APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1982, p. 1413–1418
0099-2240/82/061413-06$02.00/0
Vol. 43, No. 6

Comparison of Methods for Recovering Indigenous Viruses from Raw Wastewater Sludge

DAVID A. BRASHEAR AND RICHARD L. WARD†*

Health Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Cincinnati, Ohio 45268

Received 2 December 1981/Accepted 8 March 1982

Five general methods for recovering indigenous viruses from raw wastewater sludge were compared. Each method included elution, concentration, and disinfection steps. The elution method, found to consistently yield the greatest viral recovery, was a two-phase technique that involved blending sludge with Freon. Other methods, including two being tested as American Society for Testing Materials tentative standard methods, were less effective. Viral recoveries were generally greater (sometimes much greater) if samples were concentrated by high-speed centrifugation rather than by organic flocculation with 3% beef extract. Three cell lines were used to measure viral recoveries by the plaque assay. The efficiency of recovery was greatest on BGM cells, followed by RD and MA-104 cells.

Expansion of the human population has caused a corresponding increase in the pollution load to the environment, which has resulted in obvious deterioration of waterways. In response, Congress enacted laws to restrict the flow of wastes to streams and oceans. These laws also recommended alternative modes of disposal that would conserve the inherent beneficial properties of wastes. Disposal of wastewater and sludge on land was emphasized. However, use of this mode of disposal has potential health risks caused by disease agents that are an indigenous part of wastes. Enteric viruses present in sewage are of particular concern.

The magnitude of the health risk due to viruses during waste utilization can be quantified only if their numbers are measurable. A variety of methods have been devised to quantify viruses in wastes. Most methods used to determine virus numbers in wastewater sludges involve at least an elution step and a concentration step. The elution step is used to separate viruses from sludge particulates, and the concentration step is used to reduce the sample size to a manageable volume. In addition, a disinfection step is often required to eliminate interfering microbial contaminants.

The elution method most often used is to chemically separate viruses from sludge particulates by mixing with an eluent and then to physically separate particulates from eluted viruses by centrifugation. Many different eluents have been used for this purpose (1, 4, 6, 10–13, 15–20), but the most common is beef extract (4, 5, 12, 15, 20). Although chemical elution has been aided by physical techniques such as sonication, shaking, blending, and stirring, the eluted viruses often have the opportunity to reassociate with sludge particulates. Most viruses bound to sludge particulates are lost during centrifugation. Reextraction of the sludge pellet, as has been done in some instances (10, 11), should increase viral recoveries. Even so, natural attractions between viruses and sludge particulates continue to reduce these recoveries.

A possible method to overcome this problem is to use an eluent that prevents reassociation. Eluents that can both chemically and physically separate viruses from sludge particulates should have this property. A two-phase method involving sodium dextran sulfate and polyethylene glycol was designed for this purpose and has been used to recover viruses from wastewater and sludges with some success (8, 9).

Another two-phase extraction procedure that has been used for years in the purification of nonenveloped viruses in crude suspensions is one that involves the use of halogenated fluorocarbons, particularly trichlorotrifluoroethane (Freon). This compound is immiscible with water and causes a strong protein denaturation effect at the solvent-water interface (14). Denatured protein in this interface forms a semisolid pellet, and the upper aqueous phase contains the virus. Therefore, use of this method should physically separate enteric viruses in sludge from the vast majority of sludge particulates. Virus recoveries from wastewater sludge have been reported with this two-phase extraction procedure (20).

† Present address: Christ Hospital Institute of Medical Research, Cincinnati, OH 45219.

Although many methods have been used to quantify viruses in sludge, very few comparisons have been made between the different recovery techniques. In addition, many of the reported methods were quantified with seeded viruses. It is clear that seeded viruses and indigenous viruses have different associations with sludge particulates. Indigenous viruses should be primarily embedded in these particulates, whereas seeded viruses should be surface associated. Therefore, viral recoveries from seeded sludges may have little relevance to recoveries of indigenous viruses.

