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Reduction of Cytotoxicity in Virus Concentrates from Environmental Samples

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The reduction of cytotoxicity in virus concentrates from environmental samples was accomplished by high-speed centrifugation and by filtration of the samples through positively charged depth filters.

Contamination of water and shellfish with human enteric viruses is a recognized public health problem (2, 10). Methods have been developed for concentrating and detecting many types of enteric viruses in water (1, 8), shellfish (6, 7, 9), and sediment (3). Because current methods rely on tissue culture assays, a major problem in conducting field studies on viruses in the environment has been cytotoxicity of the virus concentrates. Various techniques, such as treatment with Freon (L. M. Stark, F. M. Wellings, and A. L. Lewis, Abstr. Annu. Meet. Am. Soc. Microbiol., p. 208, 1981) or cationic flocculants (5), have been used to reduce the cytotoxicity of these concentrates.

Detection of viruses that cannot be found with a standard plaque assay poses even greater problems. For example, human rotavirus has been detected in wastewater concentrates by an indirect immunofluorescent technique (E. M. Smith, Ph.D. thesis, Baylor College of Medicine, Houston, Tex., 1979). For further studies of rotavirus in the environment by this method, it will be necessary to reduce the total volume of concentrate to 1 to 2 ml since this is the maximum that can be assayed by a method which requires microscopic examination of each sample. This reduction in volume also increases the cytotoxicity of the concentrates. A simple method is needed to reduce this cytotoxicity in a wide variety of environmental samples. This paper reports the effect of several treatments on the cytotoxicity of virus concentrates from raw and treated wastewater and from pond sediments.

Samples were assayed for toxicity to monkey kidney cells (MA104) in 96-well microtiter plates. This cell line was chosen because it is used in the immunofluorescent assay for rotavirus. A volume of 25 μl was inoculated into each well to give a final concentration of 0.01 ml of sample per cm^2 of cell monolayer. This is comparable to the 0.05 ml/cm^2 routinely used in the rotavirus assay. After incubation for 15 min at 37°C, 0.2 ml of fresh maintenance medium was added to the inoculum in each well. The plates were examined after 18 to 24 h of incubation at 34°C in a 5% CO_2 incubator. The degree of toxicity in each well was recorded on a scale of 0 to 4 according to the percentage of cells which was affected.

The effectiveness of various treatments for reducing cytotoxicity of sewage concentrates is shown in Table 1. Filtration through positively charged depth filters (Zeta-plus, type 50S) was most effective and reduced the toxicity in 24 of 25 (96%) samples tested. The type 5S filter, which has a larger pore size, was much less effective in reducing toxicity. The negatively charged type 50D and Millipore types GS and HA were also less effective than the 50S filter.

Centrifugation at 34,000 × *g* for 1 h was the second most effective treatment tested, with four of six samples showing improvement. Centrifugation at 8,500 × *g* for 1 h improved 37% of the samples tested.

Treatment with XAD-2 (macroreticular) resin columns (Amberlite XAD-2; Eastman Kodak Co., Rochester, N.Y.) improved 6 of 11 (54%) samples. Finally, Freon treatment was the least effective technique for reducing toxicity in sewage concentrates, with only 1 of 15 samples showing improvement. Amberlite XAD-2 is a low polarity, styrene-divinylbenzene resin with excellent surface characteristics for the adsorption of lipophilic organic compounds.

The results (Table 1) indicate "all or nothing" responses to each treatment. Nothing is revealed about the initial level of toxicity or how much the toxicity is reduced in each sample. To quantify the reduction in toxicity, it was necessary to titrate the toxicity before and after treatment.

The decrease in cytotoxicity after either centrifugation or filtration through 50S filters is

TABLE 1. Effect of various treatments on cytotoxicity of sewage concentrates[a]

Treatment	No. in which toxicity eliminated/no. originally toxic		Overall efficiency (%)
	Raw sewage	Secondary effluent	
Centrifugation			
8,500 × g	5/16	—[b]	31
34,000 × g	4/6	—	67
Freon	1/15	—	6.7
XAD-2 resin	—	6/11	54
Filtration			
Type 50S	18/19	6/6	96
Type 50D	4/13	4/5	44
Type 5S	1/8	—	12
Millipore GS	4/8	—	50
Millipore HA	1/6	1/3	22

[a] Samples (20 liters) of the indicated type were concentrated by adsorption-elution by the procedure described in reference 1 to a final volume of 50 ml in 0.05 M glycine + 10% TPB. Tested undiluted in microtiter plates on MA104 cells with 0.088 ml/cm^2.

