration have been developed with poliovirus as a representative virus. This virus differs from rotaviruses in many characteristics, including adsorptive behavior and stability (2, 3). For determination of which is the most efficient method for rotavirus concentration from water it is necessary to use one of the rotaviruses as an experimental model. The simian rotavirus SA-11 has been commonly used for laboratory investigations of rotaviruses since it grows to high titer in tissue cultures and can be quantitated by plaque assay (12). Because this simian rotavirus resembles the human rotaviruses in morphology and composition and shares cross-reacting antigens (11, 18), it is useful as a model for the human disease agents.

In this study, the efficiency of simian rotavirus SA-11 concentration from tap water was investigated for two membrane filtration methods. The usefulness of organic flocculation as a second-step concentration method for rotaviruses was also determined.

MATERIALS AND METHODS

Cells. The MA-104 line of fetal rhesus monkey kidney cells was obtained from Y. Marzouk (Central Virus Laboratory, Tel Aviv, Israel). The cells were grown in RPMI-1640 medium containing 10% newborn bovine serum (Flow Laboratories, Irvine, United Kingdom); 200 μg of penicillin, 200 μg of streptomycin, 200 μg of kanamycin, 25 μg of neomycin, and 100 μg of nystatin per ml; and 20 μM glutamine.

Virus and virus assay. Simian rotavirus SA-11, kindly supplied by C. P. Gerba (Department of Microbiology, University of Arizona, Tucson), was grown and assayed in MA-104 cells under conditions similar to those described previously (12). Specifically, virus stocks were prepared in the presence of 12 μg of trypsin per ml (1:250; tissue culture grade; Difco Laboratories, Detroit, Mich.) in growth medium minus serum. The titer of virus stocks was typically 10^8 PFU/ml. Plaque assays were carried out in 50-mm-diameter tissue culture plates in the presence of 5% carbon dioxide. Facilitators added to the agar overlay to enhance plaque formation were 20 μg of trypsin and 100 μg of diethylaminoethyl dextran per ml (Sigma Chemical Co., St. Louis, Mo.), conditions experimentally determined to maximize virus titers. Plaques were formed within 3 to 5 days postinfection, and an agar overlay containing 170 μg of neutral red per ml was added to aid in plaque visualization.

Virus concentration methods. (i) Membrane filtration. Virus concentration by adsorption to and elution

TABLE 1. Concentration of rotavirus by Zeta Plus filters[a]

Expt	Virus input (PFU)	Virus eluted (PFU)	% Virus recovery efficiency[b]
1	4.0×10^6	2.7×10^6	68
2	4.0×10^6	2.1×10^6	53
3	4.0×10^6	3.9×10^6	98
4	4.0×10^6	2.0×10^6	50
5	4.3×10^5	2.6×10^5	60
6	4.3×10^5	2.6×10^5	60
7	4.3×10^5	2.1×10^5	49
8	4.3×10^5	2.4×10^5	56

[a] Filtration was done with 2 liters of tap water at pH 6.5.

[b] The mean ± standard deviation percent efficiency was 62 ± 16.

from microporous filters was as follows, with exceptions as indicated. Virus was added to Jerusalem tap water dechlorinated by the addition of 10 mg of sodium thiosulfate per liter. The pH of the input water was adjusted to various levels, and the sample was filtered through a 142-mm-diameter filter at a pressure of 0.5 kg/cm. Eluent (75 ml of 3% beef extract [pH 9.0]) was added, incubated on the filter for 15 min at room temperature, and eluted. Filters used were a Zeta Plus 60S cellulose–diatomaceous earth–"charge-modified" resin filter, with a 0.45-μm nominal pore size (AMF, Cuno Div., Meriden, Conn.), and a Cox AA type M-780 epoxy-fiberglass-asbestos filter, with a 0.45-μm nominal pore size (Cox Instrument Div., Lynch Corp., Detroit, Mich.). Virus recovery was determined by titration of the virus stock, input water, filtrate, and eluate by plaque assay.

(ii) Organic flocculation. Virus eluted from filters with 3% beef extract was reconcentrated by organic flocculation as described previously (6). Briefly, the pH of the eluate was lowered to 3.5, and the sample was stirred for 30 min at room temperature. The floc which formed was concentrated by centrifugation and suspended in a 1/20 volume of 0.15 M Na_2HPO_4 (pH 7.5). The amounts of virus in the floc supernatant were determined by plaque assay.

