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Development and Application of New Positively Charged Filters for Recovery of Bacteriophages from Water

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Electronegative and electropositive filters were compared for the recovery of indigenous bacteriophages from water samples, using the VIRADEL technique. Fiber glass and diatomaceous earth filters displayed low adsorption and recovery, but an important increase of the adsorption percentage was observed when the filters were treated with cationic polymers (about 99% adsorption). A new methodology of virus elution was developed in this study, consisting of the slow passage of the eluent through the filter, thus increasing the contact time between eluent and virus adsorbed on the filters. The use of this technique allows a maximum recovery of 71.2% compared with 46.7% phage recovery obtained by the standard elution procedure. High percentages (over 83%) of phage adsorption were obtained with different filters from 1-liter aliquots of the samples, except for Virosorb 1-MDS filters (between 1.6 and 32% phage adsorption). Phage recovery by using the slow passing of the eluent depended on the filter type, with recovery ranging between 1.6% for Virosorb 1-MDS filters treated with polyethyleneimine and 103.2% for diatomaceous earth filters treated with 0.1% Nalco.

Several procedures have been developed for the recovery and isolation of bacteriophages from aquatic environments, including the direct assay, enrichment techniques, polyethylene glycol precipitation, adsorption and elution, and differential centrifugation. Phage detection by direct assay is not suitable for samples with low phage numbers. Therefore, a concentration step is needed to detect low numbers of phages in environmental samples (7). The VIRADEL technique (virus adsorption and elution) (8) consists of the adsorption of the viruses to filters and their subsequent elution in a small volume of eluent. Optimal adsorption of viruses to electronegative filters is obtained by lowering the pH to 3.5 and by adding aluminum chloride prior to filtration. The adsorbed viruses are eluted at alkaline pH (5, 19). However, phages may be inactivated at pH 3.5 or as a result of pH changes (14, 17). Electropositive filters described by Sobsey and Jones (25) and Sobsey and Glass (23) possess a substantial advantage over the electronegative filters, since they can absorb viruses over a broader pH range without the addition of salts, and the pretreatment of the water is not required prior to virus adsorption (3). The commercial electropositive filters (Zeta-Plus, Virosorb 1-MDS, etc.) may easily be blocked by the colloidal matter contained in the sample, avoiding the processing of large volumes of water.

In the present paper, several methods for the recovery of indigenous phages from natural samples and tap water seeded with sewage were compared, using new positively charged filters.

MATERIALS AND METHODS

Samples. A trickling filter effluent from the wastewater treatment plant located at the University of Florida, Gainesville, was used.

Tap water was dechlorinated by addition of 0.1% sodium thiosulfate (Fisher Scientific Co., Fair Lawn, N.J.) and seeded with 10% trickling filter effluent (7 days old).

Bacterial strains and culture conditions used. *Escherichia coli* C3000 was used as the host strain in all phage assays. The bacterial host was grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) at 35°C overnight.

The double-layer agar technique (1) was used as the reference method for the direct phage assay. Four milliliters of soft agar composed of 2.7% Trypticase soy broth (BBL) and 0.74% agar base (Sigma Chemical Co., St. Louis, Mo.) was used as the top agar layer. Scholtens agar supplemented with 5 mmol of calcium per liter and 1.25 mM magnesium ions (11) was used as the bottom agar layer.

Concentration methods. Phages from all samples were concentrated by the VIRADEL technique (8). The following electronegative filters were used in the experiments with small volumes (<50 ml) of water: epoxyfiber glass (0.45- and 0.22-μm pore sizes; M Tech, Timonium, Md.); Whatman glass microfiber (2.7-, 1.6-, and 1.0-μm pore sizes; Schleicher & Schuell, Keene, N.H.); cellulose nitrate (0.45-μm pore size; Millipore Corp., Bedford, Mass.); polycarbonate membrane (0.45-μm pore size; Nuclepore Corp., Pleasanton, Calif.); cellulose acetate (0.45-μm pore size; Gelman Metricel, Ann Arbor, Mich.); and diatomaceous earth (DE; Sigma). DE filters were prepared as follows: epoxyfiber glass filters used as a physical support were placed onto polypropylene Swinnex (Millipore), and then an aqueous solution of DE was filtered with positive pressure through the sealed filter holder (Swinnex) until DE was deposited on the epoxyfiber glass filter.

