

Enhanced virus recovery from municipal sewage sludge with a combination of enzyme and cation exchange resin

D. Sano*, K. Fukushi*, K. Yano**, Y. Yoshida** and T. Omura*

*Department of Civil Engineering, Graduate School of Engineering, Tohoku University, Aoba-yama 06, Sendai 980-8579, Japan

**Metropolitan Research Laboratory of Public Health, 3-24-1, Hyakunin-cho, Shinjuku-ku, Tokyo 169-0073, Japan

Abstract There is a great difficulty in virus enumeration in sewage sludge because viruses in sludge are firmly captured by sludge solids. In order to determine the precise number of viruses in sludge, an enhanced virus recovery method with a combination of an enzyme and a cation exchange resin (CER) was developed. Test viruses were seeded to a sample sludge obtained from a municipal wastewater treatment plant, and the sludge were incubated with various eluents. The quantity of eluted viruses in the liquid phase was then measured by the plaque assay technique. Using the eluent containing only water, CER, and CER with enzyme exhibited 0%, 19% and 39% of virus recovery, respectively. While the conventional USEPA method exhibited a virus recovery of 21%. Furthermore, viruses eluted by the eluent containing the CER and the lysozyme included not only surface-attached viruses but also solids-embedded viruses.

Keywords Elution; cation exchange resin; enzyme; lysozyme; municipal sewage sludge; pathogenic enteric virus

Introduction

Compared with any other natural environments, pathogenic enteric viruses were detected with high frequencies from municipal sewage sludge (Yano *et al.*, 1996). Pathogenic enteric viruses include poliovirus, coxsackie virus, echovirus and enterovirus. The reasons why viruses are often detected from sewage sludge are that a large amount (10^8 to 10^{10} particles per wet gram of feces) of excreted viruses from infected individuals flow into sewage streams (Abbaszadegan *et al.*, 1999; Yano *et al.*, 1985), and that viruses tend to combine with sludge solids in the wastewater treatment process (Lund and Rønne, 1973; Hurst *et al.*, 1978). Therefore, sewage sludge should be considered as an origin source of contaminations with pathogenic viruses in natural environments. In other words, sewage sludge has to be treated with an appropriate disinfection process. However, most sewage sludges are introduced to landfills for the final disposal without receiving any disinfection processes except for incineration. There is a public health risk of virus transmission via fecal-oral route through a contaminated water by the leachate from landfills.

In order to manage the risk of virus transmission, it is necessary to determine an accurate number of viruses in sewage sludge. However, it is highly difficult to enumerate viruses in sewage sludge because of a preferential adsorption of viruses to solids (Hurst *et al.*, 1978). Since there have been great difficulties in the enumeration of viruses adsorbed to solids, a lot of investigators have studied the procedure of virus elution from the solids phase to the liquid phase (Hurst *et al.*, 1978; Pancorbo *et al.*, 1981; Sattar and Westwood, 1976). According to their studies, a 10% beef extract solution was adopted by the United States Environmental Protection Agency (USEPA) for virus elution from sewage sludge (USEPA, 1992). Benefits associated with the employment of the beef extract solution are the achievement of the virus elution at moderate pH and the opportunity for subsequent virus concentration using a simple low-pH precipitation procedure (Hurst *et al.*, 1984).

However, it is difficult to determine the precise number of viruses in sewage sludge with the USEPA method, since there are appreciable losses of viruses in both the virus elution (Sano *et al.*, 1998) and the virus concentration procedure. Therefore, an enhanced method of virus elution is necessary.

In order to enhance virus elution efficiency from sewage sludge, degradation of sludge flocs may be effective. It is expected that a lot of viruses in sewage sludge are captured by sludge polymers such as proteins, polysaccharides, lipids and nucleic acids with several attractive forces (e.g., ionic bonds, hydrophobic effects and bridging effects). Therefore, the elution of viruses bound to sludge polymers in the solids phase of sewage sludge may be achieved by a breakdown of sludge polymers and a subsequent release of viruses to the liquid phase of sewage sludge along with the degraded polymer.