Eluents. Eluents tested for their elution efficiency included the following: beef extract (Lab-Lemco, Oxoid, London, England), tryptose phosphate broth (Difco Laboratories), peptomeat (Biolife Italiana, Milan, Italy), and Triton X-100 (BDH Chemicals, Poole, United Kingdom).

RESULTS

Concentration of rotavirus by membrane filtration. The ability of microporous filters to concentrate simian rotavirus SA-11 from tap water was tested for two filter types, the electropositive Zeta Plus 60S filters and the Cox AA type M-780 filters, which are electronegative at neutral pH levels. The filtration procedures for both filters were similar, except that to enhance adsorption, the pHs of the input water were 6.5 for the Zeta Plus filters and 3.5 for the Cox filters. Virus was seeded into dechlorinated tap water, the pH was adjusted, and the sample was filtered. After filtration, the adsorbed virus was eluted with 3% beef extract (pH 9.0), and the eluates were assayed for virus.

The Zeta Plus filters efficiently concentrated rotavirus from tap water. Virus recovered in the eluate averaged 62 ± 16% of the input virus, with a range of recovery in individual experiments of 49 to 98% (Table 1). Adsorption was highly efficient, since no virus was recovered in the filtrate. For comparison, a series of experiments with poliovirus type 1 (Brunhilde strain) was performed under the same conditions, resulting in an average recovery of 73 ± 5% (data not shown). Thus, the Zeta Plus filters recovered rotavirus from tap water with an efficiency which did not differ markedly from their efficiency for poliovirus.

In contrast, the Cox filters proved to be inefficient for rotavirus concentration from tap water. In a series of eight experiments, recovery in the eluate averaged 3.1% (Table 2), a recovery much lower than that found for Zeta Plus filters. The Cox filters adsorbed the virus efficiently, since no virus was detected in the filtrate, but the virus was not eluted.

During filtration through Cox filters, the virus may have been inactivated because of the low pH of the input water. Experiments were performed to measure the rate of loss of the virus titer in the input tap water at pH 3.5. It was found that for up to 1 h at room temperature, the virus titer did not drop by more than 50% (data not shown). This was not sufficient to account for the low virus recovery, since during concentration, the time of exposure of the virus to pH 3.5 was less than 10 min. Thus, it is probable that the virus was not recovered from the filter

TABLE 2. Concentration of rotavirus by Cox filters[a]

Expt	Virus eluted (PFU)	% Virus recovery efficiency[b]
1	0.06×10^6	4.0
2	0.02×10^6	1.3
3	0.05×10^6	3.3
4	0.01×10^6	0.7
5	0.01×10^6	0.7
6	0.04×10^6	2.7
7	0.11×10^6	7.3
8	0.07×10^6	4.7

[a] Filtration was done with 2 liters of tap water at pH 3.5. Virus input was 1.5×10^6 PFU for all experiments.

[b] The mean ± standard deviation percent efficiency was 3.1 ± 2.3.

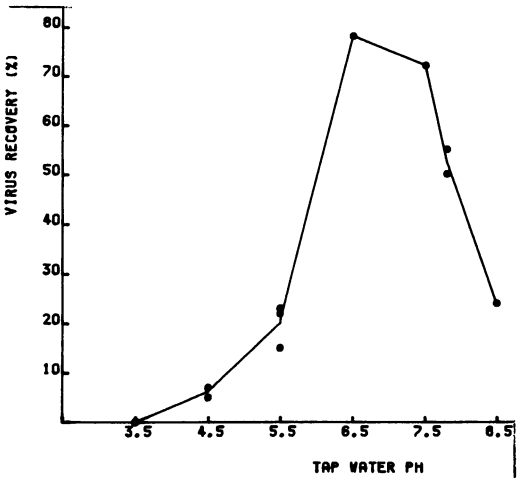

FIG. 1. Effect of input water pH on rotavirus recovery from Zeta Plus filters. Tap water (2 liters) was seeded with rotavirus (2.4×10^6 to 4.0×10^6 PFU) and adjusted to the indicated pHs. After filtration, the virus was eluted, and the amounts of virus in the eluate and filtrate were determined. No virus was detected in the filtrate; virus recovery is indicated as the percentage of input virus recovered in eluate.

either because of inefficient elution or because of inactivation of the virus on the filter.

