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A METHOD FOR RECOVERING VIRUSES FROM SLUDGES

DONALD BERMAN*, GERALD BERG and ROBERT S. SAFFERMAN

Virology Section, Biological Methods Branch, Environmental Monitoring and Support Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Cincinnati, OH 45268, U.S.A.

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Primary, activated, and anaerobic mesophilically digested sludges were salted with $MgCl_2$ (divalent cations) or $AlCl_3$ (trivalent cations) and acidified to bind indigenous unadsorbed virions to the sludge solids; the sludges were centrifuged, and the adsorbed virions were eluted from the solids with buffered 10% beef extract. The elution yields with this procedure were superior to those obtained from sludges that had been salted or acidified only. Homogenization of sludges prior to other treatment did not increase the numbers of virions recovered.

enterovirus virus recovery wastewater sludge testing procedure

INTRODUCTION

Virions adsorbed from water onto cellulose nitrate or fiberglass filters may be eluted more readily with buffered 3% beef extract than by many other methods. Elution of virions from such filters is achieved at least as efficiently with buffered 3% beef extract as with buffered beef extract concentrations up to 15% (Berg et al., 1971). Virions adsorbed to river water solids may also be eluted with buffered beef extract, but virions are more readily eluted from such solids with buffered 10% beef extract than with buffered 3% beef extract (Berg and Dahling, 1980).

This paper describes a beef extract elution method for recovering virions from primary, activated, and anaerobic mesophilically digested sludges.

MATERIALS AND METHODS

Buffered beef extract (BE)

An appropriate quantity of powdered beef extract (Lab-Lemco), 1.34 g of $Na_2HPO_4 \cdot 7$

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* Present address: Microbiological Treatment Branch, Municipal Environmental Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH 45268.

H_2O, and 0.12 g of citric acid were mixed with 100 ml of distilled water and autoclaved at 121°C for 15 min. The pH of the buffered beef extract (BE) was 7.0 ± 0.1. The eluting capacity of each new lot of BE was compared to that of lots of known eluting capacity to determine that the capability of each new extract to elute at least several enteroviruses (poliovirus 1, coxsackievirus A9, and echovirus 7) was maximal (Berg et al., 1971).

Cell cultures

Viruses recovered from sludges were assayed in a continuous line of African green monkey kidney cells designated BGM (Dahling et al., 1974). The line was propagated in a medium of equal parts Eagle's minimal essential medium (MEM) in Hanks' balanced salt solution (HBSS) with L-glutamine and non-essential amino acids (Grand Island Biological Co.) and L-15 medium (Leibovitz) with L-glutamine (KC Biological), to which were added calf serum (final concentration = 10%) and $NaHCO_3$ (final concentration = 0.075%) (Dahling and Safferman, 1979). The cells were grown in 6 oz prescription bottles. Confluent monolayers were washed once on the day of the test with Earle's balanced salt solution and then inoculated.

Assays for virions

Virions were assayed by the plaque technique (Dahling et al., 1974). The cells used in this study were in passages 126–150. Drained cultures were inoculated with 1 ml of eluate, and the cultures were incubated at room temperature for 2 h. The inoculated cultures were then overlayed with a medium each 100 ml of which contained 41.5 ml 2 X MEM in HBSS with L-glutamine and non-essential amino acids (Grand Island Biological Co.), 2 ml heat-inactivated fetal calf serum, $NaHCO_3$ (final concentration = 0.225%), $MgCl_2$ (final concentration = 0.01%), neutral red (final concentration = 0.0015%), 1 ml sterile whole milk (Real Fresh Milk, Inc.) added shortly before overlay, 10,000 units of penicillin, 10 mg of streptomycin, 1.25 mg of tetracycline, 0.1 mg of amphotericin B, and 50 ml of a 3.0% solution of autoclaved Bacto Agar (Difco). The liquid part of the medium was filtered through a 0.22 μm Millipore HA membrane filter and warmed to 37°C before the agar, cooled to 43°C, was added. Plaques were counted periodically and marked permanently when recorded.

