CONCENTRATION OF ENTEROVIRUSES FROM SEAWATER AND TAPWATER BY ORGANIC FLOCCULATION USING NON-FAT DRY MILK AND CASEIN

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Abstract. The use of casein and non-fat dry milk for the recovery of enteroviruses from seawater and tapwater was investigated. Poliovirus type 1 was recovered with high efficiency (> 70% overall recovery) from large volumes of seawater or tapwater, using 1% (w/v) non-fat dry milk buffered at pH 9.0 with 0.05M glycine. Good recovery of low numbers of poliovirus 1 from tapwater was also achieved. The method can also recover other enteroviruses (Poliovirus 2, 3, coxsackievirus B_3 and echovirus 4), but appeared inefficient in the recovery of echovirus 1.

1. Introduction

The need to monitor the occurrence of enteric viruses in environmental waters is now widely recognized. The virus concentrator developed by Wallis *et al.* [15] has been used by most investigators involved in the detection of viruses in seawater [1, 3, 11, 12] and tapwater [2]. However, various modifications have been proposed for both the concentration and reconcentration steps. One modification is the use of pleated membranes, which allow the processing of large volumes of tapwater as well as estuarine waters [2, 10]. Generally, membrane-bound viruses are eluted with glycine buffer at pH 11.5 and reconcentrated by membrane filtration when tapwater is under consideration [13].

The reconcentration step of seawater samples may, however, result in filter clogging, and this had led to the development of other reconcentration methods, such as iron [12] or aluminium hydroxide flocculation [1, 11] or polymer phase separation [3].

It has been recently proposed that organic flocculation could be an efficient method for virus detection in tapwater [8], seawater [7], wastewater effluents [9], and anaerobic sludge [5]. This method consists of eluting viruses from membrane filters or sludge solids with 3% (w/v) beef extract and then lowering the pH of the protein solution to 3.5 to flocculate the viruses. The sediment produced is then resuspended in a buffer at alkaline pH. The aim of the present investigation was to demonstrate the use of non-fat dry milk and casein in the recovery of enteroviruses from seawater and tapwater.

2. Materials and Methods

2.1. VIRUS AND VIRAL ASSAYS

Poliovirus type 1 (Sabin), poliovirus type 2 and 3 (isolated from natural sources), echovirus 1 (Farouk), echovirus 4 (Pesacek), and coxsachievirus B3 (Nancy) were used

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in this study. Virus stocks were grown in AV3 (Amnion cells) or MA104 host cells. Viruses were diluted in Eagle's M.E.M. (minimal essential medium) containing 5% fetal calf serum and assayed by the plaque method using either AV3 (human amnion cells) or MA104 (simian cells) host cells.

2.2. WATER SAMPLES

Seawater was collected from Crescent Beach, Florida, and stored at 4°C until use. The seawater had a pH of 8.2 and specific conductance of 41 000 μmhos cm^{-1}. Gainesville tapwater had pH = 8.4 and conductivity of 250 μmhos cm^{-1}. It was dechlorinated with sodium thiosulfate prior to seeding with viruses. Orthotolidine titrations were performed to determine the concentration of sodium thiosulfate needed for complete dechlorination [13].

2.3. CONCENTRATION STEP

The viruses used were suspended in 1 to 3 l of seawater at pH 3.5 with 0.0005M $AlCl_3$ and adsorbed to a series (3 μm→0.45 μm→0.25 μm) of Filterite filters (Filterite Corp. Timonium, Md.). Viruses were eluted from membrane filters with 3% (w/v) beef extract (Inolex, Glenwood, Ill.) at pH = 9.0, 0.5% (w/v) purified or isoelectric casein (Difco, Detroit, Mich.) in 0.05M glycine buffer at pH = 9.0, or 1% (w/v) non-fat dry milk (Atlantic and Pacific Tea Co., Montvale, N.J.) in 0.05M glycine at pH = 9.0. The eluates were neutralized with 1M glycine (pH 2.0) and assayed to determine the percent of viruses eluted.

In experiments dealing with large volumes of water, poliovirus type 1 was suspended in either 20 gallons (76 l) seawater or 50 gallons (189 l) tapwater and adsorbed to a 0.25 μm or 0.45 μm Filterite cartridge filter (Filterite Corp. Timonium, Md.) in the presence of 0.0005M $AlCl_3$ at pH = 3.3 to 3.5.

