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 Qβ (NBRC 20012) and MS2 (NBRC 102619) were obtained from the NITE Biological Research Center (NBRC, Chiba, Japan). The bacteriophages Qβ (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. Qβ 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 × *g*, 10 min) and then passed through a membrane filter (pore size 0.45 μm, 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 ($G = 200\ s^{-1}$, 77 rpm) and then slowly for 28 min ($G = 20\ s^{-1}$, 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, Qβ, 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, Qβ, 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 $FeCl_3$.

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 m/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, Qβ, 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 μL of sample with a QIAamp MinElute Virus Spin Kit (Qiagen K. K., Tokyo, Japan) to obtain a final volume of 20 μL. 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

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

Viruses		Oligonucleotide sequences	Positions	References
Qβ	Forward primer	5′-TCA AGC CGT GAT AGT CGT TCC TC-3′	49–71	Katayama et al., 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 et al., 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	

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/cm^3 CsCl solution using the stock solutions (see Sections 2.2 and 2.3), and layered on top of a 1.3–1.6-g/cm^3 CsCl gradient. The gradient including rNV-VLPs and bacteriophages was ultracentrifuged (100,000 × *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 (Qβ 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 and 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*

Fig. 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).

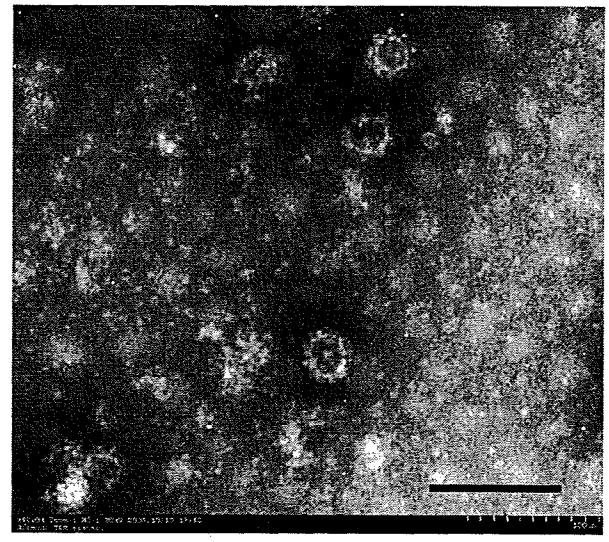

Fig. 1 – Negatively stained electron micrograph of rNV-VLPs. The scale bar corresponds to 100 nm.

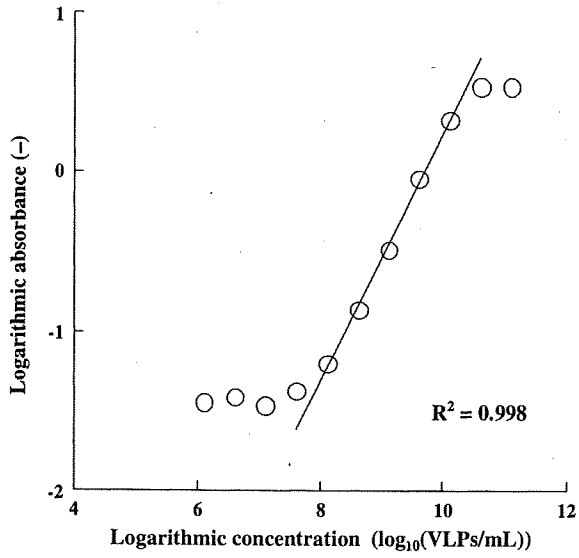

Fig. 2 – Sensitivity of the ELISA kit for the detection of rNV-VLPs.

