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**Estimation of norovirus removal performance in a coagulation–rapid sand
filtration process by using recombinant norovirus VLPs**

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Abstract

Norovirus (NV) is an important human pathogen that causes epidemic acute nonbacterial gastroenteritis worldwide. Because of the lack of a cell culture system or an animal model for this virus, studies of drinking water treatment such as separation and disinfection processes are still hampered. We successfully estimated NV removal performance during a coagulation–rapid sand filtration process by using recombinant NV virus-like particles (rNV-VLPs) morphologically and antigenically similar to native NV. The behaviors of two widely accepted surrogates for pathogenic waterborne viruses, bacteriophages Q β and MS2, were also investigated for comparison with that of rNV-VLPs. Approximately 3- \log_{10} removals were observed for rNV-VLPs with a dose of 40 μ M-Al or -Fe, as polyaluminum chloride at pH 6.8 or ferric chloride at pH 5.8, respectively. Smaller removal ratios were obtained with alum and ferric chloride at pH 6.8. The removal performance for MS2 was somewhat larger than that for rNV-VLPs, meaning that MS2 is not recommended as an appropriate surrogate for native NV. By comparison, the removal performance for Q β was similar to, or smaller than, that for rNV-VLPs. However, the removal performances for rNV-VLPs and Q β differed between the coagulation process and the following rapid sand filtration process. Therefore, Q β also is not recommended as an appropriate surrogate for native NV.

Key words: Bacteriophages, Coagulation, ELISA, Norovirus, Rapid sand filtration,
Virus-like particles

1. Introduction

Norovirus (NV), which has been previously termed Norwalk-like virus or small round structured virus, is an important human pathogen that causes epidemic acute nonbacterial gastroenteritis worldwide. This virus belongs to the genus *Norovirus* in family Caliciviridae (Zheng *et al.*, 2006). On the basis of the molecular characterization of complete gene sequences, the *Norovirus* genus has been classified into seven distinct genogroups (GI to GVII) (Phan *et al.*, 2007). Among the seven genogroups, the GI, GII, GIV, GVI, and GVII strains are found in humans (Phan *et al.*, 2007).

Acute gastroenteritis is one of the leading causes of morbidity and mortality in children in the developing countries, and NV is known to be present in a large fraction of stool samples from diarrhea hospitalizations (Ramani and Kang, 2009). NV outbreaks are caused mainly by consumption of contaminated food (Daniels *et al.*, 2000; Fankhauser *et al.*, 2002), or they can be spread person-to-person (Fankhauser *et al.*, 2002). NV outbreaks due to contaminated drinking water have also been reported (Kukkula *et al.*, 1999; Nygård *et al.*, 2003; Maunula *et al.*, 2005). The presence of NV in drinking water sources is a public health concern owing to the potential for widespread NV outbreaks. However, because of the lack of a cell culture system or an animal model for NV (Parker *et al.*, 2005; Zheng *et al.*, 2006), studies of drinking water treatments such as

separation and disinfection processes are still hampered. Accordingly, the removal performance of NV in the coagulation–rapid sand filtration process, which is commonly used in drinking water treatment facilities, has not been investigated fully.

Feline calicivirus (FCV) has been widely used as an NV surrogate in studies of drinking water treatment processes to predict the treatability of NV (Thurston-Enriquez *et al.*, 2003, 2005; Duizer *et al.*, 2004; Abbaszadegan *et al.*, 2007; Mayer *et al.*, 2008), because FCV has a similar genome organization and capsid architecture to NV and it can be easily grown in cell cultures (Thurston-Enriquez *et al.*, 2003). However, FCV belongs to the genus *Vesivirus*, family *Caliciviridae*, and it causes respiratory illness (Hashimoto *et al.*, 1999); thus, FCV is unlike an enteric virus that is spread by the fecal–oral route. More recently, murine norovirus (MNV), genus *Norovirus*, family *Caliciviridae*, has been successfully propagated in cell culture (Karst *et al.*, 2003). MNV is an enteric virus, and some researchers have reported MNV to be more suitable as a surrogate for NV than FCV in the aquatic environment (Cannon *et al.*, 2006; Bae and Schwab, 2008). However, the suitability of MNV as a surrogate for NV in physical removal processes such as coagulation and filtration processes has not been investigated.

On the other hand, expression of the NV genome in a baculovirus expression system has

made possible the production of recombinant NV virus-like particles (rNV-VLPs) (Jiang *et al.*, 1992) that are morphologically and antigenically similar to native NV (Jiang *et al.*, 1992; Green *et al.*, 1993). Because the native NV source is the stools of human volunteers infected with NV, it is difficult to obtain a large amount of NV for spiking experiments of drinking water treatment processes, but the baculovirus expression system can produce enough rNV-VLPs to conduct spiking experiments. In addition, because rNV-VLPs lack RNA, which is necessary for infection and replication in host cells, they are harmless to humans during experiments, so they are easy to handle without any special facilities. In fact, rNV-VLPs have been used with the sandy aquifer treatment process to estimate the behavior of native NV (Redman *et al.*, 1997). On the other hand, the fate of infectivity of NV in the treatment process, especially in response to disinfection processes such as chlorination, ozonation, and UV radiation, cannot be studied by using rNV-VLPs because of their lack of RNA. Thus, we can only discuss the fate of NV as particles.

Here, we used rNV-VLPs to investigate the removal performance of NV as particles during laboratory-scale coagulation–rapid sand filtration. We also experimentally investigated the behaviors of the F-specific RNA bacteriophages Q β and MS2 for comparison with those of rNV-VLPs and to assess the suitability of these

bacteriophages as surrogates for NV. This study represents the first attempt to apply rNV-VLPs to estimate the removal of native NV in a drinking water treatment process.