Since polysaccharides in sewage sludge are likely to be the backbones of sludge flocs (Jorand *et al.*, 1995), it is considered that a breakdown of polysaccharides is effective to disintegrate sludge flocs. In particular, a peptidoglycan originates from cell walls of bacteria that exist in sewage sludge at a concentration of about 10^{12} cells l^{-1} (Urbain *et al.*, 1993) and may be one of the main components of sludge flocs. Then, a degradation of the peptidoglycan may promote the disintegration of sludge flocs. Furthermore, since the peptidoglycan is not a component of a virus particle, the degradation of the peptidoglycan does not lead to a decomposition of virus particles, that is, a virus inactivation. The plaque assay technique is available for the enumeration of viruses in the liquid phase of sewage sludge. Furthermore, the decomposition of the peptidoglycan is achieved by means of a lysozyme without the virus inactivation.

Since divalent cations such as a calcium ion in sewage sludge have a role in binding sludge floc components to each other (Eriksson and Alm, 1991), the disintegration of sludge flocs is enhanced by removing divalent cations from sludge flocs. According to Keiding and Nielsen (1997), even a slight decrease in the content of calcium ions in the sludge floc with a cation exchange resin (CER) promotes desorptions of organic compounds of the sludge floc to the liquid phase. It is considered that removing calcium ions from the liquid phase of sewage sludge by the CER leads to a diffusion of calcium ions from the solid phase to the liquid phase owing to a gradient of concentration of calcium ions between the two phases. Therefore, for the purpose of removing divalent cations from the liquid phase of sewage sludge, the CER is available because it removes cations from the sludge matrix, leading to breakdown of the flocs by mixing the CER with sewage sludge (Frølund *et al.*, 1996).

The final goal of this work is to develop an enhanced method for virus enumeration in sewage sludge. In this paper, a combined effect of the CER and the lysozyme on the enhanced virus elution from sewage sludge was investigated.

Materials and methods

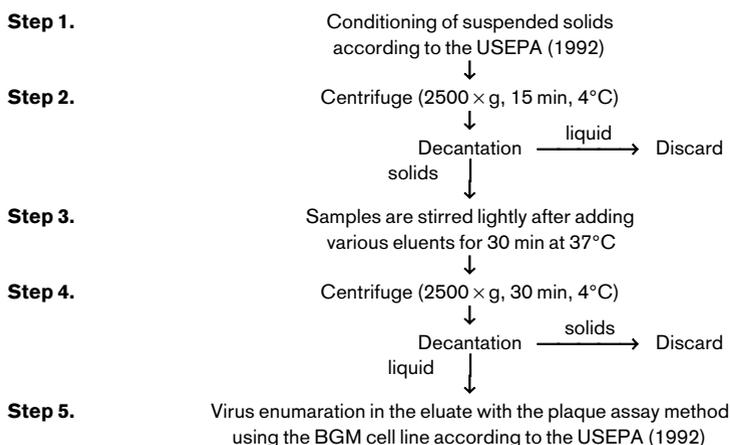
Test viruses (poliovirus 1) were seeded to a sample sludge obtained from a municipal wastewater treatment plant. Seeded sludges were incubated with various eluents. The quantity of eluted viruses to the liquid phase was measured. The ratio of the amount of eluted viruses in the liquid phase to the seeded viruses in the sludge was determined and evaluated for nine different eluents. After virus elution with water and eluents containing the CER or phosphate-buffered saline, concentrations of soluble total organic carbon (soluble TOC) and metals (magnesium, potassium and calcium) in the liquid phase of the sludge were measured, and the effect of the CER on the disintegration of sludge flocs was evaluated.