A more valid way to determine recoveries of indigenous viruses by different methods is to directly compare several methods with samples of the same sludge and with the same viral assay procedures. The purpose of this study is to make such comparisons. Two of the methods chosen for this comparative study have been selected by a task group of the American Society for Testing Materials (ASTM) to be studied as tentative ASTM standard methods for virus extraction from sludge. The first (3), which was subsequently slightly modified to include a detoxification step before being tested as an ASTM standard method, was designed for use with primary, as well as digested, sludges; the second (5) was designed specifically for anaerobically digested sludge. The other three methods that are compared differed from one another only in their eluents, i.e., water, 10% beef extract, or Freon. Raw sludge was used because it contains a large number of indigenous viruses, thus permitting the use of smaller sludge volumes to obtain significant viral recoveries than would otherwise be required. The results indicate that the Freon method permits the best viral recoveries from raw sludge of those methods tested.

MATERIALS AND METHODS

Cells. Three continuous cell lines were used for this study. BGM (African green monkey kidney) cells were obtained from D. Dahling, U.S. Environmental Protection Agency, Cincinnati, Ohio; RD (human rhabdomyosarcoma) cells were obtained from R. Crowell, Hahnemann Medical College, Philadelphia, Pa.; and MA-104 (rhesus monkey kidney) cells were purchased from Microbiological Associates, Walkersville, Md. Monolayer cultures of BGM cells were grown in L-15–Eagle minimal essential medium (50:50) supplemented with 10% fetal bovine serum and appropriate antibiotics. RD cells were grown in RPMI 1640 medium containing antibiotics and 10% bovine serum, and the MA-104 cells were grown in Eagle medium containing antibiotics, 0.3% (wt/vol) tryptose phosphate broth, 0.5% (wt/vol) glucose, and 10% fetal bovine serum.

Sludge. The entire study was performed with raw sludge obtained from the sewage treatment plant at Harrison, Ohio in August, 1980 and stored in 1-gallon (3.785-liter) containers at −80°C. This sludge was derived almost totally from domestic sources. Just before use, sludge samples were thawed and then blended at high speed for 2 min to provide equal distribution of viruses in the samples. Initial studies showed that this sludge, which was about 4% solids by weight, contained a satisfactorily large number of enteric viruses.

Elution, concentration, and disinfection methods. (i) Method 1 (Freon elution). Sludge (100 ml) was mixed with an equal volume of chilled Freon and blended for 3 min at high speed. Samples were maintained below 10°C throughout this and the following procedures unless stated otherwise. The blended mixture was centrifuged (600 × *g*, 15 min) in glass bottles to separate phases. After the upper aqueous phase was removed, the lower phases (interface plus Freon phase) were reextracted with an equal volume of phosphate-buffered saline. The combined aqueous phases were split into two equal fractions, and each was concentrated by a separate method. Viruses in one fraction were pelleted by centrifugation (140,000 × *g*, 2 h) and suspended in 5 ml of Earle balanced salt solution. The other aqueous fraction was concentrated by organic flocculation (7). For this, the sample was mixed with sufficient 20% beef extract (Oxoid Ltd.) to give a final concentration of 3% and was then adjusted to a pH of 3.5 with 5 N HCl. After being stirred for 15 min at room temperature, the flocced material was pelleted by centrifugation (1,000 × *g*, 3 min) and dissolved in 5 ml of 0.15 M Na_2HPO_4. Both concentrated samples were then disinfected with ether. For this procedure, each sample was mixed for 1 min with 5 ml of ether and centrifuged to separate phases. The top phase was removed and discarded, and residual ether was driven from the bottom phase by N_2 bubbled through the solution. Samples were then stored at −80°C until assayed.

(ii) Method 2 (water elution). Raw sludge (100 ml) was blended for 3 min at high speed with an equal volume of distilled water, and particulates were pelleted by centrifugation (2,500 × *g*, 15 min). The supernatant was split, and each fraction was concentrated by either high-speed centrifugation or organic flocculation with beef extract before being disinfected with ether, all as described for method 1. Samples were stored at −80°C until assayed.