2. Materials and methods

2.1. Source water, coagulants, and filter media

River water was sampled from the Toyohira River (Sapporo, Japan, water quality shown in Table 1) on 12 June 2008. The coagulants used for the coagulation process were two commercial aluminum coagulants, polyaluminum chloride (PACl) (PACl 250A; 10.5% Al_2O_3 , relative density 1.2 at 20 °C; Taki Chemical Co., Ltd., Hyogo, Japan) and alum (8.1% Al_2O_3 , relative density 1.3 at 20 °C; Taki Chemical Co., Ltd.), and one laboratory-made ferric chloride solution, which was prepared by dilution of reagent-grade iron (III) chloride (FeCl_3 , Wako Pure Chemical Industries, Ltd., Osaka, Japan) dissolved in Milli-Q water (Milli-Q Advantage, Millipore Corp., Billerica, MA, USA). Silica sand (effective size 0.6 mm, uniformity coefficient <1.3; Nihon Genryo Co., Ltd., Kanagawa, Japan) was used as the filter medium for the rapid sand filtration process.

2.2. rNV-VLPs

rNV-VLPs were produced by a baculovirus in silkworm, *Bombyx mori* (Katakura Industries Co. Ltd., Saitama, Japan). Subgenomic cDNA fragments of Chiba virus (AB042808, GI/4, Chiba407/1987/JP) genome were artificially synthesized and used for the expression of Chiba virus rNV-VLPs. Chiba virus, a Japanese strain in genogroup I of genus *Norovirus*, family *Caliciviridae*, was first identified as the cause of an oyster-associated outbreak of gastroenteritis that occurred in Chiba Prefecture, Japan, in 1987 (Kasuga *et al.*, 1990). Someya *et al.* (2000) have determined the complete nucleotide sequence of the Chiba virus genome. Chiba virus has a positive-sense, single-stranded RNA of 7697 bases composed of a 5' untranslated region (UTR), three open reading frames (ORFs), and a 3'UTR; the large 5'-terminal ORF (ORF1) encodes a polyprotein with 1785 amino acids that is likely processed into functional proteins. ORF2 encodes the capsid protein with 544 amino acids, and the small 3'-terminal ORF (ORF3) encodes a basic protein with 208 amino acids (Someya *et al.*, 2000). Because Bertolotti-Ciarlet *et al.* (2003) reported that expression levels of NV capsid protein are enhanced by the presence of ORF3 and 3'UTR in recombinant baculovirus, compared with expression in the absence of ORF3, 3'UTR, or both, we synthesized 2352 (positions 5346–7697) bases of Chiba virus genome containing ORF2, ORF3, and 3'UTR with *attB1*, *EcoRI*, *attB2*, and *PstI* restriction sites (total 2422 bases). The

synthesized cDNA fragment was inserted into the vector (pDONR221, Invitrogen Japan K. K., Tokyo, Japan) by the Gateway BP reaction. After *Eco*RI and *Pst*I digestion of the plasmid, the digested cDNA fragment was ligated into the baculovirus transfer vector (pM0NHT04, Katakura Industries Co. Ltd.). The transfer vector was co-transfected with the linearized genomic DNA of baculovirus (*Bombyx mori* nucleopolyhedrovirus; CPd strain, Suzuki *et al.*, 1997) into the *B. mori*-derived cell line (BmN, Maeda, 1989) to generate the recombinant baculovirus, and then the recombinant baculovirus was injected into silkworm pupae to express the rNV-VLPs. Six days after inoculation, the expressed rNV-VLPs were separated from the pupal homogenate by centrifugation and dialysis to prepare the rNV-VLP stock solution.

2.3. Bacteriophages

F-specific RNA bacteriophages Q β (NBRC 20012) and MS2 (NBRC 102619) were obtained from the NITE Biological Research Center (NBRC, Chiba, Japan). The bacteriophages Q β (Dowd *et al.*, 1998; Matsui *et al.*, 2003; Matsushita *et al.*, 2004; Shirasaki *et al.*, 2009) and MS2 (Nasser *et al.*, 1995; Redman *et al.*, 1997; Dowd *et al.*, 1998; Abbaszadegan *et al.*, 2007; Mayer *et al.*, 2008; Shirasaki *et al.*, 2009) are widely used as surrogates for pathogenic waterborne viruses in the coagulation,

coagulation–rapid sand filtration, and sandy aquifer treatment processes because of their morphological similarities to hepatitis A viruses and polioviruses, which are important to remove during the treatment of drinking water. Q β is the prototype member of the genus *Allolevivirus* in the virus family Leviviridae, and MS2 is the prototype member of the genus *Levivirus* in the Leviviridae. The genomes of these two bacteriophages contain a single molecule of linear, positive-sense, single-stranded RNA, which is encapsulated in an icosahedral protein capsid with a diameter of 24–26 nm (The Universal Virus Database of the International Committee on the Taxonomy of Viruses). Each bacteriophage was propagated for 22–24 h at 37 °C in *Escherichia coli* (NBRC 13965) obtained from NBRC. The bacteriophage culture solution was centrifuged (2000 \times g, 10 min) and then passed through a membrane filter (pore size 0.45 μ m, hydrophilic cellulose acetate; Dismic-25cs, Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The filtrate was purified by using a centrifugal filter device (molecular weight cutoff 100,000, regenerated cellulose; Amicon Ultra-15, Millipore Corp.) to prepare the bacteriophage stock solution.