Sludges

Primary, activated, and anaerobic mesophilically digested sludges were obtained from six sewage treatment plants in southwest Ohio. Sludges were transported to the laboratory within 2 h of collection, hand-shaken until well-mixed, and appropriate volumes were dispensed into beakers. Sludges were stored overnight at 4°C.

pH adjustment of sludges

In some experiments, the pH of the sludge was adjusted to 3.0–3.5. In these instances, the pH was checked for drift 15 and 30 min after adjustment and readjusted to 3.0–3.5 if necessary. There was usually little pH drift between 15 and 30 min after the initial pH adjustment.

Suspended solids

Suspended solids were determined by a standard method (American Public Health Assoc., 1975).

Membrane stacks

Membrane stacks consisted of 3.0, 1.2, 0.6 and 0.45 μm Millipore MF membrane filters, in that order, stacked together in one filter holder. The filter stacks were used to remove bacteria and fungi from BE eluates.

RESULTS

Recovering virions from sludges

Recovery of virions from primary and activated sludges. To determine whether buffered 10% BE would elute viruses from sludges as it did from river water solids (Berg and Dahling, 1980), sufficient buffered 50% BE was mixed with 100 ml volumes of primary and activated sludges to yield final BE concentrations of 10% in the sludges. The treated sludges were centrifuged, and the virions within the supernatants were assayed. Virions, often numbering in hundreds, were recovered from both primary and activated sludges.

Recovery of virions from blended primary and activated sludges. Sludge was blended in a Waring blender (Model PB5) at maximum speed for 1 or 4 min. 25 ml of buffered 50% BE were then mixed with 100 ml samples of both blended and non-blended sludge. The final concentration of BE in the samples was 10%. The mixtures were stirred on magnetic stirrers for 30 min and then centrifuged at 10,000 × *g* for 30 min. The solids were discarded, the supernatants were filtered through a membrane stack, and the virions within the supernatants were assayed.

In four comparative tests, greater numbers of virions were recovered from unblended primary sludges than from blended primary sludges (Table 1). In a similar series with activated sludges, three of five unblended sludges yielded greater numbers of virions than blended samples did. Thus, blending for 1 or 4 min did not increase the numbers of virions recovered from sludges and may have even decreased them somewhat.

TABLE 1

Recovery of indigenous virions from blended and unblended sludges

Sewage treatment plant	Type of sludge	Blending time (min)	Virions recovered	
			Blended (p.f.u./100 ml)	Unblended (p.f.u./100 ml)
Lebanon	Primary	1	562	608
Muddy Creek		1	200	212
Sycamore		4	27	70
Taft		4	512	569
Lebanon	Activated	1	112	108
Muddy Creek		1	187	229
Taft		4	205	240
		4	408	387
Sycamore		4	15	25

Recovering virions concentrated onto sludge solids

Since solids usually constitute less than 6% of the volume of sludges, and since many of the virions in sludges are adsorbed to these solids even before the sludges are formed, it seemed that the best approach to maximizing the recovery of virions from sludges should center on adsorbing the virions suspended in the liquid part of the sludges onto the solids, concentrating the solids, and then eluting the adsorbed virions from the solids. Since cations, especially in acid solution, often increase the adsorption of viruses to solids, salts were added to each sludge, the pH of the sludge was adjusted to 3.0–3.5 (Berg et al., 1971; Wallis and Melnick, 1967), the sludge solids were separated by centrifugation, and adsorbed virions were eluted from the solids.