(iii) Method 3 (beef extract elution). Sludge (100 ml) was blended for 3 min at high speed with an equal volume of 20% beef extract. This concentration was used because it has been reported to give optimal separation of viruses from particulates (2). After foaming had subsided (about 30 min), particulates were pelleted by centrifugation (2,500 × *g*, 15 min). Again, the supernatant was split, and one fraction was concentrated by high-speed centrifugation. The other fraction was concentrated by organic flocculation after dilution with sufficient distilled water to bring the beef extract concentration to 3%. Both concentrated samples were then disinfected with ether and stored at −80°C.

(iv) Method 4 (ASTM-1). This procedure (3) is one of two being studied as a tentative ASTM standard method. All steps were performed with the samples at room temperature. Sludge (100 ml) was mixed with 1 ml of 0.05 M $AlCl_3$, adjusted to pH 3 to 3.5 with HCl, and stirred for 30 min with a magnetic stirrer. After centrifugation (2,500 × *g*, 10 min), the pellet was suspended in 10% buffered beef extract (1.34 g of

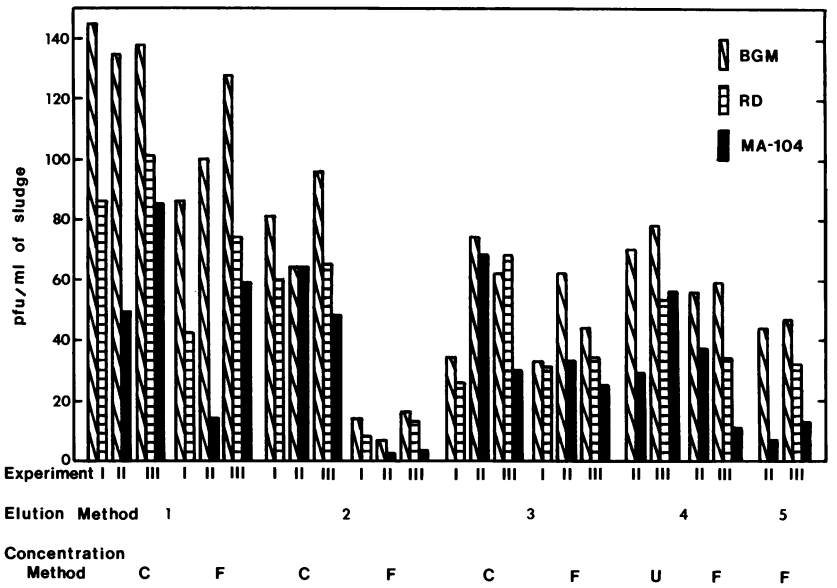

FIG. 1. Recovery of indigenous viruses from raw sludge. Viruses were eluted from raw sludge by one of five methods: (1) blending in Freon, (2) blending with distilled water, (3) blending with beef extract, (4) stirring with beef extract, or (5) blending and sonication with beef extract. Eluted viruses were then either measured directly (method 4, unconcentrated [U]) or concentrated by high-speed centrifugation (C) or organic flocculation (F). Before being assayed for recoverable PFU on BGM, RD, or MA-104 cells, all samples were disinfected by either ether treatment (methods 1 to 3), filtration (method 4), or dithizone plus chloroform treatment (method 5). Virus recoveries were all determined on the basis of the number of PFU per milliliter of sludge. The data reported here were obtained after viral extractions on three separate dates (I, II, and III).

$Na_2HPO_4 \cdot 7H_2O$ and 0.12 g of citric acid in 100 ml of 10% beef extract) and stirred (30 min) with a magnetic stirrer. After another centrifugation (10,000 × *g*, 30 min), the supernatant was passed through a sandwich of membrane filters (47-mm diameter; Millipore Corp.) of decreasing porosity as follows: AP-20 prefilter, 5.0, 1.2, 0.8, 0.65, and 0.45 μm. The filtrate was split, and one part was stored at −80°C. The other half was diluted to 3% beef extract with distilled water, concentrated by organic flocculation, and stored at −80°C.