Effect of input pH on rotavirus recovery. To determine the range of usefulness of Zeta Plus filters for rotavirus recovery from natural waters, we determined the recovery efficiency as a function of input water pH. Rotavirus was seeded into tap water, the pH was adjusted as required, and the sample was filtered. The virus was eluted, and recovery in the eluate was determined. Virus recovery efficiency was at a maximum between pH 6.5 and 7.5 (Fig. 1). Virus was not detected in the filtrate at any input pH. Thus, virus adsorption was efficient under all conditions tested, but elution was successful within a more limited pH range.

Efficiency of various eluents for rotavirus elution. To maximize the efficiency of rotavirus recovery, we compared a number of eluents for their ability to elute rotavirus from Zeta Plus filters (Table 3). Several proteinaceous media were tested, including 2.5% and 5% tryptose phosphate broth and 5% peptomeat. Peptomeat was unable to elute the virus. Tryptose phosphate broth functioned as an eluent, but 3% beef extract was more effective.

An attempt was made to improve rotavirus elution by use of the nonionic detergent Triton X-100. Triton X-100 was added to the filter and incubated for 10 min, followed by the addition of 3% beef extract. After an additional contact time of 15 min, the sample was eluted, and virus recovery was determined (Table 3). No improvement in virus recovery was found. Similarly, a mixture of Triton X-100 and beef extract was not a better eluent than beef extract alone. It is possible that higher concentrations of Triton X-100 would aid in elution, but they were not used since they damaged the cell monolayer.

To determine whether the addition of beef extract in two steps would release more virus from the filter, we performed a second elution after the first. Virus recovery did not improve (Table 3). Thus, of the eluents and procedures tested, 3% beef extract in a single step was the most simple and efficient method for rotavirus elution from Zeta Plus filters.

Effect of incubation time on elution efficiency. To test whether increased incubation time of the eluent on the filter would improve elution, we performed the following experiment. Rotavirus was added to 2-liter volumes of tap water and filtered through four Zeta Plus filters in parallel. Eluent was added and eluted at times ranging from 15 to 60 min after addition to the filter. Virus recovery did not improve with longer incubation times; after 15 min, 41% of the virus was recovered in the eluate, and 42% was recovered after 60 min. Thus, there was no advantage to extended incubation; 15 min was sufficient to elute the virus. Note that the extended exposure

TABLE 3. Efficiency of rotavirus elution[a]

Eluent[b]	No. of experiments	% Avg virus recovery[c]
3% BE	8	62 ± 16
2.5% TPB	4	28 ± 7
5% TPB	4	26 ± 10
5% PM	8	0.12 ± 0.25
2.7% BE + 0.001% TX-100	4	45 ± 8
0.01% TX-100 + 3% BE[d]	2	31 ± 5
3% BE + elution + 3% BE[e]	4	65 ± 7

[a] Virus (7.7×10^5 to 5.5×10^6 PFU) was seeded into 2 liters of tap water and filtered through Zeta Plus filters at pH 6.5. Eluent (75 ml, pH 9.0) was added and eluted after 15 min. Exceptions are as noted.

[b] BE, Beef extract; TPB, tryptose phosphate broth; PM, peptomeat; and TX-100, Triton X-100.

[c] The results are calculated as the percentage of input virus recovered in the eluate and expressed as the mean ± standard deviation of each set of experiments.

[d] The first eluent (10 ml, pH 7.0) was added to the filter and incubated for 10 min, followed by the addition of the second eluent (65 ml) and incubation for 15 min, followed by elution.

[e] Elution was done as described in *a*, followed by the addition of 25 ml of 3% BE, incubation for 15 min, and elution. The eluates were combined and titrated.

TABLE 4. Concentration of rotavirus by membrane filtration and organic flocculation[a]

Expt	% Virus recovered in:			% Organic flocculation efficiency
	Eluate	Floc	Supernatant	
1	113	44	0	39
2	89	50	0	56

[a] Virus (1.5×10^8 PFU) was added to 2 liters of tap water and filtered at pH 6.5. After elution, the virus was reconcentrated by organic flocculation. The mean percentages of virus recovered in the eluate, the floc, and the supernatant were 101, 47, and 0, respectively. The mean percent efficiency was 47.

of the virus to pH 9.0 did not reduce the virus titer, indicating that extensive inactivation was not occurring under these conditions.