The following chemicals were used to make positively charged filters: polyethyleneimine (PEI; Sigma); Nalco 7111 (Leachchem Industries Inc., Titusville, Fla.); Chitosan (Sigma); polymyxin B (Sigma); and benzyl-dimethyl-hexadecyl-

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TABLE 1. Positively charged filters used for recovery of indigenous phages from trickling filter effluents

Filter type[a]	Treatment	Reference
Cellulose nitrate	PEI, 0.5%	Millipore HA025-PEI
	Nalco, 0.1%	Millipore HA025-Nalco
	Polymyxin B, 0.1%	Millipore HA025-polymixin B
	BDHA, 0.1%	Millipore HA025-BDHA
	PEI, 0.5%	Millipore HA047-PEI
Polycarbonate	PEI, 0.5%	Nuclepore-PEI
Epoxyfiber glass (0.45 μm)	PEI, 1.0% + Nalco 0.05%	FG-PEI-Nalco
	PEI, 0.7%	FG-PEI
	BDHA, 0.1%	FG-BDHA
Epoxyfiber glass (0.22 μm)	Nalco, 0.1%	FG-Nalco
Glass microfiber (2.7 μm)	PEI, 2%	GF/D-PEI
	Chitosan, 2%	GF/D-Chitosan
Glass microfiber (1.6 μm)	PEI, 2%	GF/A-PEI
	Chitosan	GF/A-Chitosan
Glass microfiber (1.0 μm)	PEI, 2%	GF/B-PEI
	Chitosan	GF/B-Chitosan
Virosorb 1-MDS		1-MDS
	PEI, 0.5%	1-MDS-PEI
DE	Mg + Fe	mDE
	PEI, 0.5%	DE-PEI
	Nalco, 0.1%	DE-Nalco
	PEI, 0.5%, + Nalco, 0.01%	DE-PEI-Nalco

[a] The manufacturers are listed in Materials and Methods.

ammonium chloride (BDHA; Sigma). The chemicals were dissolved in deionized water and used to soak the filters for 2 h at room temperature. The filters were allowed to air dry overnight on absorbent paper towels. DE filters were treated with different chemicals and cations by the methodology described by Farrah et al. (4). Table 1 shows the different filters and treatments used in this study.

Eighteen liters of dechlorinated tap water was inoculated with 2 liters of trickling filter effluent. The mixture was tested for the recovery of indigenous phages by the VIRADEL technique with the following filters: DE treated with 0.1% Nalco, using as holder both epoxyfiber glass (0.45-μm pore size, 47-mm diameter) and Virosorb 1-MDS (0.45-μm pore size, 47-mm diameter; AMF Cuno, Meriden, Conn.) filters; DE treated with iron and magnesium salts (mDE) (4), using the above-mentioned filters as holders; single and double Virosorb 1-MDS filters (0.45-μm pore size, 47-mm diameter); single and double Virosorb 1-MDS filters (0.45-μm pore size, 47-mm diameter) treated with 0.5% PEI; and single and double cellulose nitrate filters (0.45-μm pore size, 47-mm diameter; Millipore) treated with 0.5% PEI. Aliquots of the sample (1 liter) were filtered through all filters, except Millipore filters, which allowed the passage of a mean volume of 750 ml. Samples were passed through the filters by means of either negative pressure (vacuum filtration) using a vacuum pump, positive pressure using nitrogen gas as a pressure source, or positive pressure using presterilized 100-ml syringes.

Elution process. Beef extract (Scott Laboratories, Fiskeville, R.I.) at 3% supplemented with 1 M NaCl (Fisher), adjusted to pH 9.0, was used as eluent.

The methods used for the elution process were the following: standard procedure, consisting of the passage of 10 ml of the eluent through the filter at approximately 1 ml/s (2); and drop-by-drop method, which consists of the slow passage of the eluent through the filter (at 0.5 ml/min) by means of positive pressure.

The titers of the phage (PFU per milliliter) in the eluents and filtrates were calculated for all cases, and the results were expressed as the mean percentage of phage recovery of triplicate determinations for each experiment.

RESULTS

Table 2 shows the adsorption and recovery percentages of phage from trickling filter effluent, using different filters together with the VIRADEL technique.

Phage adsorption to DE filters without treatment is low, with an average of 20.9%. Adsorption increased to >90% when the same filters were treated with cations (magnesium and iron), PEI, or Nalco. The recovery of the adsorbed phages depends on the elution procedure used. The standard elution procedure (filtration of 10 ml of 3% beef extract, pH 9, supplemented with 1 M NaCl) achieved recoveries that varied between 5.9 and 46.7% depending on the filters used. With the drop-by-drop method, virus recoveries were between 9.3% and >71% in comparison with the reference method (direct assay by the double agar layer).