Table 1 Eluents used for virus elution from sewage sludge

Virus elution runs	Eluents used for the virus elution from sludge
E1 (control)	Water*
E2	Phosphate-buffered saline (PBS)
E3	Water with cation exchange resin (CER)
E4	Maintenance medium (MM)
E5	Water with lysozyme
E6	MM with CER
E7	MM with lysozyme
E8	MM with lysozyme and CER
E9 (reference)	10% beef extract solution**

*Ultra pure water

**recommended eluent by USEPA (1992)

**Figure 1** Summary of the procedure used for the virus elution from sewage sludge**Eluents used for virus elution from sewage sludge**

The CER used for the virus elution from sewage sludge was the Amberlite weakly acidic cation exchanger (IRP-88, SIGMA, St. Louis, U.S.A.). The CER exchanges potassium ions for cations that have higher electronegativities than the potassium ion such as magnesium ion and calcium ion. The lysozyme (SIGMA, St. Louis, U.S.A.) used for the virus elution from sludge was isolated from chicken egg white. Table 1 shows the eluents used for the virus elution from sludge. Since more than 10 meq l⁻¹ of the CER often affected the viability of a cell culture that was used in the virus assay, concentration of the CER in eluent was set up at 10 meq l⁻¹. The activity of lysozyme in eluent was 4.7 × 10⁸ units l⁻¹ and the activity was sufficient to degrade the total amount of the peptidoglycan in sewage sludge. The concentrations of sodium chloride, potassium chloride, disodium phosphate (anhydrous) and monopotassium phosphate (anhydrous) in Eluent 2 (phosphate-buffered saline: PBS) were 8,000 mg l⁻¹, 200 mg l⁻¹, 1,150 mg l⁻¹ and 200 mg l⁻¹, respectively. Elution 4 (maintenance medium: MM) contained 0.94% of minimum essential medium (Nissui pharmaceutical corporation limited, Tokyo, Japan), 1% of fetal bovine serum (JRH Bioscience, Lenexa, U.S.A.), 1% of L-glutamine solution (GIBCO BRL, N.Y., U.S.A.), 1% of antibiotic-antimycotic solution (GIBCO BRL, N.Y., U.S.A.) and 1.5% of NaHCO₃ solution (GIBCO BRL, N.Y., U.S.A.).

Sample sludge and test virus

Sample sludge was collected from the secondary clarifier in a municipal WWTP in Sendai, Japan. Averages of % total solids and % volatile solids of the sample sludge were 0.63 (standard deviation: SD = 0.14), 79 (SD = 4), respectively. Virus strain used in this paper was oral polio vaccine (poliovirus 1).

Procedure of virus elution from sewage sludge

The procedure for the virus elution from sewage sludge is summarized and illustrated in Figure 1. Viruses were seeded to the sample sludge at concentrations of approximately 10^2 PFU ml⁻¹ in Step 1. Preliminary experiments in our research group confirmed that there were insignificant concentrations of indigenous viruses in the sample sludge compared with the viruses seeded to the sludge. Step 3 was conducted at a temperature value of $37 \pm 1^\circ\text{C}$. Other steps were carried out at $23 \pm 1^\circ\text{C}$.

Virus assay

Virus infectious units were measured by means of the plaque assay method with the Buffalo green monkey (BGM) cell line according to the USEPA (1992). Prior to the assay, samples that were obtained at Step 5 in the virus elution procedure (Figure 1), were filtered with the 0.22- μm pore size membrane filter (Millipore, Bedford, U.K.) and the antibiotic-antimycotic solution added was at a concentration of approximately 1% in order to remove bacteria from samples. One millilitre of samples was then inoculated into each BGM cell line that grew on the flat bottom of 6-well multidishes (Nalge Nunc, 152795, Roskilde, Denmark). After the inoculation, samples in the 6-well dishes were cultivated in a CO₂ incubator at a CO₂ concentration of 5% for 90 min at $37 \pm 1^\circ\text{C}$. The samples were then discarded and 4 ml of warm (about 38°C) overlay agar solution was added to each well. The overlay agar solution contained 0.94% of minimum essential medium, 1.25% of agar (Nacalai tesque, Kyoto, Japan), 1% of fetal bovine serum, 1% of L-glutamine solution, 1% of antibiotic-antimycotic solution and 1.5% of NaHCO₃ solution. After the addition of the overlay agar solution, the BGM cells were cultivated in the CO₂ incubator again. After 24 hours, in order to stain the BGM cells, 1 ml of neutral red solution of 0.015% (GIBCO BRL, N.Y., U.S.A.) was added to each well. After addition of the neutral red solution, the BGM cells were cultivated in the CO₂ incubator for more than 12 hours. The neutral red solution was then discarded and plaques that were formed on the BGM cells were counted. The plaques were counted until no new plaques appeared (about 2 days after discarding the neutral red solution).