2.4. Coagulation experiments

Batch coagulation experiments were conducted with 400 mL of rNV-VLPs and

bacteriophage-spiked river water in glass beakers at 20 °C. The rNV-VLPs (see section 2.2) and the stock solutions (see section 2.3) of both bacteriophages were simultaneously added to the beaker at approximately 10^{11} VLPs/mL and 10^8 plaque-forming units (PFU)/mL, respectively, and mixed with an impeller stirrer. PACl, alum, or FeCl_3 was injected into the water as a coagulant at 20 μM (0.54 mg-Al/L or 1.12 mg-Fe/L), 40 μM (1.08 mg-Al/L or 2.24 mg-Fe/L), or 60 μM (1.62 mg-Al/L or 3.36 mg-Fe/L). In the PACl and alum experiments, the pH of the water was immediately adjusted to, and maintained at, 6.8 using hydrochloric acid or sodium hydroxide. In contrast, in the FeCl_3 experiments, the pH was adjusted to one of three levels, 5.8 (minimum pH level established by drinking water quality standards in Japan), 6.3, or 6.8. The water was stirred rapidly for 2 min ($G = 200 \text{ s}^{-1}$, 77 rpm) and then slowly for 28 min ($G = 20 \text{ s}^{-1}$, 17 rpm). The water was then left at rest for 20 min to settle the floc particles generated. Samples were taken from the beaker before coagulant dosing (C_{c0}) and after settling (C_{cs}) for quantification of the rNV-VLP, Q β , and MS2 concentrations. Statistical analysis by *t*-test (two-tailed) based on a 0.05 level of significance was performed to determine whether the removal performances of rNV-VLPs, Q β , and MS2 differed when the conditions of the coagulation process were different. Because of the absence of experimental replication for any coagulation condition with FeCl_3 , statistical

analysis could not be performed for FeCl₃.

2.5. Rapid sand filtration experiments

After the coagulation experiments, filtration experiments were carried out with a glass column (diameter 0.8 cm, length 20 cm) packed with silica sand. Silica sand was washed with Milli-Q water and dried at 105 °C for 1 h. The cleaned silica sand was gradually added into the glass column to achieve a 10 cm filter depth. Next, to saturate the filter media, Milli-Q water was pumped through the column by a peristaltic pump for 15 min, and then the excess Milli-Q water was drained off from the column just before the filtration experiment. Approximately 350 mL of the supernatant of the settling sample (see section 2.4) was withdrawn from the beaker by the peristaltic pump, and transferred to another glass beaker as raw water for the sand filtration experiments. The raw water was continuously mixed with a magnetic stirrer at 200 rpm during the filtration experiment, and it was fed into the column at a constant flow rate (120 mL/day) by the peristaltic pump. Samples were taken from the beaker (C_{r0}) and column filtrate (C_{rf}) after 15 and 30 min of filtration time for quantification of the rNV-VLP, Q β , and MS2 concentrations. Statistical analysis as described above was also performed on the results of the coagulation–rapid sand filtration process.

2.6. rNV-VLP assay

rNV-VLPs were detected by using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (NV-AD (II), Denka Seiken Co., Ltd., Tokyo, Japan).

The assay was performed according to the manufacturer's instructions. Optical densities at wavelengths of 450 nm and 630 nm in a 96-well microplate were measured with a microplate reader (MTP-300, Corona Electric Co., Ltd., Ibaraki, Japan).

2.7. Bacteriophage assay

Viral RNA of bacteriophages was quantified by real-time reverse transcription-polymerase chain reaction (RT-PCR) method. This method detects viruses regardless of their infectivity. For quantification of bacteriophages in the samples, viral RNA was extracted from 200 μ L of sample with a QIAamp MinElute Virus Spin Kit (Qiagen K. K., Tokyo, Japan) to obtain a final volume of 20 μ L. The extracted RNA solution was added to a High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems Japan Ltd., Tokyo, Japan) for the reverse transcription (RT) reaction, which was conducted at 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 s, followed by cooling to 4 °C in the thermal cycler (Thermal Cycler Dice Model

TP600, Takara Bio Inc., Shiga, Japan). The cDNA solution was then amplified by a TaqMan Universal PCR Master Mix with UNG (Applied Biosystems Japan Ltd.), 400 nM of each primer (HQ-SEQ grade, Takara Bio Inc.), and 250 nM of TaqMan probe (Applied Biosystems Japan Ltd.). The oligonucleotide sequences of the primers and the probes are shown in Table 2. Amplification was conducted at 50 °C for 2 min, 95 °C for 10 min, and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min in an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems Japan Ltd.).

The standard curve for the real-time RT-PCR method was based on the relationship between the infectious bacteriophage concentration of a freshly prepared stock solution measured by the plaque forming unit (PFU) method (Adams, 1959) and the number of cycles of PCR amplification.

2.8. Electron microscopy

Negative-stain electron microscopy was used to analyze the presence, integrity, and morphology of the rNV-VLPs. Ten microliters of rNV-VLP stock solution (see section 2.2) was put on a 400-mesh copper grid with collodion membrane (Nissin EM Corp., Tokyo, Japan) and adsorbed to the grid for 1 min. Excess solution on the grid was drained from the side of the grid with filter paper, and rNV-VLPs were negatively

stained with 10 μL of 2% phosphotungstic acid (pH 5.5) for 45 s. After the excess stain was drained off, the grid was examined with a transmission electron microscope (TEM, H-7650, Hitachi High-Technologies Corp., Tokyo, Japan). The particle diameter of rNV-VLPs was expressed as the mean and standard deviation of 10 randomly chosen particles on the electron micrograph.

2.9. Cesium chloride density gradient

The densities of rNV-VLPs and bacteriophages were analyzed in a cesium chloride (CsCl) density gradient. rNV-VLPs and both bacteriophages were simultaneously suspended at approximately 10^{12} VLPs/mL and 10^8 PFU/mL, respectively, in a 1.2-g/cm³ CsCl solution using the stock solutions (see section 2.2, 2.3), and layered on top of a 1.3- to 1.6-g/cm³ CsCl gradient. The gradient including rNV-VLPs and bacteriophages was ultracentrifuged ($100,000 \times g$, 18 h) in a preparative centrifuge (CP80MX, Hitachi Koki Co., Ltd., Tokyo, Japan) with a P28S swing rotor, and then divided into 1-mL fractions. The density of each fraction was measured with an electronic balance (Mettler Toledo AG245 Balance, Mettler-Toledo K. K., Tokyo, Japan), and rNV-VLP and bacteriophage (Q β and MS2) concentrations were quantified by ELISA and real-time RT-PCR method, respectively.