Recovery of virions from acidified primary and activated sludges and from $MgCl_2$-salted, acidified primary and activated sludges

$MgCl_2 \cdot 6H_2O$ was mixed into one of two paired 100 ml samples of primary sludge (final $MgCl_2$ molarity = 0.05). The pH of each sample was then adjusted to 3.0–3.5 with 5 N HCl. Paired 100 ml samples of activated sludge were treated similarly. The paired samples were mixed with magnetic stirrers for 30 min and centrifuged at 2500 × *g* for 15 min. Supernatants were discarded, the solids of each sample were suspended in 100 ml quantities of buffered 10% BE, and the suspensions were mixed on magnetic stirrers for 30 min and centrifuged at 10,000 × *g* for 30 min. The supernatants were filtered through membrane stacks and the virions within the supernatants were assayed.

In six of seven tests, the presence of $MgCl_2$ increased the yields of virions from acidified primary sludges above those obtained from acidified sludges to which $MgCl_2$ had not been added (Table 2). Similar results were obtained with three of five paired samples of

TABLE 2

Effect of divalent cations (Mg^{2+} as $MgCl_2$) on recovery of indigenous virions from sludges acidified to pH 3.0–3.5

Sewage treatment plant	Type of sludge	Total suspended solids (g/100 ml)	Virions recovered	
			Acidified sludge without $MgCl_2$ (p.f.u./100 ml)	Acidified sludge with $MgCl_2$ (p.f.u./100 ml)
Taft	Primary	1.98	468	558
		0.27	188	152
Sycamore		3.95	80	107
Mill Creek		3.07	220	312
		1.13	427	450
Lebanon		1.16	713	910
Muddy Creek		1.00	360	427
Taft	Activated	0.63	380	292
Sycamore		0.50	27	27
Lebanon		0.56	313	353
Muddy Creek		1.09	247	280
Mill Creek		0.99	87	113

activated sludges. With one of these paired samples, there was no difference. The numbers of virions recovered from acidified primary and activated sludges (to which $MgCl_2$ had not been added) with the technique described were almost always considerably greater than those recovered from parallel samples by the 50% BE extraction procedure described above (data not shown).

Recovery of virions from $MgCl_2$-salted and from $MgCl_2$-salted, acidified anaerobic mesophilically digested sludges

$MgCl_2 \cdot 6H_2O$ was mixed into two 100 ml samples of anaerobic mesophilically digested sludge (final $MgCl_2$ molarity = 0.05). One of the samples was subsequently acidified with 5 N HCl to pH 3.0–3.5. Each sludge sample was mixed for 30 min and centrifuged at 2500 × *g* for 15 min. The supernatant was discarded, the solids were suspended in 100 ml of buffered 10% BE, and the suspended solids were mixed for 30 min and centrifuged at 10,000 × *g* for 30 min. The supernatant was filtered through a membrane stack, and the virions within the supernatant were assayed.

With four of the five paired samples tested, greater yields of virions were obtained from $MgCl_2$-salted anaerobic mesophilically digested sludges subsequently acidified to pH 3.0–3.5 than from salted anaerobic mesophilically digested sludges that had not been acidified (Table 3).

TABLE 3

Effect of acidification to pH 3.0–3.5 on recovery of indigenous virions from anaerobic mesophilically digested sludges to which divalent cations (Mg^{2+} as $MgCl_2$) had been added

Sewage treatment plant	Total suspended solids (g/100 ml)	Virions recovered	
		$MgCl_2$ added, sludge not acidified (p.f.u./100 ml)	$MgCl_2$ added sludge acidified (p.f.u./100 ml)
Mill Creek	6.67	63	87
	5.23	132	345
Lebanon	2.30	303	217
Sycamore	0.17	30	80
Dayton	6.03	72	77

Recovery of virions from $AlCl_3$-salted and from $AlCl_3$-salted, acidified anaerobic mesophilically digested sludges

$AlCl_3 \cdot 6H_2O$ was mixed into two 100 ml samples of anaerobic mesophilically digested sludge (final $AlCl_3$ molarity = 0.0005). One of the samples was subsequently acidified with 5 N HCl to pH 3.0–3.5. Each sludge sample was mixed for 30 min and centrifuged at 2500 × *g* for 15 min. The supernatant was discarded, the solids were suspended in 100 ml of buffered 10% BE, and the suspended solids were mixed for 30 min and centrifuged at 10,000 × *g* for 30 min. The supernatant was filtered through a membrane stack, and the virions within the supernatant were assayed.