Viruses were eluted with 1 l of 1% (w/v) NFDM (non-fat dry milk) in 0.05M glycine (pH = 9.0). The eluates obtained were neutralized and assayed on host cells.

2.4. RECONCENTRATION STEP: ORGANIC FLOCCULATION

Casein and NFDM flocculate at pH = 4.6 to 4.7, which is the isoelectric point of casein [6]. The filter eluates were flocculated at pH = 4.5 to 4.6 by the addition of 1M glycine buffer (pH = 2.0) and then centrifuged at 4500 rpm for 4 min. For large volumes, eluates could be allowed to settle and only the lower portion centrifuged. The centrifugation step may be carried out at a lower speed with no effect on virus recovery. The pellet was redissolved in 0.15M Na_2HPO_4 at pH = 9.0. The concentration factor ranged from 10 (small volume) to 40 (large volume). The final sample as sonicated at 35 W power output for 10 to 15 s. (Sonifier Cell Disruptor, model W185D, Heat Systems Ultrasonics, Inc., Plainview, N.Y.).

When using Casein or NFDM it was found to be advantageous to add a small quantity (1 squirt) of an antifoam agent (Dow Corning Corp., Midland, Mich.) to inhibit foam

TABLE I

Effect of antifoam[1] on poliovirus type 1 assay, using MA104 host cells[2]

Treatment	PFU ml^{-1}			
	Run 1[3]	Run 2	Run 3	Mean
Control (M.E.M.)	453	392	370	405
M.E.M. + antifoam	435	442	419	432

[1] Antifoam spray purchased from Dow Corning Corp., Midland, Mich.
[2] 14.9 ml of M.E.M. was added to each of 6 test tubes and 0.1 ml of poliovirus was added to give a final concentration of approximately 400 PFU ml^{-1}. Three tubes were treated (one squirt) with antifoam while the three others did not receive antifoam. All 6 tubes were vortexed for 10 s and the fluids assayed on MA104 host cells.
[3] Each run represents the mean of 4 to 5 tissue culture bottles.

production during filtration and pellet resuspension. The use of an antifoam agent did not reduce polio virus numbers or cause cytotoxicity to MA104 host cells (Table I).

When large volumes of water were processed, the final concentrates were dialyzed against phosphate buffered saline (PBS) for 18 to 24 h at 4°C prior to virus assay.

Organic flocculation with beef extract was performed according to Katzenelson *et al.* [8].

3. Results

3.1. USE OF PURIFIED CASEIN FOR THE RECOVERY OF POLIOVIRUS 1 FROM SMALL VOLUMES OF SEAWATER

A first set of experiments was undertaken to study the effect of pH on the elution of poliovirus type 1 from membrane filters (Filterite) with 0.5% (w/v) purified casein buffered with 0.05M glycine. It was observed (Table II) that poliovirus elution was maximum (100%) at pH = 10.0 and decreased to 64% at pH = 11.5. Furthermore, virus elution was relatively high (76%) even at pH = 8.0.

TABLE II

Effect of pH on elution of poliovirus type 1 from membrane filters with 0.5% purified casein[1]

pH of Casein	% elution[2]
8	76
9	74
10	100
11.5	64

[1] Poliovirus 1 suspended in 1 l of seawater, was adsorbed to a series (3 μm → 0.45 μm → 0.25 μm) of Filterite filters in the presence of 0.0005M $AlCl_3$ at pH = 3.5. The adsorbed virus was eluted with 0.5% purified casein (30 ml) adjusted to various pH's with 0.5M glycine.
[2] Each number represents the mean for 2 to 4 samples.

TABLE III

Comparison of beef extract to casein for the recovery of viruses from seawater[1]

Eluent	Total number of viruses adsorbed to membrane filters PFU	Viruses eluted from membrane filter % of adsorbed virus	Organic flocculation (reconcentration) % recovery	Overall recovery efficiency
3% Beef extract pH = 9.0	3.50×10^6	79	65	51
	2.59×10^6	92	67	61
0.5% Purified casein pH = 10.0	5.30×10^6	82	66	55
	4.43×10^6	125	53	66

[1] Poliovirus 1, suspended in 3 l of seawater at pH 3.5 with 0.0005M $AlCl_3$, was adsorbed to a series (3 μm → 0.45 μm → 0.25 μm) of Filterite filters in the presence of 0.0005M $AlCl_3$ at pH = 3.5. Adsorbed virus was eluted with 3% beef extract (pH = 9.0) or 0.5% purified casein in 0.05M glycine (pH = 10.0). The eluates were then concentrated by organic flocculation.