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 (Fig. 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 (Fig. 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 (Fig. 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; 1.36–1.37 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/cm^3, 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 Qβ and MS2 concentrations, quantified by real-time RT-PCR method, were 1.42 and 1.40 g/cm^3, respectively, roughly corresponding to the densities previously reported for Qβ (1.46 g/cm^3, Engelberg-Kulka et al., 1979) and MS2 (1.38 g/cm^3, 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*

Fig. 4 shows the removal ratios ($\log_{10}[C_{c0}/C_{cs}]$) of the model viruses (rNV-VLPs, Qβ, 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, Qβ, 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-$\log_{10}$) of rNV-VLPs or either bacteriophage was observed in the absence of a coagulant. Even with 20 μM-Al of PACl, no removal was observed. In contrast, the coagulation process removed model viruses at PACl doses of 40 and 60 μM-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-$\log_{10}$ at a PACl dose of 20 μM-Al, whereas with a dose of 40 μM-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 μM-Al also increased the removal ratios of Qβ and MS2 to approximately 2-$\log_{10}$, although no

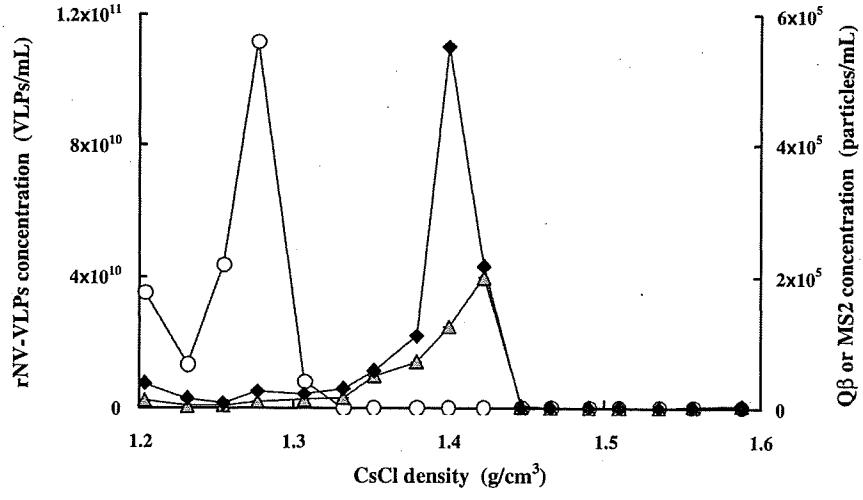

Fig. 3 – Densities of rNV-VLPs, Qβ, and MS2 analyzed by CsCl density gradient. Circles, triangles, and diamonds represent rNV-VLPs, Qβ, and MS2, respectively.

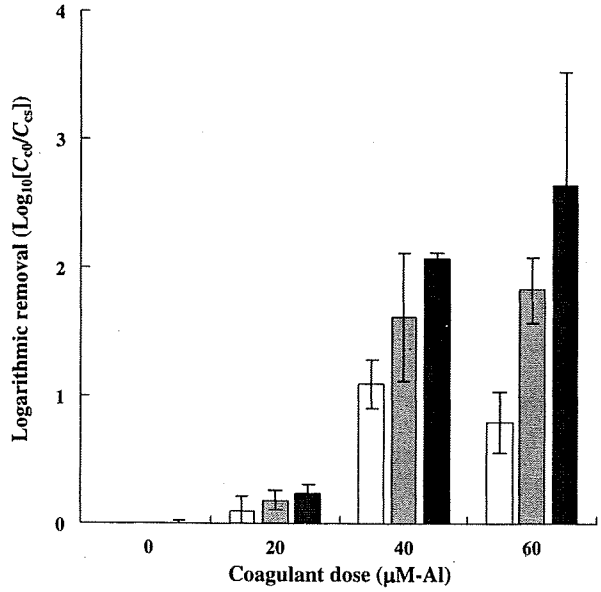

Fig. 4 – Effect of coagulant dose on rNV-VLP, Qβ, and MS2 removals after settling in the coagulation process with PACl at pH 6.8. White, gray, and black columns represent rNV-VLPs, Qβ, and MS2, respectively. Values are means and standard deviation of two or three replications.