Virus recovery efficiency

Virus recovery efficiencies from sewage sludge were determined with the following equation (1).

$$\begin{aligned} & \text{virus recovery efficiencies (\%)} \\ &= \frac{\text{PFU of the supernatant (100ml) after virus elution (PFU)}}{\text{initial PFU of sludge (100ml) after virus inoculation (PFU)}} \times 100 \end{aligned} \quad (1)$$

Total organic carbon and metal analysis

Samples for the measurement of concentrations of the soluble TOC and metals (magnesium, potassium and calcium) were obtained after Step 4 in identical procedure for virus elution from sewage sludge (Figure 1) except for the virus inoculation.

TOC analysis. Concentrations of the soluble TOC in samples were measured with a total organic carbon analyzer (TOC-5000, Shimadzu, Kyoto, Japan).

Metal analysis. Concentrations of metals (magnesium, potassium and calcium) in samples were measured with an inductively coupled plasma-mass spectrum analyzer (ICP-MS) (4500 series, Hewlett Packard, CA., U.S.A.). Prior to measurements of metal concentrations, samples were digested with nitric acid in order to decompose proteins in samples with a microwave oven (O-I-Analytical, Texas, U.S.A.) according to the Standard Methods (APHA, 1995).

Results and discussion

Sludge flocs disintegration with the cation exchange resin

Figure 2 shows fractions of the soluble TOC released to supernatants of sewage sludge with various eluents. Figure 3 shows fractions of metals (magnesium, potassium and calcium) in supernatants. The fractions of the soluble TOC (Figure 2) are equivalent to percentages of a mass of the soluble TOC in the supernatant to a mass of volatile solids (VS) of the sample sludge. The soluble TOC is the concentration of organic carbon compounds in the liquid phase of the sewage sludge. When organic carbon compounds, such as proteins and polysaccharides, are vigorously released to the liquid phase of the sewage sludge, the fraction of the soluble TOC becomes large. Therefore, the fraction of the soluble TOC represents the degree of the disintegration of the sludge floc matrix. The fractions of metals (Figure 3) are equal to percentages of a mass of each metal in supernatants relative to a total mass of each metal in sludge.

The fraction of the soluble TOC at E 3 (water with CER) was about 3.4 times the fraction of the soluble TOC at E 1 (Water) (Figure 2). Furthermore, the fraction of the soluble TOC at E 3 was about 3.4 times the fraction of the soluble TOC in the supernatant of the sample sludge (Figure 2). These results indicate that release of organic carbon compounds from the solid phase to the liquid phase was promoted at E 3. On the other hand, fractions of the magnesium and the calcium at E 3 were about one twelfth and one eighth of those at E 1, respectively. This result indicates that the CER at E 3 removed divalent cations (magnesium ion and calcium ion) from the liquid phase. According to Keiding and Nielsen (1997), organic

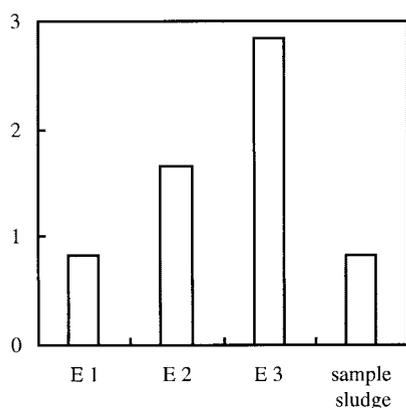


Figure 2 Comparison of fractions of the soluble TOC in supernatants after the virus elution by various eluents and in the supernatant of the sample sludge. Fractions are equivalent to percentages of the mass of the soluble TOC in the supernatant to mg VS of the sample sludge