Since elution was highly efficient at pH = 10.0, it was decided to compare the recovery efficiency of 0.5% purified casein with that of 3% beef extract (pH = 9.0). It was found that the overall recovery (elution followed by organic flocculation) for both methods was similar and was always higher than 50% (Table III).

3.2. USE OF NON-FAT DRY MILK (NFDM) FOR POLIOVIRUS DETECTION IN SEAWATER

Since casein is the major protein found in milk, it would be economically advantageous to use a biological fluid such as NFDM to elute viruses from membrane filters. Table IV shows the effect of pH on elution of poliovirus type 1 from membrane filters using 1% (w/v) NFDM in 0.05M glycine buffer. Virus elution was maximum (> 100%) at pH = 9.0 and decreased to 35% and 37% at pH 8 and 7, respectively. The effect of NFDM concentration on virus elution and overall recovery was also investigated (Table V). It was found that viral elution was the highest (71%) in the presence of 1% (w/v) NFDM. Virus recovery by organic flocculation was always high and ranged from 81 to 100%.

TABLE IV

Effect of pH of elution of poliovirus type 1 from Filterite filters using 1% (w/v) non-fat dry milk (NFDM)[1]

Ph of NFDM eluent	% elution[2]
7.0	43
8.0	35
9.0	116
10.0	86
11.5	69

[1] Poliovirus 1 (Sabin) suspended in 1 l of seawater was adsorbed to a series (3.0 μm → 0.45 μm → 0.25 μm) of Filterite filters in the presence of 0.0005M $AlCl_3$ at pH = 3.5. Viruses were eluted with 10 ml of 1% NFDM in 0.5M glycine adjusted to the various pH's with 0.5M glycine.

[2] Each number represents the mean of 2 to 4 trials.

TABLE V

Concentration of poliovirus type 1 from seawater using various concentrations of non-fat dry milk (NFDM)[1,2]

Treatment	Total PFU adsorbed ($\times 10^6$)	% elution	Organic flocculation % recovery	% overall recovery
1.00% NFDM[3]	50.7	71	100	71
0.75% NFDM	1.86	58	84	48
0.50% NFDM	1.36	35	94	33
0.25% NFDM	1.22	35	82	28

[1]Poliovirus type 1, suspended in seawater, was adsorbed to a series (3.0 μm → 0.45 μm → 0.25 μm) of Filterite filters in the presence of 0.0005M $AlCl_3$ at pH = 3.5. Viruses were eluted with the various concentrations of NFDM in 0.05M glycine, pH = 9.0. Organic flocculation was undertaken by adding 0.5M glycine to the filter eluate until a pH of 4.5 to 4.6 was reached. The floc formed was pelleted by centrifugation and the pellet redissolved in 0.15M Na_2HPO_4, pH = 9.0.

[2]Each number represents the mean value obtained from duplicate trials.

[3]Trials using 1% NFDM used seawater samples of 20 gallons (76 l) processed through 0.45 μ Filterite filters. Other trials used 1 to 3 l seawater samples and were processed as described above.

The overall recovery of poliovirus type 1 by this method was 71% in the presence of 1% NFDM at pH = 9.0. It is worth noting that these results pertain to 20 gallon (75.6 l) samples of seawater (Table V). The performance of 1% (w/v) NFDM at pH 9.0 was then investigated for other types of virus.

3.3. CONCENTRATION OF OTHER ENTEROVIRUSES FROM SEAWATER

The concentration of six enteroviruses (Polio 1, Polio 2, Polio 3, coxsackie B3, Echo 1, and Echo 4) by the NFDM technique is shown in Table VI. It is evident that this method is efficient for the recovery of the three strains of poliovirus as well as coxsackievirus B3. The technique displayed at 31% overall recovery of echovirus 4, but was inefficient as

TABLE VI

Concentration of six enteroviruses from seawater by the non-fat dry milk (NFDM) technique[1,2]

Virus	Virus input PFU ($\times 10^6$)	% elution from filters	Organic flocculation % recovery	% overall recovery
Polio 1[3]	31.6 – 69.8	71	100	71
Polio 2	29.9 – 30.8	76	78	59
Polio 3	61.1 – 69.6	111	72	75
Coxsackie B3	0.46	87	75	65
Echo 1	17.0	78	10	8
Echo 4	0.8 – 1.6	72	43	31

[1]The various viruses, suspended in 1 to 3 l of seawater, were adsorbed to a series of (3.0 μm → 0.45 μm → 0.25 μm) of filterite filters in the presence of 0.0005M $AlCl_3$ at pH = 3.5. The filters were eluted with 1% NFDM in 0.05M glycine, pH = 9.0. Eluates were further concentrated by organic flocculation.