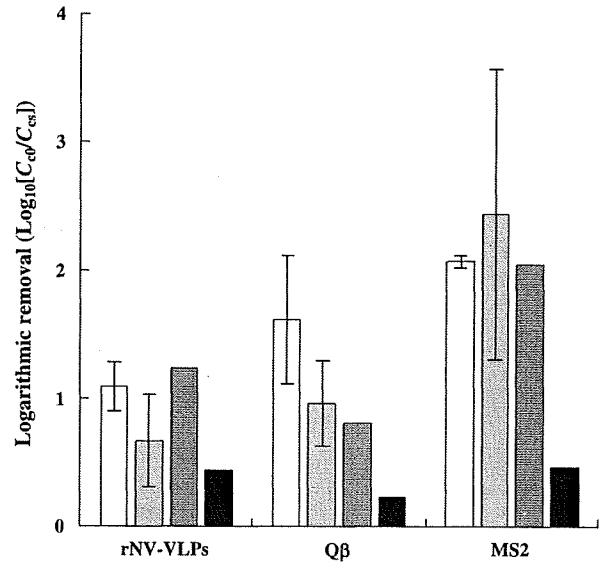

Fig. 5 – Effect of coagulant type on the removals of rNV-VLPs, Qβ, and MS2 after settling in the coagulation process. White, light gray, dark gray, and black columns represent PACl (pH 6.8), alum (pH 6.8), $FeCl_3$ (pH 5.8), and $FeCl_3$ (pH 6.8), respectively. Values are means and standard deviation of two or three replications for PACl and three or four replications for alum. Values for $FeCl_3$ were determined from one experiment. Coagulant dose was 40 μM-Al or -Fe.

significance ($P = 0.06$) was observed for Qβ (40 μM-Al) and MS2 (60 μM-Al). Accordingly, a PACl dose of 40 μM-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 μM-Al or -Fe were compared among rNV-VLPs, Qβ, and MS2 after settling (Fig. 5). Because the optimal coagulation pH for $FeCl_3$ is generally lower than that for aluminum coagulants (American Water Works Association, 1990), the coagulation pH for $FeCl_3$ was adjusted to 5.8 and 6.3 (data not shown) as well as to 6.8. The coagulation processes with PACl and $FeCl_3$ (pH 5.8) obtained approximately 1-log_{10} removals of rNV-VLPs. These removals are somewhat larger than those obtained with alum and $FeCl_3$ (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 $Al_2(OH)_2^{4+}$, $Al_3(OH)_4^{5+}$, and $Al_{13}O_4(OH)_{24}^{7+}$ (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.

$FeCl_3$ (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 $FeCl_3$ than with alum. Chang et al. (1958) also reported effective removal of coxsackie virus with $FeCl_3$, and floc particles formed with $FeCl_3$ were more compact and settled more rapidly than those formed with alum. Therefore, the difference in the characteristics of the floc particles between $FeCl_3$ (pH 5.8) and alum led to the difference in rNV-VLP removal performances in the present coagulation process. However, $FeCl_3$ 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 $FeCl_3$; 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 Qβ obtained with PACl was higher than that obtained with alum or $FeCl_3$. By comparison, approximately 2-log_{10} removals of MS2 were achieved with all coagulant types except $FeCl_3$ (pH 6.8). Consequently, PACl more effectively removed the model viruses than alum or $FeCl_3$ in the present coagulation process. Additionally, it was difficult to remove the model viruses with $FeCl_3$ at pH 6.8, although the removal performances for rNV-VLPs and MS2 with $FeCl_3$ at pH 5.8 were equal to those with PACl.

Differences in the removal performances among rNV-VLPs, Qβ, and MS2 were observed in the coagulation process: although the removal ratio of Qβ was similar to that of rNV-VLPs at a coagulant dose of 40 μM-Al or -Fe, the removal ratio of MS2 was approximately 1-$\log_{10}$ larger than that of rNV-VLPs (Fig. 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 Qβ or MS2 in the filtered river water at pH 6.8 (Fig. 6), a result that is not in accordance with a previous result in which rNV-VLPs were more negative than MS2 in an 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 Qβ was similar to that for rNV-VLPs at a coagulant dose of 40 μM-Al or -Fe (Fig. 5), the removal performance for Qβ was somewhat larger than that for rNV-VLPs at PACl dose of 60 μM-Al (Fig. 4). Therefore, Qβ also is not recommended as an appropriate surrogate for native NV.