Efficiency of organic flocculation for rotavirus concentration. Virus isolation from water requires concentration to a volume small enough to be handled conveniently by the system used for virus detection. For large volumes of water, this generally means the use of a second concentration step to attain a final volume of a few milliliters. Organic flocculation is a method which has been shown to be highly efficient for poliovirus concentration (6, 7). It is particularly applicable when beef extract is used as the filter eluent, since it simply requires the reduction of the pH of the sample to 3.5 for floc formation. The floc is then concentrated by centrifugation and resuspended in a small volume of phosphate buffer. To determine the efficiency of this technique for rotavirus concentration, we performed organic flocculation on the eluates of Zeta Plus filters. The organic flocculation step had an average efficiency of 47% (Table 4). In these experiments, because of the high recovery from the filters, the efficiency of the two steps together was 47%. The supernatant which remained after the organic floc was removed was also assayed for virus, and none was detected, indicating that the rotavirus adsorbed efficiently to the floc.

TABLE 5. Concentration of rotavirus by organic flocculation[a]

Expt	Virus input (PFU)	% Virus recovered in:	
		Floc	Supernatant
1	2.1×10^7	57	0.01
2	2.3×10^7	65	0.52

[a] Virus was seeded into 75 ml of 3% beef extract. Organic flocculation was performed, and the indicated fractions were assayed for virus. The mean percentages of virus recovered in the floc and the supernatant were 61 and 0.26, respectively.

TABLE 6. Recovery of small quantities of rotavirus from tap water[a]

Expt	Virus input (PFU)	Virus recovered (PFU)	% Recovery[b]
1	70	37	53
2	70	69	99
3	70	61	87
4	70	82	117
5	150	29	19
6	150	25	17
7	15	17	113
8	15	14	93

[a] Virus was seeded into 2 liters of tap water, filtered through Zeta Plus filters at pH 6.5, eluted, and reconcentrated by organic flocculation.

[b] The mean ± standard deviation percent recovery was 75 ± 40.

To determine whether there was virus loss as a result of using beef extract which had served as an eluant, we performed organic flocculation with virus added to fresh beef extract. An average of 61% of the virus was recovered from the organic floc, and <1% remained in the supernatant (Table 5), a slightly improved recovery.

Although organic flocculation successfully concentrated rotavirus, there was some virus loss. This may have been due to inactivation or incomplete resuspension after flocculation. To improve virus dispersion, we sonicated the resuspended concentrate for up to 10 min in a water bath sonicator. This treatment did not increase the virus titer (data not shown). Since the virus was exposed to pH 3.5 for 45 min during organic flocculation, the stability of rotavirus in beef extract (pH 3.5) was investigated. Virus was seeded into 3% beef extract, the pH was adjusted to 3.5, and samples were removed and titrated at various times. By 30 min, the virus titer had dropped to 70% of the initial value (data not shown), possibly accounting for most of the virus loss.

Recovery of small quantities of rotavirus from tap water. Viruses are found in small amounts in natural waters, and a useful concentration method must recover small numbers of infectious particles with high efficiency. The ability of a combination of the membrane filtration and organic flocculation methods to concentrate small quantities of rotavirus was tested in the following experiments. Rotavirus (15 to 150 PFU) was seeded into 2-liter volumes of tap water, the water was filtered through Zeta Plus filters, and the virus was eluted and reconcentrated by organic flocculation. Virus recovery in the eluate was not determined because of the small amounts of seeded virus. Virus recovery in the

TABLE 7. Concentration of rotavirus from large volumes of tap water[a]

Exp	Input water vol (liters)	% Virus recovered in:		% Organic flocculation efficiency
		Eluate	Organic floc	
1	64.5	24	14	57
2	33	30	22	73
3	33	15	12	78

[a] Virus (4×10^5 PFU) was seeded into the indicated amount of tap water, filtered through Zeta Plus filters at pH 6.5, eluted, and reconcentrated by organic flocculation. The mean ± standard deviation percentages of virus recovered in the eluate and the organic floc were 23 ± 8 and 16 ± 7, respectively. The mean ± standard deviation percent efficiency was 69 ± 11.

organic floc averaged 75% (Table 6), with a range of 17 to 117%. This wide range may have been due to the large statistical error associated with assaying for small numbers of viruses. The results indicate a high recovery efficiency for the two methods when used with low virus concentrations.