[2]Each number represents the mean of duplicate trials.

[3]The polio 1 trials used seawater samples of 20 gallons (76 l) processed through 0.45 μm Filterite filters. Other trials used 1 to 3 l seawater samples and were processed as described above.

TABLE VII

Concentration of poliovirus type 1 from 50 gallons (189 l) of tapwater by the non-fat dry milk (NFDM) technique[1]

Total virus input PFU	% elution from filters	Organic flocculation % recovery	Final volume (ml)	% overall recovery
High virus input				
7.12×10^7	72	110	80	79
7.17×10^7	119	68	55	81
Low virus input[2]				
641	–	–	28	72
569	–	–	30	78

[1] Poliovirus type 1, suspended in 50 gallons (189 l) of dechlorinated tapwater, was adsorbed to a 0.25 μm or 0.45 μm Filterite cartridge filter in the presence of 0.0005M $AlCl_3$ at pH = 3.5. Viruses were eluted from the filters using 1% NFDM in 0.05M glycine pH = 9.0. Filter eluates were brought to pH = 4.5–4.6 with 1M glycine (pH = 2.0) and the resultant floc centrifuged at 4500 rpm for 4 min. The pellets were then resuspended in 10–67 ml Na_2HPO_4, pH = 9.0.

[2] Concentrates from low virus input trials were dialyzed for 18 to 24 h against PBS at 4°C prior to direct assay on host cells.

regards to echovirus 1. The poor performance of the technique as regards to echorivus 1 is mainly due to our inability to concentrate this particular virus by organic flocculation (Table VI).

3.4. CONCENTRATION OF POLIOVIRUS TYPE 1 FROM LARGE VOLUMES OF TAPWATER BY THE NFDM TECHNIQUE

Fifty gallon (189 l) samples of tapwater were seeded with high ($7.12 \times 10^7 - 9.17 \times 10^7$ PFU) and low (569–641 PFU) inputs of poliovirus and processed by membrane filtration (Filtrite cartridge filters) followed by the NFDM technique (i.e., elution with 1% NFDM at pH 9.0, followed by organic flocculation). The results of those experiments are displayed in Table VII. At high virus input, the overall recovery of poliovirus from two tapwater samples was 79% and 81%. At low virus input, virus recovery was still high and ranged from 72 to 78%.

Figure 1 shows a general scheme of the NFDM technique for the recovery of viruses from seawater or tapwater. The concentration factor may be as high as 6300 when 50 gallons (189 l) of water are processed.

4. Discussion

Organic flocculation is becoming increasingly popular in virus detection methodology. Hence, beef extract has been used for the detection of poliovirus in tapwater [8], seawater [7], wastewater effluents [9], and anaerobic sludge [5]. This study has essentially shown than an efficient recovery of poliovirus type 1 from seawater or tapwater can be achieved through elution of pleated membranes with 0.5% casein or 1% non-fat dry milk (NFDM)