(v) Method 5 (ASTM-2). This procedure, described by Glass et al. (5), is also being tested as a tentative ASTM standard method. All steps except sonication were performed with the samples at room temperature. Sludge (200 ml) was blended (2 min, high speed) with 4.8 g of dehydrated beef extract and transferred to a beaker containing 0.1 ml of antifoam 10 (General Electric Co.). After being stirred until the foam had subsided (approximately 30 min), the sample was transferred to centrifuge bottles, shaken briefly, and sonicated (100 W, 2 min) in an ice bath. It was then centrifuged (10,000 × *g*, 30 min), and the supernatant was concentrated by organic flocculation and detoxified. For this, the virus sample in 2.5 ml of 0.15 M Na_2HPO_4 was mixed for 1 min with chloroform (4 ml) containing 10 μg of dithizone (diphenylthiocarbazone) per ml. After centrifugation (10,000 × *g*, 30 min), the upper aqueous phase was mixed with 1 drop of 0.1% $CaCl_2$. Air was passed through the solution for 10 min, followed by the addition of 0.25 ml of 15× phosphate-buffered saline plus sufficient antibiotics to make the final concentration 200 U of penicillin and 200 μg of streptomycin per ml. The sample was then stored at −80°C.

Determination of viral recoveries. Recovery of indigenous viruses from sludge was measured by the plaque assay on BGM, RD, and MA-104 cells. Stored samples were thawed and sonicated (100 W, 15 s) just before analysis. Appropriate dilutions of viral samples were made into nutrient broth, and 0.2-ml volumes were layered onto cells in 25-cm^2 plastic tissue culture flasks (five replicates per dilution). After an adsorption period (2 h), each flask was overlaid with Eagle medium containing 1% agar, 2% fetal calf serum, 1% nonfat milk, and 0.0015% neutral red. Flasks were incubated at 37°C and observed daily during a 2-week period for plaque formation. Confirmation of plaques in the initial stages of this study was shown to be nearly 100%. For this reason and because of the very large number of plaques that it would have been necessary to test, viral confirmation was not routinely performed.

RESULTS

Five general methods for recovering indigenous viruses from sludge are compared in this study. Each is described above. The procedures for methods 1, 2, and 3 were repeated on three separate dates spaced over a period of several months, and the procedures for methods 4 and 5 were performed in parallel on the last two of these dates. A summary of the results is shown in Fig. 1.

TABLE 1. Relative recoveries of indigenous viruses from raw sludge by different extraction methods

Elution/concentration method	Relative recovery[a] on cell line:			
	BGM	RD	MA-104	Avg
1. Freon/centrifugation	1.00	1.00	1.00	1.00
Freon/flocculation	0.73	0.61	0.49	0.61
2. Water/centrifugation	0.58	0.67	0.94	0.73
Water/flocculation	0.09	0.11	0.05	0.08
3. Beef extract/centrifugation	0.41	0.48	0.87	0.59
Beef extract/flocculation	0.34	0.35	0.48	0.39
4. $AlCl_3$ and beef extract/unconcentrated	0.55	0.52	0.63	0.57
$AlCl_3$ and beef extract/flocculation	0.42	0.34	0.44	0.40
5. Beef extract, blending, and sonication/flocculation	0.34	0.32	0.15	0.27

[a] Values are the average viral recoveries for each method as calculated from the results presented in Fig. 1 and are determined relative to recoveries obtained by the Freon/centrifugation technique.

It is immediately apparent that the Freon extraction technique (method 1) was the most efficient method of recovering viruses in this study. It also appears that organic flocculation is a less effective method of concentrating viruses than is high-speed centrifugation. This is especially evident after distilled water elution (method 2). It is interesting to note that the procedures chosen to be tested as tentative standard methods (methods 4 and 5) were no more effective than the other elution-concentration techniques and were much less effective than the Freon method. These conclusions can be more easily visualized when average viral recoveries, as determined on each cell line, are presented in tabular form (Table 1).

It also appears from the data of Fig. 1 that the BGM cell line has the greatest plaquing efficiency of the three lines studied. Again, this result is more evident when the average relative recoveries on each cell line are presented as a table (Table 2).