The results of adsorption and recovery of phage with membrane filters are also shown in Table 2. Adsorption of the phages depended on both the filter type and the filter treatment, percentages of about 40% for the untreated filters and 73% for the treated filters being achieved. However, the recovery of phage was very low for all methods, and only Millipore-PEI and double Virosorb 1-MDS filters displayed a phage recovery >40%, using the drop-by-drop elution method.

To verify the results obtained from small volumes (50 ml) of water, several filter types were selected and tested with a higher volume (1 liter) of sample. Table 3 shows phage adsorption and recovery from 1 liter of tap water seeded with indigenous phages. All methods tested achieved very high (>80%) phage adsorption percentages, except the Virosorb 1-MDS filters (single or double), which adsorbed <35%. With respect to phage recovery, filtration through DE plus Nalco (epoxyfiber glass or Virosorb as holders) and mDE (Virosorb as holder) achieved the highest recoveries (>50%).

TABLE 2. Comparison of several filters for the concentration of indigenous phages from trickling filter effluent, using two elution methods[a]

Filter type	Adsorption (%)[b]	% Recovery[c]	
		Standard elution	Drop-by-drop elution
Millipore HA025-PEI	99.4 ± 0.5	42.9 ± 3.5	47.6 ± 6.7
Millipore HA025-Nalco	58.4 ± 8.3	8.5 ± 2.8	10.1 ± 1.4
Millipore HA025-BDHA	97.6 ± 1.1	0.8 ± 0.2	7.9 ± 0.3
Millipore HA025-polymyxin B	53.2 ± 6.7	0.7 ± 0.5	6.9 ± 1.6
Millipore HA047-PEI	99.9 ± 0.0	NT	21.6 ± 6.1
Gelman Metricel	40.2 ± 9.1	5.1 ± 2.3	NT
Nuclepore-PEI	5.8 ± 3.1	0.5 ± 0.0	NT
FG-PEI-Nalco	98.6 ± 1.3	12.8 ± 6.0	15.2 ± 3.0
FG-BDHA	5.2 ± 2.8	4.5 ± 1.1	6.3 ± 0.6
FG-PEI	99.9 ± 0.1	NT	1.7 ± 0.4
FG-Nalco	39.0 ± 6.5	24.1 ± 4.5	34.0 ± 6.4
Double GF/B	49.3 ± 5.1	0.8 ± 0.5	1.7 ± 0.4
Double GF/B-Chitosan	53.6 ± 12.2	0.1 ± 0.0	0.7 ± 0.5
Double GF/B-PEI	99.9 ± 0.0	8.4 ± 5.0	20.6 ± 8.4
Double GF/A	41.3 ± 6.3	0.1 ± 0.0	0.1 ± 0.0
Double GF/A-Chitosan	26.6 ± 5.8	0.1 ± 0.0	0.5 ± 0.0
Double GF/A-PEI	99.9 ± 0.0	10.7 ± 3.1	25.4 ± 7.4
Double GF/D	39.8 ± 3.9	0.1 ± 0.1	0.4 ± 0.1
Double GF/D-Chitosan	27.2 ± 4.5	0.1 ± 0.0	0.3 ± 0.1
Double GF/D-PEI	97.1 ± 2.2	4.5 ± 2.4	8.0 ± 4.1
Single 1-MDS	54.9 ± 13.3	18.1 ± 7.4	25.9 ± 10.0
Double 1-MDS	98.2 ± 0.3	39.2 ± 5.1	49.0 ± 14.4
DE	20.9 ± 4.9	5.9 ± 2.8	9.3 ± 3.4
mDE	91.0 ± 1.6	28.8 ± 11.1	60.7 ± 12.5
DE-PEI	98.0 ± 1.4	12.7 ± 7.3	18.7 ± 7.3
DE-Nalco	91.9 ± 1.9	46.7 ± 15.4	71.2 ± 14.8
DE-PEI-Nalco	99.9 ± 0.0	14.3 ± 4.1	19.0 ± 9.0

[a] All data are the means of five different experiments carried out in triplicate.
[b] Adsorption rate = 100 − (phage titer in filtrate/phage titer in the sample by direct assay) × 100.
[c] Considering 100% the recovery percentage obtained by the reference method (direct assay by the double-agar-layer technique). NT, Not tested.

DISCUSSION

The methods based on adsorption to microporous filters and subsequent elution are still considered efficient in the concentration of viruses from water samples. Several concentration procedures have been developed for enteric virus quantification by using electronegative filters as adsorbents (8). The adsorption of viruses to this type of filter requires a decrease in pH of the sample or the addition of cations or both. It was shown previously that these concentration procedures were not appropriate for phage concentration, since low pH values inhibited several types of bacteriophages (16–18).