With all five paired samples tested, greater yields of virions were obtained from $AlCl_3$-salted anaerobic mesophilically digested sludges subsequently acidified to pH 3.0–3.5 than from salted anaerobic mesophilically digested sludges that had not been acidified (Table 4).

Relative effectiveness of $MgCl_2$ and $AlCl_3$ for recovering indigenous viruses from acidified primary, activated, and anaerobic mesophilically digested sludges

$MgCl_2 \cdot 6H_2O$ and $AlCl_3 \cdot 6H_2O$ were mixed into separate 100 ml samples of sludges (final $MgCl_2$ molarity = 0.05, final $AlCl_3$ molarity = 0.0005). The pH of each sludge sample was adjusted to 3.0–3.5 with 5 N HCl, and each sample was mixed for 30 min and centrifuged at 2500 × *g* for 15 min. The supernatant of each was discarded, the solids were suspended in 100 ml of buffered 10% BE, and the suspended solids were mixed for 30 min and centrifuged at 10,000 × *g* for 30 min. Each supernatant was filtered through a membrane stack, and the virions within the supernatant were assayed.

With three of four primary sludges, $MgCl_2$ produced greater recoveries of virions than $AlCl_3$ did. With two of three activated sludges, $AlCl_3$ produced greater recoveries of virions than $MgCl_2$ did. With three of five anaerobic mesophilically digested sludges, $MgCl_2$ produced greater yields of virions than $AlCl_3$ did (Table 5). Thus, $MgCl_2$ (final molarity = 0.05) and $AlCl_3$ (final molarity = 0.0005) produced about equal results.

TABLE 4

Effect of acidification to pH 3.0–3.5 on recovery of indigenous virions from anaerobic mesophilically digested sludges to which trivalent cations (Al^{3+} as $AlCl_3$) had been added

Sewage treatment plant	Total suspended solids (g/100 ml)	Virions recovered	
		$AlCl_3$ added, sludge not acidified (p.f.u./100 ml)	$AlCl_3$ added, sludge acidified (p.f.u./100 ml)
Mill Creek	6.67	50[a]	63[a]
	5.23	151	377
Lebanon	2.30	216	336
Sycamore	0.17	27	76
Dayton	6.03	49	69

[a] $AlCl_3$ was added as a solid (1.2 g $AlCl_3$ $6H_2O$/100 ml sludge). The final $AlCl_3$ concentration was 0.05 M.

TABLE 5

Relative effectiveness of Mg^{2+} (as $MgCl_2$) and Al^{3+} (as $AlCl_3$) for recovering indigenous virions from acidified (pH 3.0–3.5) sludges

Sludge	Sewage treatment plant	Total suspended solids (g/100 ml)	Virions recovered	
			$MgCl_2$ added (p.f.u./100 ml)	$AlCl_3$ added (p.f.u./100 ml)
Primary	Muddy Creek	4.01	2320	2260[a]
	Sycamore	6.30	2116	1964
		6.81	1885	1595
		2.70	910	990
Activated	Muddy Creek	1.32	817	821[a]
	Sycamore	0.61	160	145
		0.92	125	185
Anaerobic mesophilically digested[b]	Mill Creek	5.23	345	377
		6.67	87	63[a]
	Lebanon	2.30	217	336
	Sycamore	0.17	80	76
	Dayton	6.03	77	69

[a] Final $AlCl_3$ concentration was 0.05 M.

[b] Data obtained from Tables 3 and 4.

DISCUSSION

Since the solids of domestic sludges are in significant part fecal material, it seemed that disruption of the solids by blending might increase the numbers of indigenous virions exposed to subsequent elution with beef extract. This was not the case. It is possible that the shear forces exerted by the blender were not strong enough to expose significant numbers of surfaces to the beef extract eluant. It is also possible that shearing exposed new receptors that bonded virions previously free in suspension so strongly that they were not eluted by the beef extract or, alternatively, that the process of blending inactivated some virions. The small reduction in the numbers of virions recovered by elution from blended sludges offers some support for the latter possibilities.