DISCUSSION

Numerous methods for recovering viruses from sludge have been reported, but few comparisons of these methods have been made. Five general methods are compared in this study. The first three (methods 1 to 3) are modified versions of published techniques (3, 18, 20). The other two (methods 4 and 5) are published procedures (3, 5) that are being tested as tentative ASTM standard methods.

The distinguishing feature of the first three methods is the eluent. Each was selected because it had been used by other investigators with apparent success. To promote maximal

TABLE 2. Relative plaquing efficiencies of extracted viruses on different cell lines

Elution/concentration method	Relative plaquing efficiency[a] on cell line:		
	BGM	RD	MA-104
1. Freon/centrifugation	1.00	0.66	0.49
Freon/flocculation	1.00	0.53	0.30
2. Water/centrifugation	1.00	0.71	0.75
Water/flocculation	1.00	0.69	0.28
3. Beef extract/centrifugation	1.00	0.93	0.70
Beef extract/flocculation	1.00	0.85	0.55
4. $AlCl_3$ and beef extract/unconcentrated	1.00	0.68	0.57
$AlCl_3$ and beef extract/flocculation	1.00	0.57	0.43
5. Beef extract, blending, and sonication/flocculation	1.00	0.68	0.22

[a] Values are the average viral recoveries on each cell line (calculated from the results in Fig. 1) and are determined for each method relative to recoveries obtained on BGM cells. The average relative recoveries on cell lines BGM, RD, and MA-104 were 1.00, 0.70, and 0.48, respectively.

dissociation of viruses from particulates, sludge was blended with each eluent.

Two separate techniques for concentrating viruses were also incorporated into these first three methods. Although others could have been used, high-speed centrifugation and organic flocculation with 3% beef extract were selected because of their efficiencies as proven by many investigators.

The three cell lines used to recover viruses (BGM, RD, and MA-104) were chosen because they can be easily grown and maintained in tissue culture and they remain viable for extended periods under agar and permit replication of enteric viruses. However, they have been reported to have different plaquing efficiencies with different virus types (B. E. Moore, C. A. Turk, C. Villareal, and C. A. Sorber, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, Q40, p. 207; S. E. Oglesbee, D. A. Wait, and A. F. Meinhold, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, Q113, p. 219). If different virus types are also extracted with dissimilar efficiencies, relative viral recoveries on these three cell lines may vary between extraction methods. This, in fact, was observed (see Fig. 1 and Table 1).

The results show that the two-phase extraction technique with Freon followed by virus concentration through high-speed centrifugation was the most effective method studied. However, viral recovery by this method decreased an average of only 39% on the three cell lines when organic flocculation with beef extract was substituted as the method of concentration. This is important because most laboratories have access to a low-speed centrifuge needed for organic flocculation, whereas many do not have an ultracentrifuge required for high-speed centrifugation.

The Freon extraction technique used here not only effectively separates viruses from sludge particulates but also has the added advantage of removing dissolved materials that cause cell toxicity. Others have reported using this compound on environmentally derived viral samples for this purpose alone (L. M. Stark, F. M. Wellings, and A. L. Lewis, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, Q51, p. 209; T. W. Hejkal and V. C. Rao, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, Q46, p. 208). In fact, the detoxification step added to the published method (3) being tested as a tentative ASTM standard method involves the use of Freon. In this study and others conducted in our laboratory, no cytotoxicity was observed in any sample treated with Freon, even when these samples were tested undiluted. This was often not true with the other methods.

The BGM cells were consistently found to allow the greatest viral recoveries in these experiments. However, it should be noted that only one sample of raw sludge was used throughout this study. Other sludges will have a different assortment of enteric viruses. Therefore, it is unclear whether BGM cells will detect greater viral numbers than RD and MA-104 cells in all sludge samples. It also has not been determined whether the Freon extraction technique used here will allow efficient viral recoveries from other sludges and other types of environmental samples.