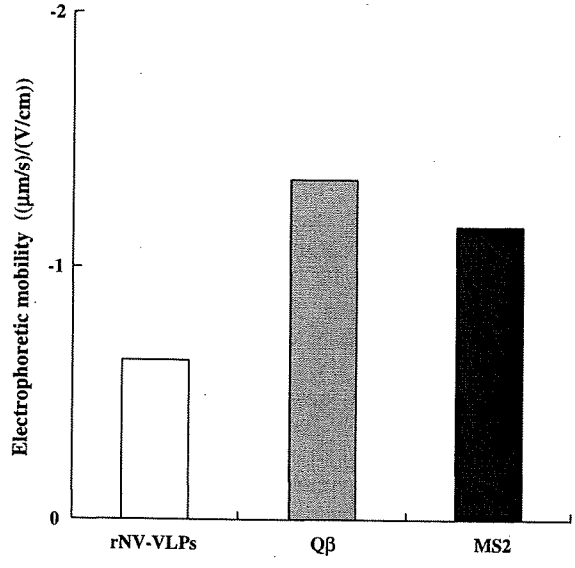

Fig. 6 – Electrophoretic mobility of rNV-VLPs, Qβ, and MS2 in filtered river water.

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

Fig. 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 Fig. 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-$\log_{10}$) of rNV-VLPs or either bacteriophage was observed in the absence of a coagulant. Even with the PACl dose of 20 μM-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 μM-Al compared with the coagulation process alone (Fig. 4). Therefore, the entrapped model viruses in the suspended aluminum floc particles were effectively removed

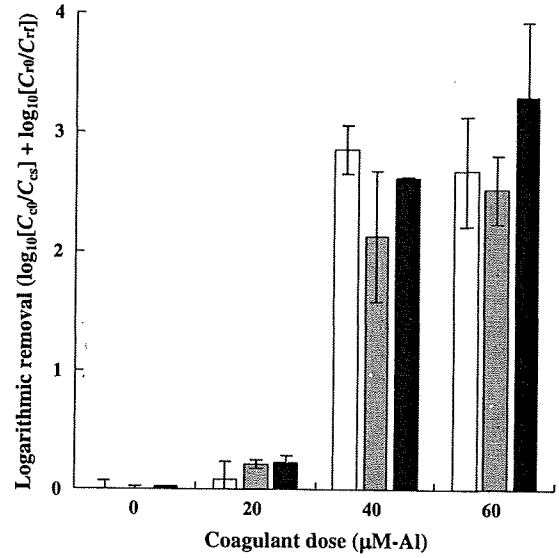

Fig. 7 – Effect of coagulant dose on rNV-VLP, Qβ, and MS2 removals in the coagulation–rapid sand filtration process with PACl at pH 6.8. White, gray, and black columns represent rNV-VLPs, Qβ, and MS2, respectively. Values are means and standard deviation of two or three replications.

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 Qβ and MS2 at PACl doses of 40 and 60 μM-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 Qβ, MS2, and φX174 (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 Qβ 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-$\log_{10}$ at a PACl dose of 20 μM-Al, whereas, with a dose of 40 μM-Al or more, the removals significantly ($P < 0.05$) increased to approximately 2–3-$\log_{10}$ for rNV-VLPs, Qβ, and MS2 in the present coagulation–rapid sand filtration process. Accordingly, a PACl dose of 40 μM-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, Qβ, and MS2 were compared in the coagulation–rapid sand process between a 40 μM-Al and a 40 μM-Fe of coagulant dose (Fig. 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 Qβ with $FeCl_3$ (pH 5.8) was the highest obtained, followed by those obtained with PACl, alum, and $FeCl_3$ (pH 6.8). A similar trend was observed with MS2. Consequently, $FeCl_3$ (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 $FeCl_3$ is used as the coagulant compared with the use of PACl or alum.