2.10. Electrophoretic mobility

The electrophoretic mobility of rNV-VLPs and bacteriophages was measured in filtered river water. River water in a stirred ultrafiltration cell (Model 8400, Millipore Corp.) was filtered through an ultrafiltration membrane (molecular weight cutoff 100,000, regenerated cellulose; Ultrafiltration Disks, YM-100, Millipore Corp.) to exclude the large particles, and the pH was adjusted to 6.8 with HCl. The river water samples were kept for 1 day at 20 °C to stabilize the pH. Just before the measurement of electrophoretic mobility, the rNV-VLPs and each bacteriophage were suspended at approximately 10^{10} VLPs/mL or 10^{10} PFU/mL in the filtered river water using the stock solution (see sections 2.2, 2.3). The electrophoretic mobility of the rNV-VLPs and both bacteriophages was measured with an electrophoretic light-scattering spectrophotometer (Zetasizer Nano ZS; 532 nm green laser, Malvern Instruments Ltd., Malvern, Worcestershire, UK) at 25 °C and at a 17° measurement angle.

3. Results and discussion

3.1 Characteristics of the produced rNV-VLPs

Figure 1 shows an electron micrograph of rNV-VLPs produced by the

baculovirus–silkworm expression system. The presence of particles was confirmed on the electron micrograph, and rNV capsid proteins spontaneously self-assembled into VLPs during expression. The rNV-VLP particle diameter was 35.7 ± 3.2 nm, measured on 10 randomly chosen particles on the electron micrograph. This value roughly corresponds to the particle diameter (approximately 38 nm) previously reported for native NV (Someya *et al.*, 2000).

A commercially available ELISA kit was used for quantification of rNV-VLPs. The sensitivity of the ELISA kit was determined by using 0.5- \log_{10} -fold serial dilutions of the rNV-VLP stock solution (see section 2.2) with river water (Figure 2). An excellent linear correlation between the rNV-VLP concentration and absorbance was observed in the range from 10^8 to 10^{10} VLPs/mL. The rNV-VLP quantification limit with the ELISA kit was approximately 10^8 VLPs/mL (Figure 2). This result suggests that the rNV-VLPs produced here could be quantified by the commercially available ELISA kit.

The CsCl density of the fraction with the highest rNV-VLP concentration, quantified by ELISA, was 1.28 g/cm^3 (Figure 3), meaning that the rNV-VLPs produced had a density of 1.28 g/cm^3 . Although this value was somewhat smaller than that of native NV (1.38 g/cm^3 , Jiang *et al.*, 1992; $1.36\text{--}1.37 \text{ g/cm}^3$, Utagawa *et al.*, 1994), probably owing to the lack of RNA, it is similar to the previously reported values for rNV-VLPs (1.31 g/cm^3 ,

Jiang *et al.*, 1992; 1.27 g/cm³, Katayama *et al.*, 2006). In addition, the density of rNV-VLPs was smaller than that of either bacteriophage: the CsCl densities of the fractions with the peak Q β and MS2 concentrations, quantified by real-time RT-PCR method, were 1.42 and 1.40 g/cm³, respectively, roughly corresponding to the densities previously reported for Q β (1.46 g/cm³, Engelberg-Kulka *et al.*, 1979) and MS2 (1.38 g/cm³, Kuzmanovic *et al.*, 2003).

These results suggest that rNV-VLPs were successfully produced by the baculovirus–silkworm expression system, and that the produced rNV-VLPs were morphologically similar to native NV. Accordingly, it is possible to estimate removal performance for NV as particles of the coagulation–rapid sand filtration process by using the rNV-VLPs produced here.

3.2. Simultaneous removal of rNV-VLPs and bacteriophages in the coagulation process

Figure 4 shows the removal ratios ($\log_{10}[C_{c0}/C_{cs}]$) of the model viruses (rNV-VLPs, Q β , and MS2) after settling in the coagulation process with PACl at pH 6.8 (the measurement errors were less than 0.1- \log_{10} for rNV-VLPs, Q β , and MS2). Because of the small sizes of the model viruses and the stability resulting from electrical repulsion in the river water, no removal ($<0.1\text{-}\log_{10}$) of rNV-VLPs or either bacteriophage was

observed in the absence of a coagulant. Even with 20 $\mu\text{M-Al}$ of PACl, no removal was observed. In contrast, the coagulation process removed model viruses at PACl doses of 40 and 60 $\mu\text{M-Al}$. This result indicated that the stably monodispersed model viruses in the river water were destabilized by the addition of PACl and became adsorbed to/entrapped in the aluminum floc particles generated during the coagulation process, and then the aluminum floc particles with the adsorbed/entrapped model viruses settled from suspension by gravity during the settling process. The removal ratios of rNV-VLPs were only $<0.3\text{-log}_{10}$ at a PACl dose of 20 $\mu\text{M-Al}$, whereas with a dose of 40 $\mu\text{M-Al}$ or more the removal ratios significantly ($P < 0.05$) increased to approximately 1-log_{10} for rNV-VLPs. Increasing PACl dose to more than 40 $\mu\text{M-Al}$ also increased the removal ratios of Q β and MS2 to approximately 2-log_{10} , although no significance ($P = 0.06$) was observed for Q β (40 $\mu\text{M-Al}$) and MS2 (60 $\mu\text{M-Al}$). Accordingly, a PACl dose of 40 $\mu\text{M-Al}$ is recommended to remove the model viruses in the present coagulation process. Similar trends were observed when alum and FeCl_3 were used as the coagulant (data not shown).