LITERATURE CITED

1. **Abid, S. H., C. Lue-Hing, and S. Sedita.** 1978. Development of a method for concentrating enteroviruses in anaerobically digested sludge. The Metropolitan Sanitary District of Greater Chicago report no. 78-13. The Metropolitan Sanitary District of Greater Chicago, Chicago, Ill.
2. **Berg, G., and D. R. Dahling.** 1980. Method for recovering viruses from river water solids. Appl. Environ. Microbiol. **39**:850–853.
3. **Berman, D., G. Berg, and R. S. Safferman.** 1981. A method for recovering viruses from sludges. J. Virol. Methods **3**:283–291.
4. **Farrah, S. R., P. R. Scheuerman, and G. Bitton.** 1981. Urea-lysine method for recovery of enteroviruses from sludge. Appl. Environ. Microbiol. **41**:455–458.
5. **Glass, J. S., R. J. van Sluis, and W. A. Yanko.** 1978. Practical method for detecting poliovirus in anaerobic digester sludge. Appl. Environ. Microbiol. **35**:983–985.
6. **Hurst, C. J., S. R. Farrah, C. P. Gerba, and J. L. Melnick.** 1978. Development of quantitative methods for the detection of enteroviruses in sewage sludges during activation and following land disposal. Appl. Environ. Microbiol. **36**:81–89.
7. **Katzenelson, E., B. Fattal, and T. Hostovesky.** 1976. Organic flocculation: an efficient second-step concentration method for the detection of viruses in tap water. Appl. Environ. Microbiol. **32**:638–639.
8. **Lund, E., and C. E. Hedstrom.** 1966. The use of an aqueous polymer phase system for enterovirus isolations from sewage. Am. J. Epidemiol. **84**:287–291.
9. **Lund, E., and V. Ronne.** 1973. On the isolation of virus from sewage treatment plant sludges. Water Res. **7**:863–871.
10. **Moore, B. E., B. P. Sagik, and C. A. Sorber.** 1978. Land application of sludges: minimizing the impact of viruses on water resources, p. 154–167. *In* B. P. Sagik and C. A. Sorber (ed.), Proceedings of the conference on risk assessment and health effects of land application of municipal wastewater and sludges. University of Texas, San Antonio.
11. **Nath, M. W., and J. C. Johnson.** 1980. Enumeration and inactivation of enteric viruses in sludge. Virginia Water Resources Research Center bulletin no. 131. Virginia Water Resources Research Center, Blacksburg, Va.
12. **Nielsen, A. L., and B. Lydholm.** 1980. Methods for the isolation of virus from raw and digested wastewater sludge. Water Res. **14**:175–178.
13. **Pancorbo, O. C., P. R. Scheuerman, S. R. Farrah, and G. Bitton.** 1981. Effect of sludge type on poliovirus association with and recovery from sludge solids. Can. J. Microbiol. **27**:279–287.
14. **Philipson, L.** 1967. Water-organic solvent phase systems. Methods Virol. **2**:236–243.
15. **Sattar, S. A., and J. C. N. Westwood.** 1976. Comparison of four eluents in the recovery of indigenous viruses from raw sludge. Can. J. Microbiol. **22**:1586–1589.
16. **Sattar, S. A., and J. C. N. Westwood.** 1979. Recovery of viruses from field samples of raw, digested, and lagoon-dried sludges. Bull. W.H.O. **57**:105–108.

17. **Subrahmanyan, T. P.** 1977. Persistence of enteroviruses in sewage sludge. Bull. W.H.O. **55**:431–434.
18. **Turk, C. A., B. E. Moore, B. P. Sagik, and C. A. Sorber.** 1980. Recovery of indigenous viruses from wastewater sludges, using a bentonite concentration procedure. Appl. Environ. Microbiol. **40**:423–425.
19. **Ward, R. L., and C. S. Ashley.** 1976. Inactivation of poliovirus in digested sludge. Appl. Environ. Microbiol. **31**:921–930.
20. **Wellings, F. M., A. L. Lewis, and C. W. Mountain.** 1976. Demonstration of solids-associated virus in wastewater and sludge. Appl. Environ. Microbiol. **31**:354–358.