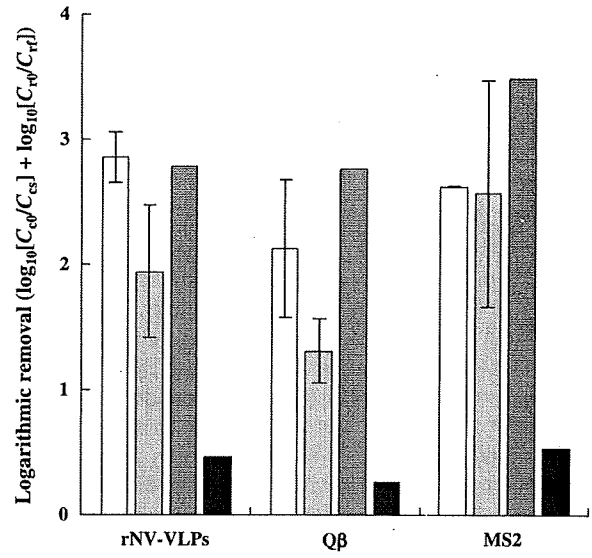

Fig. 8 – Effect of coagulant type on removal of rNV-VLPs, Qβ, and MS2 in the coagulation–rapid sand filtration process. White, light gray, dark gray, and black columns represent PACl (pH 6.8), alum (pH 6.8), $FeCl_3$ (pH 5.8), and $FeCl_3$ (pH 6.8), respectively. Values are means and standard deviation of two or three replications for PACl and three or four replications for alum. Values for $FeCl_3$ were determined from one experiment. Coagulant dose was 40 μM-Al or -Fe.

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 Qβ was similar to, or smaller than, that for rNV-VLPs at a coagulant dose of 40 μM-Al or -Fe (Fig. 8). However, the removal performances for rNV-VLPs and Qβ differed between each unit process, i.e., the coagulation process removed Qβ rather than rNV-VLPs, while vice versa in the following rapid sand filtration process. Accordingly, Qβ 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 $FeCl_3$ (pH 5.8) at a coagulant dose of 40 μM-Al or -Fe achieved approximately 3-$\log_{10}$ removals of rNV-VLPs—larger than those achieved with alum or $FeCl_3$ (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.

Acknowledgements

We thank Masatoshi Okamatu, Norikazu Isoda (Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University), and Daisuke Tomabechi (Department of Global Epidemiology, Research Center for Zoonosis Control, Hokkaido University), who instructed us in the methods of electron microscopic analysis and CsCl density gradient analysis. This research was supported in part by a Grant-in-Aid for the Encouragement of Young Scientists (no. 19760368, 2007) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; a Grant-in-Aid (no. 19310801, 2007) from the Ministry of Health, Labor, and Welfare of Japan; the Hokkaido University Clark Memorial Foundation (2007); and a Kajima Foundation Research Grant (2007).

REFERENCES

Abbaszadegan, M., Mayer, B.K., Ryu, H., Nwachuku, N., 2007. Efficacy of removal of CCL viruses under enhanced coagulation conditions. Environmental Science and Technology 41 (3), 971–977.

Adams, M.H., 1959. Bacteriophages. Interscience Publishers, New York, pp. 450–454.

Al-Shakhshir, R.H., Regnier, F.E., White, J.L., Hem, S.L., 1995. Contribution of electrostatic and hydrophobic interactions to the adsorption of proteins by aluminium-containing adjuvants. Vaccine 13 (1), 41–44.

American Water Works Association, 1990. In: Pontius, E.W. (Ed.), Water Quality and Treatment: a Handbook of Community Water Supplies, fourth ed. McGraw-Hill, New York, pp. 300–305.

Bae, J., Schwab, K.J., 2008. Evaluation of murine norovirus, feline calicivirus, poliovirus, and MS2 as surrogates for human norovirus in a model of viral persistence in surface water and groundwater. Applied and Environmental Microbiology 74 (2), 477–484.

Bertolotti-Ciarlet, A., Crawford, S.E., Hutson, A.M., Estes, M.K., 2003. The 3′ end of Norwalk Virus mRNA contains determinants that regulate the expression and stability of the viral capsid protein VP1: a novel function for the VP2 protein. Journal of Virology 77 (21), 11603–11615.

Cannon, J.L., Papafragkou, E., Park, G.W., Osborne, J., Jaykus, L.A., Vinjé, J., 2006. Surrogates for the study of norovirus stability and inactivation in the environment: a comparison of murine norovirus and feline calicivirus. Journal of Food Protection 69 (11), 2761–2765.

Chang, S.L., Stevenson, R.E., Bryant, A.R., Woodward, R.L., Kabler, P.W., 1958. Removal of coxsackie and bacterial viruses in water by flocculation: 1. Removal of coxsackie and bacterial viruses in water of known chemical content by flocculation with aluminum sulfate or ferric chloride under various testing conditions. American Journal of Public Health and the Nations Health 48 (1), 51–61.