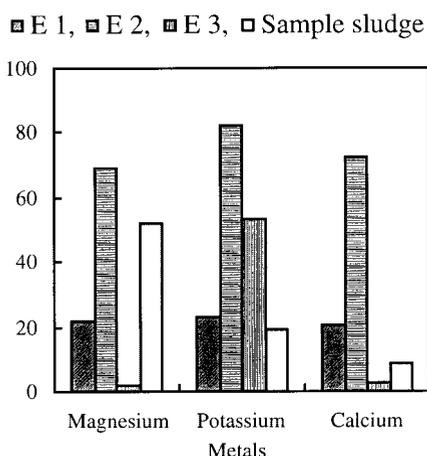


Figure 3 Comparison of fractions of metals in supernatants. Fractions were equal to percentages of a mass of each metal in supernatants to total mass of each metal in sludge

compounds weakly attached to surfaces of sludge flocs were dispersed by the CER, since the CER captured calcium ions which bound organic compounds to sludge flocs. In our experiment, significant low concentrations of magnesium and calcium ions in the liquid phase were observed (Figure 3). It is expected that some divalent cations were diffused to the liquid phase and captured by the CER. As a result, organic compounds attached to surfaces of sludge flocs were dispersed to the liquid phase (Figure 2).

In the case at E 2 (PBS), the fraction of the soluble TOC was about two times the control (E 1). Therefore, dispersions of organic carbon compounds from the solid phase occurred at E 2, which has a high ionic strength owing to 0.14 M of sodium chloride. This result agrees with results of Bruus *et al.* (1992). They reported that the addition of cation (sodium, potassium or magnesium ions) to thickened sludge resulted in a dispersion of calcium ions to the liquid phase from the solid phase and a subsequent release of organic compounds to the liquid phase. The amount of released organic compounds was correlated with the amount of added cations. They suggested that the release of organic compounds was achieved owing to exchanges of divalent cations in sludge flocs for excess amount of monovalent cations, which have poor bridging strength compared with divalent cations. In our experiment, high amounts of magnesium and calcium were obtained at E 2 compared with E 1 and E 3 (Figure 3). Therefore, it is considered that the disintegration of sludge flocs at E 2 occurred because of partial replacements of divalent cations in sludge flocs with excess amount of monovalent cations in PBS. However, since the fraction of the soluble TOC at E 3 (water with CER) was about 1.7 times the fraction of the soluble TOC at E 2 (PBS) (Figure 2), sludge-disintegration ability of CER at E 3 was superior to that of PBS at E 2. That is, for the disintegration of sludge, removing divalent cations from the liquid phase by the CER was more effective than the replacement of divalent cations in sludge flocs with excess amount of monovalent cations in PBS.

Virus recovery efficiencies with various eluents from sludge

Table 2 shows virus recovery efficiencies from sewage sludge with various eluents and averages of pH values in sludge-eluent mixtures during the virus elution. Virus recovery efficiency at E 3 (water with CER) was 19%. It is expected that eluted viruses at E 3 were released to the liquid phase along with the dispersion of organic carbon compounds by the CER at E 3 (Figure 2). Therefore, it is considered that most of the eluted viruses at E 3 were surface-attached viruses and most of the solids-embedded viruses remained in sludge flocs,

Table 2 Virus recovery efficiencies from sewage sludge and averages of pH values in sludge-eluent mixtures during virus elution from sewage sludge

Virus elution runs	Eluents used for the virus elution from sludge	Virus recovery of efficiency (%)	Number of replicates	Averages of pH values*
E1 (control)	Water	N.D.**	4	4.06
E2	Phosphate-buffered saline (PBS)	10	8	6.62
E3	Water with cation exchange resin (CER)	19	8	6.87
E4	Maintenance medium (MM)	11	6	6.56
E5	Water with lysozyme	N.D.**	4	4.06
E6	MM with CER	10	7	N.T.†
E7	MM with lysozyme	19	4	N.T.†
E8	MM with lysozyme and CER	39	8	N.T.†
E9 (reference)	10% beef extract solution	21	14	6.79

*Averages of pH values in sludge-eluent mixtures with an eluent during the virus elution

**No virus was detected in the supernatant

†Not tested

because released organic carbon compounds were considered as surface-attached. Since there was little difference between virus recovery efficiencies at E 3 and E 9 (10% beef extract solution), it is expected that most of the viruses eluted at E 9 had attached to sludge flocs weakly. Furthermore, in practical wastewater treatment processes, it is inferred that at least about 20% of viruses captured by sludge flocs are surface-attached viruses.