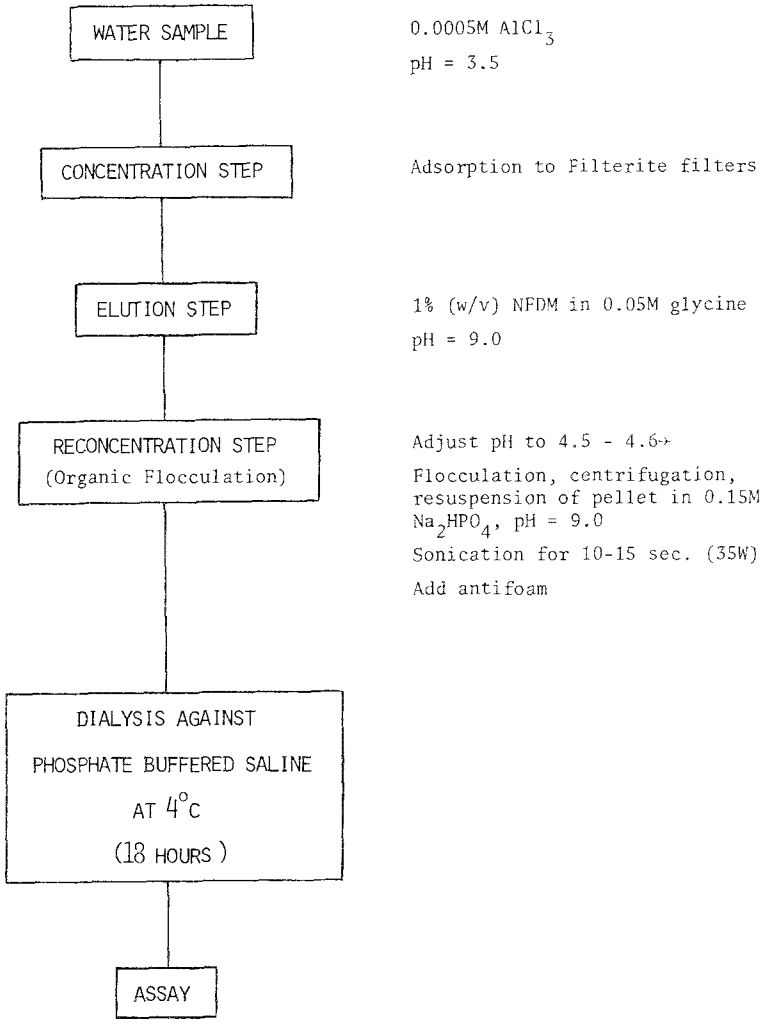

Fig. 1. Scheme for recovering viruses by the NFDM Technique.

at pH 10 or 9, respectively. These biological fluids have been buffered at their respective pH with 0.05M glycine. It is well known that 0.05M glycine, at pH 9 or 10, does not efficiently elute viruses from membrane filters [2, 14] or from sediments [4]. Since, in our experiments, elution has been carried out mostly at pH 9, it is concluded that viruses were desorbed mainly through the action of casein or NFDM. The NFDM technique operates best at pH 9.0 and this is an advantage over those methods that use glycine buffer at pH 11.5. This high pH may be harmful to certain enteric viruses such as adenoviruses [3]. Long exposure to glycine buffer at pH 11.5 (> 10 min) may also be detrimental to poliovirus type 1 [4] which has been used in most of the 'seeded' experiments dealing with

virus recovery from environmental samples. Organic flocculation with casein or beef extract gave similar results as regards to poliovirus 1 recovery from seawater. These results agree with those obtained by Katzenelson [7] in experiments pertaining to poliovirus recovery from seawater with the beef extract method.

High overall recoveries were achieved with 1% (w/v) NFDM at pH 9.0 in experiments dealing with poliovirus concentration from seawater or tapwater. The NFDM technique is also capable of detecting low numbers of poliovirus in relatively large volumes of tapwater (189 l).

We have also studied the concentration of six enteroviruses (Poliovirus 1, 2, 3, coxsackievirus B3, and Echovirus 1 and 4) by the NFDM technique (Table VI). It was shown that the elution step was efficient for all the viruses tested (71 to more than 100% recovery). Problems were, however, encountered during the organic flocculation step where poor recovery of Echovirus 1 and lower recovery of Echovirus 4 were observed. Echovirus 1 adsorbed poorly to the milk protein flocs and this confirms an earlier study showing poor adsorption of the same virus to soils (Goyal, S. M. and J. L. Melnick. 1978, Abstract. Amer. Soc. Microbiol. Ann. Meeting). The same study has also shown intratypic differences among echovirus isolates. The poor recovery of some viruses by organic flocculation with beef extract was recently observed by Williams and Jakubowski (1978, Abstract. Amer. Soc. Microbiol. Ann. Meeting). We are presently looking at means to improve the recovery of echovirus 1 by the NFDM technique.

It is worth stressing that organic flocculation with milk protein (casein, NFDM) is less expensive than with beef extract. At 1978 prices, a pound of NFDM costs only U.S. $1.50 as compared to U.S. $25.00 per pound of beef extract. Non-fat dry milk is furthermore readily available and may be purchased from any neighborhood grocery store at a relatively low price.

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