Daniels, N.A., Bergmire-Sweat, D.A., Schwab, K.J., Hendricks, K.A., Reddy, S., Rowe, S.M., Fankhauser, R.L., Monroe, S.S., Atmar, R.L., Glass, R.I., Mead, P., 2000. A foodborne outbreak of gastroenteritis associated with Norwalk-like viruses: first molecular traceback to deli sandwiches contaminated during preparation. The Journal of Infectious Diseases 181 (April), 1467–1470.

Dowd, S.E., Pillai, S.D., Wang, S., Corapcioglu, M.Y., 1998. Delineating the specific influence of virus isoelectric point and size on virus adsorption and transport through sandy soils. Applied and Environmental Microbiology 64 (2), 405–410.

Duizer, E., Bijkerk, P., Rockx, B., Groot, A., Twisk, F., Koopmans, M., 2004. Inactivation of caliciviruses. Applied and Environmental Microbiology 70 (8), 4538–4543.

Engelberg-Kulka, H., Israeli-Reches, M., Dekel, L., Friedmann, A., 1979. Qβ-defective particles produced in a streptomycin-resistant *Escherichia coli* mutant. Journal of Virology 29 (3), 1107–1117.

Fankhauser, R.L., Monroe, S.S., Noel, J.S., Humphrey, C.D., Bresee, J.S., Parashar, U.D., Ando, T., Glass, R.I., 2002. Epidemiologic and molecular trends of "Norwalk-like Viruses" associated with outbreaks of gastroenteritis in the United States. The Journal of Infectious Diseases 186 (July), 1–7.

Green, K.Y., Lew, J.F., Jiang, X., Kapikian, A.Z., Estes, M.K., 1993. Comparison of the reactivities of baculovirus-expressed recombinant Norwalk virus capsid antigen with those of the native Norwalk virus antigen in serologic assays and some epidemiologic observations. Journal of Clinical Microbiology 31 (8), 2185–2191.

Gregory, J., Dupont, V., 2001. Properties of flocs produced by water treatment coagulants. Water Science and Technology 44 (10), 231–236.

Hashimoto, M., Roerink, F., Tohya, Y., Mochizuki, M., 1999. Genetic analysis of the RNA polymerase gene of caliciviruses from dogs and cats. Journal of Veterinary Medical Science 61 (6), 603–608.

Jiang, X., Wang, M., Graham, D.Y., Estes, M.K., 1992. Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein. Journal of Virology 66 (11), 6527–6532.

Karst, S.M., Wobus, C.E., Lay, M., Davidson, J., Virgin, H.W., 2003. STAT1-dependent innate immunity to a Norwalk-like virus. Science 299 (5612), 1575–1578.

Kasuga, K., Tokieda, M., Ohtawara, M., Utagawa, E., Yamazaki, S., 1990. Small round structured virus associated with an outbreak of acute gastroenteritis in Chiba. Japanese Journal of Medical Science and Biology 43 (4), 111–121.

Katayama, H., Shimazaki, A., Ohgaki, S., 2002. Development of virus concentration method using negatively charged membrane by alkaline elution after acid rinse. Journal of Japan Society on Water Environment 25 (8), 469–475 (in Japanese).

Katayama, K., Hansman, G.S., Oka, T., Ogawa, S., Takeda, N., 2006. Investigation of norovirus replication in a human cell line. Archives of Virology 151 (7), 1291–1308.

Kukkula, M., Maunula, L., Silvennoinen, E., Bonsdorff, C.H., 1999. Outbreak of viral gastroenteritis due to drinking water contaminated by Norwalk-like Viruses. The Journal of Infectious Diseases 180 (December), 1771–1776.

Kuzmanovic, D.A., Elashvili, I., Wick, C., O'Connell, C., Krueger, S., 2003. Bacteriophage MS2: molecular weight and spatial distribution of the protein and RNA components by small-angle neutron scattering and virus counting. Structure 11 (11), 1339–1348.

Maeda, S., 1989. Gene transfer vectors of a baculovirus, *Bombyx mori*, and their use for expression of foreign genes in insect cells. In: Mitsuhashi, J. (Ed.), Invertebrate Cell System Applications. CRC Press, Boca Raton, Florida, pp. 167–181.