In the case of the virus recovery at E 1 (water), no viruses were detected from the supernatant. The reason for no virus detection at E 1 was the low pH value (4.06) in the sludge-eluent mixture at E 1. At low pH, most carboxyl groups on surfaces of sludge solids are occupied by hydrogen ions and the electrostatic repulsive force between solids decreases. No viruses were recovered at E 5 (water with lysozyme) either. The reason for no virus detection is that the pH value of the sludge-eluent mixture at E 5 (4.06) is out of the optimum pH range of the lysozyme (pH value of 6 to 8). Virus recovery efficiencies at E 2 (PBS) and E 4 (MM) were 10% and 11%, respectively (Table 2). The pH values of sludge mixtures at E 2 or E 4 were stable at pH values of about 6.6 (Table 2). Commonly, at pH values of around 7.0, hydrogen ions are likely to be dissociated from carboxyl groups on the surfaces of sludge solids, and sites of negative charge on surfaces of sludge solids increase. As a result, electrostatic repulsive force increases and then the release of viruses to the liquid phase is promoted. Therefore, one reason for the virus elution at E 2 and E 4 was a neutral pH value. Hurst *et al.* (1984) suggested that a moderate pH is required for the virus elution from sewage sludge.

The virus recovery efficiency at E 2 (PBS) was 10%. This value was a half of the virus recovery efficiency at E 3 (water with CER). Although the pH values of sludge-eluent mixtures at E 2 and E 3 were 6.62 and 6.87, respectively (Table 2), the fraction of the soluble TOC at E 3 was about 1.7 times that at E 2. Since the release of organic carbon compounds was more vigorously promoted by the CER at E 3 than at E 2, the virus recovery efficiency at E 3 was about two times that at E 2.

Virus recovery efficiencies at E 6 (MM with CER) and E 7 (MM with lysozyme) were 10%, and 19%, respectively (Table 2). Since the virus recovery efficiency at E 3 (water with CER) was 19%, it is considered that most of viruses eluted at E 6 or E 7 were also virus surface attached to sludge flocs. However, virus recovery efficiency at E 8 (MM with lysozyme and CER) was 39%, which was about four times that at E 6 and about two times that at E 7. This result indicates that the disintegration of sludge flocs by the combined effect of the CER and the lysozyme promoted virus elution from sewage sludge. In the case at E 7 (MM with lysozyme), eluted viruses may reattach to degraded sludge floc components. Then, the readsorption of viruses may affect the infectious abilities of viruses and virus detection with the plaque assay using the BGM cells. However, in the case at E 8 (MM with lysozyme and CER), the readsorption between degraded floc components and viruses may be discouraged owing to removing divalent cations from the liquid phase by the CER. Since the virus recovery efficiency at E 9 (10% beef extract solution) was 21%, about two times the viruses could be recovered from the sludge at E 8 (MM with lysozyme and CER) compared with the USEPA method. Then, it is considered that recovered viruses at E 8 included not only surface-attached viruses but also some solids-embedded viruses. Furthermore, The elution of solids-embedded viruses was accomplished with the disintegration of sludge flocs by the combined effect of the CER and the lysozyme.

Conclusions

A novel virus elution method was proposed and experimentally tested. Most viruses were not recovered by water. Using water with CER, 19% of seeded viruses were recovered from sewage sludge. In addition to the CER, lysozyme exhibited a drastic improvement of the virus recovery efficiency of 39%. This value of the virus recovery efficiency was almost

two times more effective than the conventional USEPA method. By investigating metal concentrations in the liquid phase of sludge-eluent mixtures, it is expected that surface-attached viruses can be released from sludge solids by the CER. The lysozyme with the CER eluted not only surface-adsorbed viruses but also some solids-embedded viruses. The development of this novel method enables us to determine the real number of pathogenic viruses in sewage sludge and to evaluate true infectious risks to humans caused by such viruses.

Acknowledgements

This article has been funded in part by the Japan Science & Technology Corporation under assistance program of Core Research for Evolutional Science & Technology (CREST).

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