Concentration of rotavirus from large volumes of water. The ability of Zeta Plus filters to concentrate rotavirus from large volumes of tap water was tested in the following series of experiments. Virus was added to 33 or 64.5 liters of water, filtered, eluted, and reconcentrated by organic flocculation. The largest volume of water which could be filtered through a single filter was 64.5 liters, with a filtration time of 2 h. The amounts of virus in the eluate and in the organic floc were measured (Table 7). The average virus recovery of 16% was lower than that found with smaller volumes of input water. Since recovery of the virus after organic flocculation was relatively high, virus loss occurred during the membrane filtration step. Thus, the low virus recovery was probably a result of reduced adsorption efficiency or of inefficient elution from the filter.

DISCUSSION

Several studies have investigated methods for rotavirus concentration from tissue culture harvests (4, 10) and water (8, 9, 13, 17). The techniques used for tissue culture harvests are not directly applicable to the handling of large volumes of water. Successful concentration of rotavirus from up to 100 liters of water by ultrafiltration has been reported (8), but this technique is both unwieldy for field virus concentration and time-consuming. The use of Talc-Celite layers also has been reported to be efficient for rotavirus concentration from water (9), but this method is more difficult to use than membrane filtration.

Field isolation of rotavirus from sewage has been recently reported by two laboratories. Steinmann (17) concentrated rotavirus from sewage by filtration through Seitz electropositive filters, followed by reconcentration by ultracentrifugation. Rotavirus was detected by immunological techniques which did not distinguish between viable and noninfectious viruses. Unfortunately, the efficiency of the methods used was not reported, so that comparisons with other techniques are difficult. In another study (13), membrane filtration with Filterite filters was used to concentrate rotavirus from sewage, resulting in successful isolation of viable virus. Simian rotavirus SA-11 was used to measure the efficiency of the technique, and recovery efficiency from 20 liters of tap water averaged 54.6%. The major disadvantages of these filters are the need for reduction of the input water pH to 3.5 and the relative sensitivity of the filters to interference by contaminants in the water (14).

Zeta Plus filters have several advantages over Filterite filters, including relative insensitivity to changes in water quality (14) and efficient virus recovery at a more neutral pH (16). As reported here, the Zeta Plus filters were more successful in recovering rotavirus from tap water than were the electronegative Cox filters with which they were compared. The Cox filters used have been included as one of the filter types recommended for use as a tentative standard method for virus concentration (1) and have been found to efficiently recover poliovirus from water when beef extract is used as an eluent (6, 7). Although it was found here that these filters adsorbed rotavirus, elution was highly inefficient, and these filters would not be appropriate for rotavirus concentration.

In contrast, Zeta Plus filters both adsorbed and eluted rotavirus, resulting in recovery of the majority of the input virus. The filters were useful at a neutral pH range, obviating the need for pH adjustment in the filtration of many natural waters. It was of interest that the input pH of the water influenced elution rather than adsorption efficiency. That the chemical nature of the input water may interfere with virus elution has also been observed with polyvalent cations (15). Thus, the chemical condition of the water may influence the virus-filter bond or the state of virus aggregation and thus, virus elution.

Proteinaceous media used as virus eluents have been generally found to be more efficient than other materials (4, 7, 14, 15). Of the media and procedures tested here, 3% beef extract (pH 9.0) was the most efficient for rotavirus elution. It should be noted that it was not sufficient for the eluent to be proteinaceous, since the two other media tested were less effective than was beef extract.

Organic flocculation as a second concentration step after filter elution with beef extract has been included as a tentative standard method for virus concentration (1). Rotavirus recovery, however, was found to be slightly lower after organic flocculation than that reported for poliovirus (6, 7). The virus adsorbed to the organic floc, but not all of the input virus was recovered. Virus loss may have been due to inactivation, since the rotavirus titer is not stable at pH 3.5 in water (2) or, as reported here, in beef extract. The efficiency of this method for rotavirus concentration may be improved by minimizing the amount of time the virus is exposed to the low pH or by using a slightly higher pH. In general, 50% or more of the virus was recovered, and the technique was useful both as a second-step concentration method and on its own as a small-volume concentration method.

One problem encountered with the Zeta Plus membrane filtration method was a decreased efficiency when large volumes of water were processed, as a result of inefficient adsorption or elution. The inefficient elution may have been due to the buildup of layers of material on the filter, physically inhibiting elution or preventing access of the eluent to the adsorbed virus. This problem may be overcome by the use of filters with an increased absorptive capacity and a larger pore size. Further investigations are needed in this direction.

In summary, the methods described here for rotavirus concentration are simple, rapid, and appropriate for the simultaneous isolation of other enteric viruses. The ability of the Zeta Plus filters to recover naturally occurring rotaviruses from turbid waters requires further study.

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