[b] —, Not done.

shown in Table 2 for several types of concentrates. Filtration through 50S filters gave 1.2- to 4.4-fold reductions in cytotoxicity, depending on the type of concentrate. Generally, as the degree of cytotoxicity in the original sample increased, the reduction in toxicity by 50S filtration decreased. Centrifugation of concentrates from sewage pond sediments at 8,500 × g for 1 h and assay of the supernatant gave a 3.4-fold decrease in toxicity.

An important consideration is whether a treatment which reduces cytotoxicity also reduces the number of infectious virus particles in the sample. Table 3 shows the effect of filtration through 50S filters on the titers of poliovirus 1 (strain LSc) and simian rotavirus SA11 suspended in various media. Both poliovirus 1 and SA11 adsorbed to the filters when suspended in glycine buffer. When tryptose phosphate broth (TPB) or beef extract was added, adsorption was inhibited. Addition of 5% TPB to the medium completely inhibited poliovirus adsorption, but only 70% of the initial SA11 was recovered at this level. In the presence of 10% TPB, 86% of the input SA11 was recovered. Beef extract completely inhibited adsorption of poliovirus at a concentration of 1%, but 3% beef extract was necessary to satisfactorily inhibit adsorption of SA11. Beef extract (3%) is a common eluent used in many virus concentration methods, and 10% TPB in 0.05 M glycine has been used for rotavirus concentration in our laboratory. These results demonstrate that either of these eluents may be filtered through 50S filters without substantial loss of virus.

Suspensions of poliovirus 1 and SA11 in 500 ml of beef extract were concentrated by a low-pH organic flocculation technique (4) to determine whether viruses in this concentrate would pass through a 50S filter. Recoveries for poliovirus 1 and SA11 were 84 and 86%, respectively. Addition of up to 3% beef extract did not influence the recovery of either virus. Results with indigenous enterovirus (data not shown) present in raw sewage concentrates indicated no appreciable loss of these viruses by filtration through 50S filters.

The 50S filters also removed more than 99.9% of *Escherichia coli* from suspensions containing 10^4 cells per ml of 0.05 M glycine with or without TPB or beef extract.

The cytotoxic substances present in these samples were not identified. They were, however, nondialyzable and were concentrated by the adsorption-elution method for virus concentration. This indicated that they were negatively charged molecules with a molecular weight of ≥12,000.

TABLE 2. Decrease in cytotoxicity after centrifugation or filtration through type 50S filters

Sample type[a]	Concn factor	Lowest dilution at which no toxicity was observed[b]			Mean reduction in toxicity[d]
		Unfiltered	Centrifuged[c]	50S filtrate	
Raw sewage concentrate					
100 liter	5,000	80	—	67	1.2
20 liter	4,000	19	—	8	2.3
20 liter	400	8	—	1.6	4.4
Pond sediment[e] concentrate	500 g → 10 ml	5	—	2	2.5
Pond sediment[e] concentrate	500 g → 10 ml	13	4	—	3.4

[a] Samples were concentrated by the procedure described in reference 1.

[b] Geometric mean of four or more samples.

[c] At 8,500 × g for 1 h. —, Not done.

[d] Mean untreated divided by mean treated cytotoxicity titer.

[e] From a wastewater stabilization pond receiving raw domestic sewage.

TABLE 3. Recovery of virus after filtration through 50S filters

Suspending medium[a]	% Recovered in filtrate	
	Poliovirus 1	SA11
0.05 M glycine only	43	9.4
0.05 M glycine + 1% TPB	91	54
0.05 M glycine + 5% TPB	107	70
0.05 M glycine + 10% TPB	94	86
0.05 M glycine + 1% beef extract	133	70
0.05 M glycine + 3% beef extract	116	84
Concentrate only[b]	84	86
Concentrate + 1% beef extract	83	85
Concentrate + 3% beef extract	84	87

[a] Suspending media at pH 7.0 to 7.5.

[b] Acid precipitation of 500 ml of 3% beef extract suspended to 13 ml with 0.05 M glycine.

In conclusion, treatment of virus concentrates from environmental samples by high-speed centrifugation (≥8,500 × *g*) and filtration through positively charged depth filters (Zeta-plus, type 50S) substantially reduced cytotoxicity, although the level of toxicity reduction depended on the type of sample. Additionally, the 50S filters reduced bacterial contamination (data not shown) and adsorbed negligible amounts of virus when TPB or beef extract was present in the media. A combination of centrifugation and 50S filtration may provide an effective means for increasing the sensitivity of virus detection methods by decreasing the cytotoxicity of final concentrates.

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