Many of the virions indigenous to a sludge are adsorbed on and within the solids of the sludge, probably on and within the fecal matter with which the virions enter the sewage. The liquid portion of sludges also contains some virions; therefore, the approach we chose to use to recover the virions in sludges was one of binding to the sludge solids those virions free in suspension which allowed us then to deal only with the solids, which constitute a small percentage of the sludge. Thus, we added divalent (Mg^{2+}) or trivalent (Al^{3+}) cations to the sludges to bridge the virions to sludge particles and to neutralize the negative charges on the virions and on the sludge solids particles so that electrostatic forces could help further to bond the virions to the sludge solids. Moreover, the pH of the sludges was reduced to 3.0–3.5, below the isoelectric point of many viruses, and this probably neutralized negative charges on the virions and thereby promoted adsorption of the virions to the sludge solids (Mix, 1974). The addition of divalent cations (Mg^{2+} as $MgCl_2$, final molarity = 0.05) to primary sludges that were subsequently acidified to pH 3.0–3.5 appeared to increase the numbers of indigenous virions subsequently eluted beyond those obtained from sludges that were only acidified. The same seemed to be the case with activated sludges. Moreover, acidification to pH 3.0–3.5 of anaerobic mesophilically digested sludges to which either divalent (Mg^{2+} as $MgCl_2$, final molarity = 0.05) or trivalent (Al^{3+} as $AlCl_3$, final molarity = 0.0005) cations had been added almost always increased the number of virions eluted subsequently. Thus, either $MgCl_2$ or $AlCl_3$, added in appropriate quantities to sludges subsequently acidified to pH 3.0–3.5, generally raised the yields of virions subsequently eluted above those obtained in the absence of either divalent or trivalent cations or in the absence of acidification. Few virions were ever recovered from the supernatants of centrifuged, acidified, salted sludges. With buffered 10% BE we have also recovered virions from salted, acidified, anaerobic thermophilically digested sludges. Acidification of sludges to pH 3.0–3.5 destroyed about 99% of the bacteria present and thereby reduced the risk of later contaminating cell cultures used to detect and quantify the virions recovered.

The volumes of buffered BE used to elute virions from centrifuged sludge solids were equal to the original volumes of the sludge samples tested. Thus, the procedures described are recovery methods and not concentration methods. Large volumes of buffered BE were used for elutions, primarily because extracts from concentrated sludge

solids were often toxic to cell cultures. To reduce the numbers of cell cultures required for assays, buffered BE eluates can be reconcentrated by the Katzenelson procedure (Katzenelson et al., 1976). Moreover, preliminary data indicate that treatment of centrifuged solids with Freon removes much of the toxicity and may allow elutions to be undertaken with lesser quantities of buffered BE than we have used thus far. Data demonstrating the effectiveness of the reconcentration and toxicity reduction procedures will be published at a later date.

The method described herein, that of fixing suspended virions in a sludge to the sludge solids by introducing divalent (Mg^{2+}) or trivalent (Al^{3+}) cations into the sludge and acidifying the sludge to pH 3.0–3.5, and eluting the virions from the centrifuged solids with buffered 10% BE, was designed to recover enteroviruses but will probably recover at least also the rotaviruses (Farrah et al., 1978). The cell culture or animal host system in which the virions are detected is, of course, a major factor that determines which virions will be recovered. In our laboratory, the method we described in this paper was superior to the beef extract elution procedures that we and others described earlier (Wolf et al., 1974; Glass et al., 1978; Nielsen and Lydholm, 1980) and better than other procedures that have been described also (Lund and Hedstrom, 1966; Malina et al., 1975; Turk et al., 1980; Wellings et al., 1976). A comparative study will be published elsewhere.

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