Matsui, Y., Matsushita, T., Sakuma, S., Gojo, T., Mamiya, T., Suzuoki, H., Inoue, T., 2003. Virus inactivation in aluminum and polyaluminum coagulant. Environmental Science and Technology 37 (22), 5175–5180.

Matsushita, T., Matsui, Y., Inoue, T., 2004. Irreversible and reversible adhesions between virus particles and hydrolyzing precipitating aluminum: a function of coagulation. Water Science and Technology 50 (12), 201–206.

Maunula, L., Miettinen, I.T., Bonsdorff, C.H., 2005. Norovirus outbreaks from drinking water. Emerging Infectious Diseases 11 (11), 1716–1721.

Mayer, B.K., Ryu, H., Abbaszadegan, M., 2008. Treatability of U.S. Environmental Protection Agency Contaminant Candidate List viruses: removal of coxsackievirus and echovirus using enhanced coagulation. Environmental Science and Technology 42 (18), 6890–6896.

Nasser, A., Weinberg, D., Dinoor, N., Fattal, B., Adin, A., 1995. Removal of hepatitis A virus (HAV), poliovirus and MS2 coliphage by coagulation and high rate filtration. Water Science and Technology 31 (5–6), 63–68.

Nygård, K., Torvén, M., Ancker, C., Knauth, S.B., Hedlund, K.O., Giesecke, J., Andersson, Y., Svensson, L., 2003. Emerging genotype (GGIIb) of norovirus in drinking water, Sweden. Emerging Infectious Diseases 9 (12), 1548–1552.

O'Connell, K.P., Bucher, J.R., Anderson, P.E., Cao, C.J., Khan, A.S., Gostomski, M.V., Valdes, J.J., 2006. Real-time fluorogenic reverse transcription-PCR assays for detection of bacteriophage MS2. Applied and Environmental Microbiology 72 (1), 478–483.

Parker, T.D., Kitamoto, N., Tanaka, T., Hutson, A.M., Estes, M.K., 2005. Identification of genogroup I and genogroup II broadly reactive epitopes on the norovirus capsid. Journal of Virology 79 (12), 7402–7409.

Phan, T.G., Kaneshi, K., Ueda, Y., Nakaya, S., Nishimura, S., Yamamoto, A., Sugita, K., Takanashi, S., Okitsu, S., Ushijima, H., 2007. Genetic heterogeneity, evolution, and recombination in noroviruses. Journal of Medical Virology 79 (9), 1388–1400.

Ramani, S., Kang, G., 2009. Viruses causing childhood diarrhoea in the developing world. Current Opinion in Infectious Diseases 22 (5), 477–482.

Rao, V.C., Symons, J.M., Ling, A., Wang, P., Metcalf, T.G., Hoff, J.C., Melnick, J.L., 1988. Removal of hepatitis A virus and rotavirus by drinking water treatment. Journal American Water Works Association 80 (2), 59–67.

Redman, J.A., Grant, S.B., Olson, T.M., 1997. Filtration of recombinant Norwalk virus particles and bacteriophage MS2 in quartz sand: importance of electrostatic interactions. Environmental Science and Technology 31 (12), 3378–3383.

Shirasaki, N., Matsushita, T., Matsui, Y., Urasaki, T., Ohno, K., 2009. Comparison of behaviors of two surrogates for pathogenic waterborne viruses, bacteriophages Qβ and MS2, during the coagulation process. Water Research 43 (3), 605–612.

Someya, Y., Takeda, N., Miyamura, T., 2000. Complete nucleotide sequence of the Chiba virus genome and functional expression of the 3C-like protease in *Escherichia coli*. Virology 278 (2), 490–500.

Stewart, T.A., Trudell, D.E., Alam, T.M., Ohlin, C.A., Lawler, C., Casey, W.H., Jett, S., Nyman, M., 2009. Enhanced water purification: a single atom makes a difference. Environmental Science and Technology 43 (14), 5416–5422.

Suzuki, T., Kanaya, T., Okazaki, H., Ogawa, K., Usami, A., Watanabe, H., Kadono-Okuda, K., Yamakawa, M., Sato, H., Mori, H., Takahashi, S., Oda, K., 1997. Efficient protein production using a *Bombyx mori* nuclear polyhedrosis virus lacking the cysteine proteinase gene. Journal of General Virology 78 (12), 3073–3080.