For this reason, investigations on the concentration of phages from water have been centered around the use of electropositive or electronegative charged modified filters which made possible the performance of the assays at neutral pH. Our results showed that the commercial electropositive filters such as Virosorb 1-MDS, tested by several authors (9, 20, 21), possess low adsorption and recovery rates of indigenous phages. In practice, the concentration of phages or other viruses under environmental conditions is less efficient than in laboratory conditions. This may be attributed to the presence of suspended solids and/or dissolved organic compounds in the water. Suspended solids of >0.2-μm size exert little effect, but dissolved organic compounds significantly affect virus adsorption to electropositive filters (24). Havelaar (10) suggested that the virus elution process was affected negatively by the ionic strength of the sample, which is inversely proportional to the elution efficiency of the phages.

TABLE 3. Coliphage concentration from 1 liter of water seeded with sewage, using different filter types[a]

Filters	Holder	Adsorption (%)	Recovery (%)
DE-Nalco	Epoxyfiber glass	92.8 ± 3.14	103.2 ± 6.98
	Virosorb 1-MDS	83.2 ± 5.67	62.4 ± 13.48
mDE	Epoxyfiber glass	97.6 ± 1.34	32.0 ± 6.48
	Virosorb 1-MDS	97.6 ± 1.94	52.8 ± 12.23
Single 1-MDS		1.6 ± 1.60	7.2 ± 2.80
Double 1-MDS		32.0 ± 13.29	12.0 ± 6.05
Single 1-MDS-PEI		99.2 ± 1.08	1.6 ± 1.13
Double 1-MDS-PEI		99.2 ± 0.75	8.8 ± 4.96
Single Millipore-PEI		99.2 ± 0.34	43.6 ± 8.09
Double Millipore-PEI		99.2 ± 0.60	24.0 ± 4.55

[a] Elution by the drop-by-drop method. Values are means ± standard deviations of four different experiments.

The charge-modified cellulose filters displayed high phage adsorption percentages. Thus, in Table 2, it can be seen that Millipore-PEI and Millipore-BDHA filters exhibited adsorption percentages >97%, higher than those obtained with Virosorb 1-MDS filters (54.9%). This implies that the interactions between filters and viruses are electrostatic in nature, being affected by the chemical composition of the filter. With regard to phage elution, epoxyfiber glass and cellulose nitrate charge-modified filters have overall recoveries of about 14 and 19%, respectively, with intervals between 1.7 and 47.6%. For Virosorb 1-MDS filters, the average recovery was 25.9 or 49%, depending on the number of filters (single or double, respectively). These results are similar to those reported by Havelaar (10) with these filters; Havelaar obtained mean recoveries between 31 and 76% depending on the beef extract concentration and pH of the eluent used. In the same way, our results are in agreement with those obtained with 1-MDS cartridges by Nupen and Bateman (13), who reported a mean recovery of phage of 31%, with a range between 18 and 42%.

Rose et al. (15), studying the comparative efficiency of fiber glass and Virosorb 1-MDS filters for the recovery of coliphages from primary wastewater effluents, obtained a higher recovery of phages with fiber glass filters (17 versus 14% on Virosorb). However, these results were reversed when the samples used were from secondary effluents (22% of recovery from fiber glass versus 61% from Virosorb). The differences in the recovery rates of both types of filters may be due to the turbidity and organic matter concentration of the samples. It is possible that a high organic content interfered with adsorption and with the recovery rate of the viruses from the water sample (6, 22). Sobsey et al. (26) also detected that the water quality affected both the adsorption to and the recovery of the animal viruses on both fiber glass and Virosorb 1-MDS filters.

The development of techniques for the recovery of bacteriophages from large volumes of water is very important, since these viruses can serve as models or indicators for the removal or inactivation of the enteroviruses in the wastewater treatment processes. The techniques most generally used have been filtration and ultrafiltration, but the isolation and recovery of the viruses by means of these techniques have not been evaluated completely. Nupen et al. (12), comparing the recovery efficiency of coliphages by ultrafiltration, observed that this process depended on the amount of suspended matter contained in the sample. The results obtained in the present study (Table 3) indicate that the phage recovery percentages varied with the filter type used. The mean recovery of coliphages, considering all filters, from 1 liter of water is 35.5%, with a range between 0.8 and 103.7%. These results are very similar to those obtained by Nupen et al. (12): mean value of 36%, but with a narrower range (between 26 and 44%); these authors used only one type of filter but different water samples.

In short, the best method tested for the adsorption and recovery of phages from low (50 ml) and higher (1 liter) volumes of water was the DE-plus-Nalco filter placed on epoxyfiber glass or Virosorb 1-MDS filters as holders and elution by the drop-by-drop technique.

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