To investigate the effect of coagulant type on the removals of model viruses, the removal performances of the coagulation process with a coagulant dose of 40 $\mu\text{M-Al}$ or -Fe were compared among rNV-VLPs, Q β , and MS2 after settling (Figure 5). Because

the optimal coagulation pH for FeCl₃ is generally lower than that for aluminum coagulants (American Water Works Association, 1990), the coagulation pH for FeCl₃ was adjusted to 5.8 and 6.3 (data not shown) as well as to 6.8. The coagulation processes with PACl and FeCl₃ (pH 5.8) obtained approximately 1-log₁₀ removals of rNV-VLPs. These removals are somewhat larger than those obtained with alum and FeCl₃ (pH 6.8) although no significant difference between PACl and alum was observed ($P > 0.05$). The efficacy of PACl for the removal of negatively charged colloids, including viruses, compared with that of alum is attributed to soluble polycationic species, which are abundant in PACl. Because the widely accepted mechanism of negatively charged colloid removal is by charge neutralization by soluble polycationic species such as Al₂(OH)₂⁴⁺, Al₃(OH)₄⁵⁺, and Al₁₃O₄(OH)₂₄⁷⁺ (Stewart *et al.*, 2009), the difference in the abundances of polycationic species probably can explain the differences in the characteristics of the aluminum floc particles generated during the coagulation process between PACl and alum. In fact, Gregory and Dupont (2001) reported that aluminum floc particles formed with PACl are larger, stronger, and more readily separated by the settling process than those formed with alum. Accordingly, PACl could more effectively remove the rNV-VLPs than alum.

FeCl₃ (pH 5.8) also removed rNV-VLPs more effectively than alum in the present

coagulation process. Rao *et al.* (1988) investigated the removal performance for hepatitis A virus and poliovirus during the coagulation process, and demonstrated that greater removals of both viruses were obtained with FeCl₃ than with alum. Chang *et al.* (1958) also reported effective removal of coxsackie virus with FeCl₃, and floc particles formed with FeCl₃ were more compact and settled more rapidly than those formed with alum. Therefore, the difference in the characteristics of the floc particles between FeCl₃ (pH 5.8) and alum led to the difference in rNV-VLP removal performances in the present coagulation process. However, FeCl₃ could not effectively remove rNV-VLPs at pH 6.8: the removal ratio of rNV-VLPs decreased as pH increased, with removal performance in the order pH 5.8 > 6.3 > 6.8. Abbaszadegan *et al.* (2007) and Mayer *et al.* (2008) investigated the effect of pH on the removal of five viruses, including FCV, during enhanced coagulation processes with FeCl₃; they reported that the removal ratios of these viruses were improved as pH decreased (optimum pH range 5–6). Our findings are consistent with these previous results.

The removal performance for Q β obtained with PACl was higher than that obtained with alum or FeCl₃. By comparison, approximately 2-log₁₀ removals of MS2 were achieved with all coagulant types except FeCl₃ (pH 6.8). Consequently, PACl more effectively removed the model viruses than alum or FeCl₃ in the present coagulation

process. Additionally, it was difficult to remove the model viruses with FeCl₃ at pH 6.8, although the removal performances for rNV-VLPs and MS2 with FeCl₃ at pH 5.8 were equal to those with PACl.

Differences in the removal performances among rNV-VLPs, Q β , and MS2 were observed in the coagulation process: although the removal ratio of Q β was similar to that of rNV-VLPs at a coagulant dose of 40 μ M-Al or Fe, the removal ratio of MS2 was approximately 1-log₁₀ larger than that of rNV-VLPs (Figure 5). In general, the surface charge on virus particles is often invoked to explain virus removal by physicochemical water treatment processes, including coagulation processes (Matsushita *et al.*, 2004): more negatively charged viruses may resist aggregation, with the result that they are more difficult to destabilize and aggregate by charge neutralization during the coagulation process than less negatively charged viruses. However, rNV-VLPs were less negative than Q β or MS2 in the filtered river water at pH 6.8 (Figure 6), a result that is not in accordance with a previous result in which rNV-VLPs were more negative than MS2 in a NaCl solution at around pH 7 (Redman *et al.*, 1997), possibly owing to the difference in the electrolyte solution. The difference in electrophoretic mobility among the model viruses did not strongly affect removal performances in our study. We imagine that the differences in other characteristics of the model viruses, such as

hydrophobicity, affected the removal performances of the coagulation process. Because the hydrophobic force contributes to the adsorption of protein on aluminum phosphate adjuvant (Al-Shakhshir *et al.*, 1995), the difference in hydrophobicity among surface proteins of the model viruses might cause differences in the interaction between surface proteins and the floc particles generated during the coagulation process. Further investigation is needed.

Much discussion of possible surrogates for pathogenic waterborne viruses in the aquatic environment has not yet resulted in any overall agreement. MS2 has been widely used as a surrogate for pathogenic waterborne viruses in coagulation treatments (Nasser *et al.*, 1995; Abbaszadegan *et al.*, 2007; Mayer *et al.*, 2008; Shirasaki *et al.*, 2009), mainly because of its morphological similarities to those viruses. However, as described above, the removal performance for MS2 was approximately 1-log_{10} larger than that for rNV-VLPs in the present coagulation process, meaning that the removal performances for native NV would be overestimated if MS2 were selected as a surrogate for native NV. Accordingly, MS2 is not recommended as an appropriate surrogate for native NV. By comparison, although the removal performance for Q β was similar to that for rNV-VLPs at a coagulant dose of 40 μM -Al or Fe (Figure 5), the removal performance for Q β was somewhat larger than that for rNV-VLPs at PACl dose of 60 μM -Al (Figure

4). Therefore, Q β also is not recommended as an appropriate surrogate for native NV.

3.3 Simultaneous removal of rNV-VLPs and bacteriophages in the coagulation–rapid sand filtration process

Figure 7 shows the removal ratios ($\log_{10}[C_{c0}/C_{cs}] + \log_{10}[C_{r0}/C_{rf}]$) for model viruses in the coagulation–rapid sand filtration process with PACl at pH 6.8. Because the removal ratios of model viruses in the coagulation–rapid sand filtration process were almost constant during the filtration, the removal ratios in Figure 7 are represented by the averages of the values after 15 and 30 min filtration. Even though the rapid sand filtration process was introduced after the coagulation process, no removal ($<0.1\text{-log}_{10}$) of rNV-VLPs or either bacteriophage was observed in the absence of a coagulant. Even with the PACl dose of 20 $\mu\text{M-Al}$, no removal was observed. In contrast, improvement of removal ratios of the model viruses were observed in the rapid sand filtration process at PACl doses of 40 and 60 $\mu\text{M-Al}$ compared with the coagulation process alone (Figure 4). Therefore, the entrapped model viruses in the suspended aluminum floc particles were effectively removed by the subsequent rapid sand filtration process. Even monodispersed model viruses might have been adsorbed to the negatively charged sand surface by the electrostatic attractive force/van der Waals attractive force, because the

surface charges of the model viruses probably changed from negative to neutral or positive by charge neutralization during the coagulation process. The removal ratio of rNV-VLPs was increased more by the introduction of the rapid sand filtration process than were those of Q β and MS2 at PACl doses of 40 and 60 μ M-Al: an approximately 2- \log_{10} improvement was obtained for rNV-VLPs, whereas approximately 0.5- \log_{10} improvements were obtained for the two bacteriophages. In the sandy aquifer treatment process, many factors affect virus adsorption. Dowd *et al.* (1998) demonstrated that the larger bacteriophages PRD1 and PM2 (60–63 nm) show greater retardation than the smaller bacteriophages Q β , MS2, and ϕ X174 (24–27 nm). Our findings agree with this previous result: the removal ratio of rNV-VLPs (35.7 ± 3.2 nm) was larger than those of Q β and MS2 (24–26 nm), as described above. Accordingly, a difference in the particle diameter between rNV-VLPs and the two bacteriophages possibly affected the removal performance in the present rapid sand filtration process. In addition, Redman *et al.* (1997) reported that the removal ratio of less negatively charged rNV-VLPs was higher than that of more negatively charged ones owing to the reduction of electrostatic repulsive interactions between rNV-VLPs and the negatively charged sand surface. Although the electrophoretic mobility of rNV-VLPs and both bacteriophages after the coagulation process was not measured, a difference in the surface charge between

rNV-VLPs and the bacteriophages after the coagulation process also might have affected removal performance in the present rapid sand filtration process.

Ultimately, the removal ratios of model viruses were only $<0.3\text{-log}_{10}$ at a PACl dose of $20\ \mu\text{M-Al}$, whereas, with a dose of $40\ \mu\text{M-Al}$ or more, the removals significantly ($P < 0.05$) increased to approximately $2\text{--}3\text{-log}_{10}$ for rNV-VLPs, Q β , and MS2 in the present coagulation–rapid sand filtration process. Accordingly, a PACl dose of $40\ \mu\text{M-Al}$ is recommended to remove the model viruses in this coagulation–rapid sand filtration process. Similar trends were observed when alum and FeCl_3 were used as the coagulant (data not shown).

To investigate the effect of coagulant type on the removals of model viruses, the removal performances for rNV-VLPs, Q β , and MS2 were compared in the coagulation–rapid sand process between a $40\ \mu\text{M-Al}$ and a $40\ \mu\text{M-Fe}$ of coagulant dose (Figure 8). The coagulation–rapid sand filtration process with PACl and FeCl_3 (pH 5.8) achieved approximately 3-log_{10} removals of rNV-VLPs, whereas the removal ratios of rNV-VLPs obtained with alum and FeCl_3 (pH 6.8) were smaller; in particular, only a 0.5-log_{10} removal was observed with FeCl_3 (pH 6.8). In addition, a significant difference between PACl and alum was observed ($P < 0.05$). By comparison, the removal ratio of Q β with FeCl_3 (pH 5.8) was the highest obtained, followed by those

obtained with PACl, alum, and FeCl₃ (pH 6.8). A similar trend was observed with MS2. Consequently, FeCl₃ (pH 5.8) more effectively removed model viruses than PACl or alum in the present coagulation–rapid sand filtration process, although a decreased coagulation pH is required when FeCl₃ is used as the coagulant compared with the use of PACl or alum.

The removal performance for MS2 was somewhat larger than that for rNV-VLPs in the present coagulation–rapid sand filtration process. Accordingly, MS2 is not recommended as an appropriate surrogate for native NV. By comparison, the removal performance for Q β was similar to, or smaller than, that for rNV-VLPs at a coagulant dose of 40 μ M-Al or Fe (Figure 8). However, the removal performances for rNV-VLPs and Q β differed between each unit process, i.e., the coagulation process removed Q β rather than rNV-VLPs, while vice versa in the following rapid sand filtration process. Accordingly, Q β also is not recommended as an appropriate surrogate for native NV, even though its removal ratio in total unit process was similar to, or smaller than, that of rNV-VLPs at any condition. To propose appropriate surrogates, further investigation is needed.

According to the U.S. Environmental Protection Agency (USEPA) National Primary Drinking Water Standards (USEPA, 2001), enteric viruses must be removed or

inactivated by 4- \log_{10} from source water by filtration, disinfection, or a combination of these technologies. This 4- \log_{10} removal ratio was not obtained for rNV-VLPs by the present coagulation–rapid sand filtration process alone. To achieve 4- \log_{10} removal or inactivation of native NV, further separation or disinfection processes will be required after coagulation–rapid sand filtration. On the other hand, virucidal activity of PACl during the coagulation process has been reported by our research group (Matsui *et al.*, 2003; Matsushita *et al.*, 2004; Shirasaki *et al.*, 2009): an approximately 4- \log_{10} difference between the total (infectious + inactivated) concentration measured by real-time RT-PCR method and the infectious concentration measured by the PFU method has been observed during the coagulation process (Shirasaki *et al.*, 2009), indicating that some of the bacteriophages were probably inactivated by PACl. Because rNV-VLPs lack RNA, which is necessary for infection and replication in host cells, we cannot discuss the fate of infectivity of NV in the treatment process. If PACl exhibits virucidal activity for native NV as well as for bacteriophages, >4- \log_{10} removal (including inactivation) of native NV might be achieved during coagulation–rapid sand filtration.

4. Conclusions

(1) Application of rNV-VLPs in laboratory-scale experiments enabled us to estimate the removal performance of the coagulation–rapid sand filtration process for NV as particles.

(2) The coagulation–rapid sand filtration process with PACl and FeCl₃ (pH 5.8) at a coagulant dose of 40 μM-Al or -Fe achieved approximately 3-log₁₀ removals of rNV-VLPs—larger than those achieved with alum or FeCl₃ (pH 6.8).

(3) The removal performance for MS2 was somewhat larger than that for rNV-VLPs in the coagulation–rapid sand filtration process. Accordingly, MS2 is not recommended as an appropriate surrogate for native NV. By comparison, the removal performance for Qβ was similar to, or smaller than, that for rNV-VLPs. However, the removal performances for rNV-VLPs and Qβ differed between the coagulation process and the following rapid sand filtration process. Therefore, Qβ also is not recommended as an appropriate surrogate for native NV.

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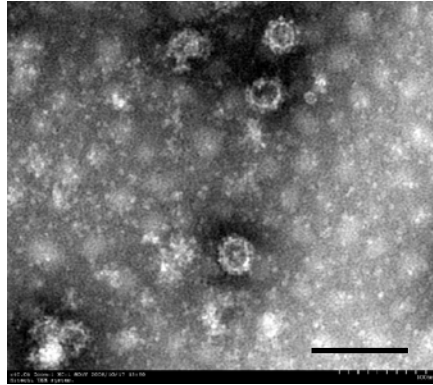


Figure 1. Negatively stained electron micrograph of rNV-VLPs. The scale bar corresponds to 100 nm.

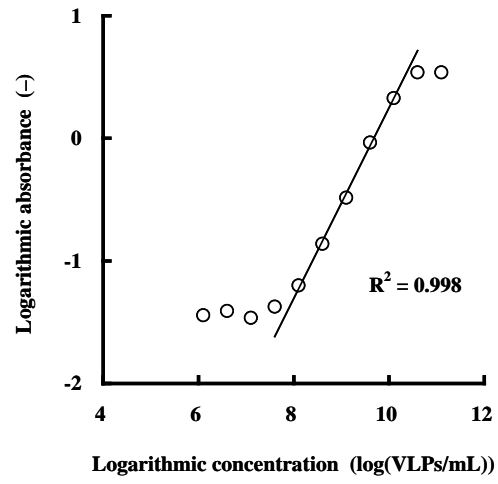


Figure 2. Sensitivity of the ELISA kit for the detection of rNV-VLPs.

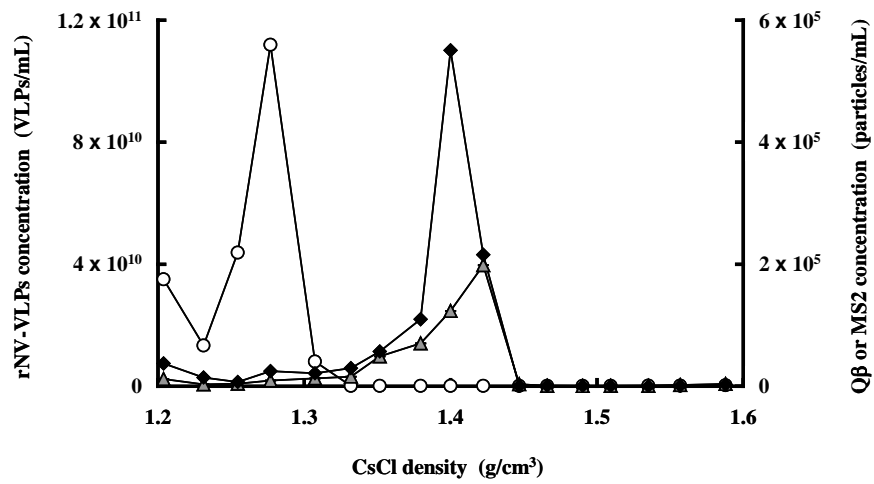


Figure 3. Densities of rNV-VLPs, Qβ, and MS2 analyzed by CsCl density gradient.

Circles, triangles, and diamonds represent rNV-VLPs, Qβ, and MS2, respectively.

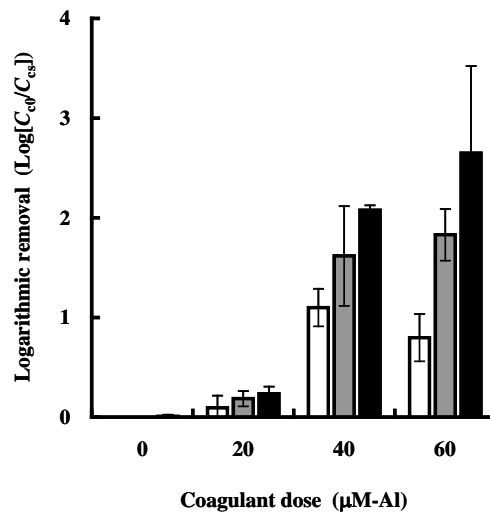


Figure 4. Effect of coagulant dose on rNV-VLP, Q β , and MS2 removals after settling in the coagulation process with PACl at pH 6.8. White, gray, and black columns represent rNV-VLPs, Q β , and MS2, respectively. Values are means and standard deviation of two or three replications.

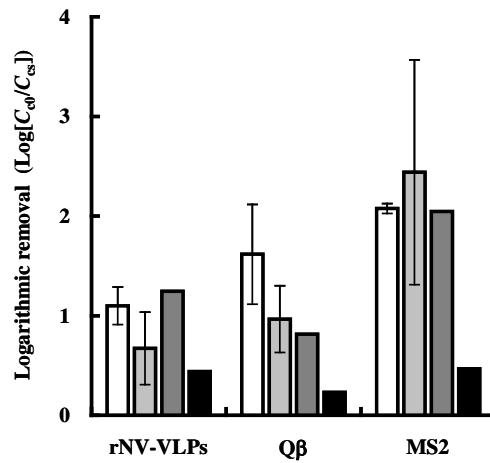


Figure 5. Effect of coagulant type on the removals of rNV-VLPs, Qβ, and MS2 after settling in the coagulation process. White, light gray, dark gray, and black columns represent PACl (pH 6.8), alum (pH 6.8), FeCl₃ (pH 5.8), and FeCl₃ (pH 6.8), respectively. Values are means and standard deviation of two or three replications for PACl and three or four replications for alum. Values for FeCl₃ were determined from one experiment. Coagulant dose was 40 μM-Al or -Fe.

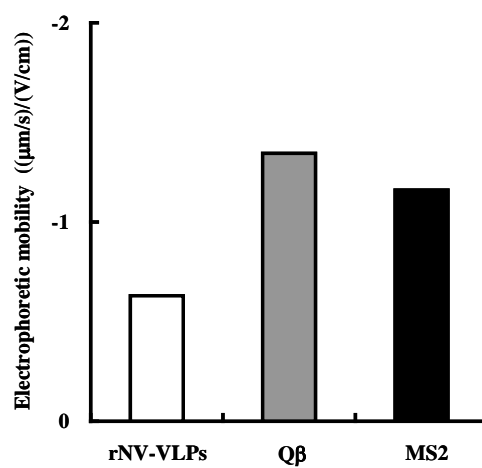


Figure 6. Electrophoretic mobility of rNV-VLPs, Qβ, and MS2 in filtered river water.

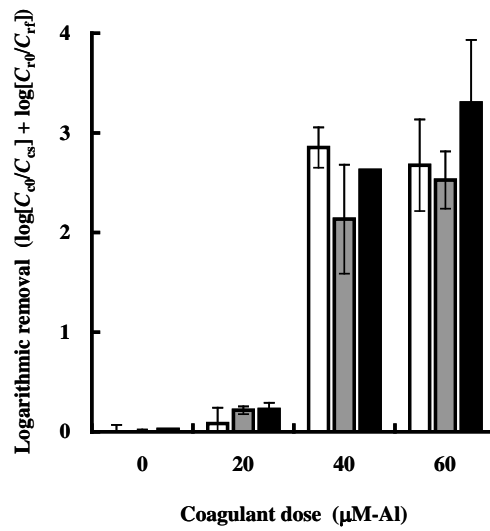


Figure 7. Effect of coagulant dose on rNV-VLP, Qβ, and MS2 removals in the coagulation–rapid sand filtration process with PACl at pH 6.8. White, gray, and black columns represent rNV-VLPs, Qβ, and MS2, respectively. Values are means and standard deviation of two or three replications.

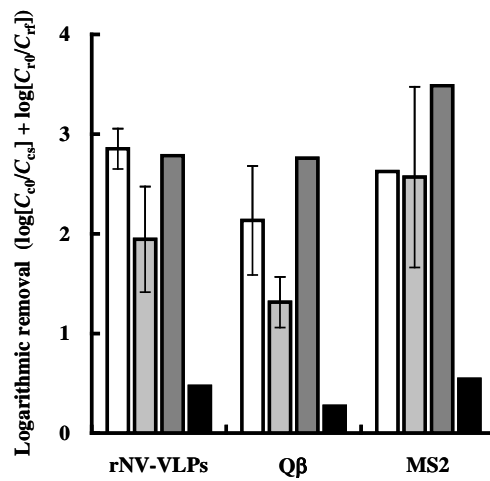


Figure 8. Effect of coagulant type on removal of rNV-VLPs, Q β , and MS2 in the coagulation–rapid sand filtration process. White, light gray, dark gray, and black columns represent PACl (pH 6.8), alum (pH 6.8), FeCl₃ (pH 5.8), and FeCl₃ (pH 6.8), respectively. Values are means and standard deviation of two or three replications for PACl and three or four replications for alum. Values for FeCl₃ were determined from one experiment. Coagulant dose was 40 μ M-Al or -Fe.

Table 1. Water quality of the Toyohira River.

pH	7.2
DOC (mg/L)	0.76
OD260 (cm ⁻¹)	0.019
Turbidity (NTU)	0.63
Alkalinity (mg-CaCO ₃ /L)	17.2

Table 2. Oligonucleotide sequences of the primers and probes used in real-time RT-PCR quantification of Q β and MS2.

Viruses		Oligonucleotide sequences	Positions	References
Q β	Forward primer	5'-TCA AGC CGT GAT AGT CGT TCC TC-3'	49-71	Katayama <i>et al.</i> , 2002
	Reverse primer	5'-AAT CGT TGG CAA TGG AAA GTG C-3'	187-208	
	TaqMan probe	5'-CGA GCC GCG AAC ACA AGA ATT GA-3'	147-169	
MS2	Forward primer	5'-GTC GCG GTA ATT GGC GC-3'	632-648	O'Connell <i>et al.</i> , 2006
	Reverse primer	5'-GGC CAC GTG TTT TGA TCG A-3'	690-708	
	TaqMan probe	5'-AGG CGC TCC GCT ACC TTG CCC T-3'	650-671	