The Universal Virus Database of the International Committee on Taxonomy of Viruses. http://phene.cpmc.columbia.edu/index.htm (accessed 17.04.08).

Thurston-Enriquez, J.A., Haas, C.N., Jacangelo, J., Gerba, C.P., 2003. Chlorine inactivation of adenovirus type 40 and feline calicivirus. Applied and Environmental Microbiology 69 (7), 3979–3985.

Thurston-Enriquez, J.A., Haas, C.N., Jacangelo, J., Gerba, C.P., 2005. Inactivation of enteric adenovirus and feline calicivirus by ozone. Water Research 39 (15), 3650–3656.

U.S. Environmental Protection Agency, 2001. National Primary Drinking Water Standards, Office of Water. U.S. Environmental Protection Agency, Washington, DC. EPA816-F-01–007.

Utagawa, E.T., Takeda, N., Inouye, S., Kasuga, K., Yamazaki, S., 1994. 3′-Terminal sequence of a small round structured virus (SRSV) in Japan. Archives of Virology 135 (1–2), 185–192.

Zheng, D.P., Ando, T., Fankhauser, R.L., Beard, R.S., Glass, R.I., Monroe, S.S., 2006. Norovirus classification and proposed strain nomenclature. Virology 346 (2), 312–323.

遺伝子組換えノロウイルス外套タンパク粒子(rNV-VLPs)を用いたヒトノロウイルスの浄水処理性評価

白崎　伸隆[1]*・松下　拓[1]・松井　佳彦[1]・
大芝　淳[1]・浦崎　稔史[1]・大野　浩一[1]

[1]北海道大学大学院工学研究科環境創生工学専攻（〒060-8628北海道札幌市北区北13条西8丁目）

＊ E-mail: *nobutaka@eng.hokudai.ac.jp*

本研究では，細胞培養系が確立されていないヒトノロウイルスの凝集沈澱処理性を，ヒトノロウイルスの外套タンパク粒子（rNV-VLPs）を用いることによって評価した．また，病原性ウイルスの代替指標として広く用いられている大腸菌ファージQβ，MS2との処理性比較を行った．凝集沈澱処理におけるrNV-VLPsの除去率は，40 μM-Alのポリ塩化アルミニウム（PACl）添加濃度を用いた場合，約1 logであった．また，得られたrNV-VLPsの除去率は，MS2の除去率よりも低く，Qβの除去率と同程度であった．従って，MS2に比べてQβがヒトノロウイルスの凝集沈澱処理性を検討する際の指標と成り得る可能性が示唆された．

Key Words : *bacteriophages, drinking water treatment, ELISA, norovirus, virus-like particles*

1. はじめに

近年，その感染事例が世界中で年々増加しているヒトノロウイルスに代表される水系感染症を引き起こす可能性のあるウイルスは100種以上を数える[1]．これら病原性ウイルスの中でも，ヒトノロウイルスを含むカリシウイルス科のウイルスおよびピコルナウイルス科のA型肝炎ウイルスは，将来水道水質基準に組み込むべき項目として米国環境保護局（U.S.EPA）の汚染物質候補リスト（Contaminant Canndidate List 3）に挙げられている[2]．A型肝炎を引き起こすA型肝炎ウイルスは，細胞培養系が確立されているため，古くから様々な浄水処理実験が行われており[3-5]，数多くの知見が蓄積されている．その一方で，非細菌性急性胃腸炎を引き起こすヒトノロウイルスに関しては，未だ効率的な細胞培養系が確立されていないため[6]，添加実験による浄水処理性の検討を行うために必要なウイルス量を確保することが極めて困難であり，また，塩素等の消毒処理における処理性，すなわち感染力価の低下を定量することもできない．そのため，培養可能な病原性ウイルスと比べて浄水処理性の検討がほとんどなされていないのが現状である．こういった問題から，同じカリシウイルス科に属し，細胞培養系が確立されているマウスノロウイルス[7,8]，ネコカリシウイルス[9-12]，イヌカリシウイルス[11]等がヒトノロウイルスの代替指標として提案され，主に塩素，オゾン，紫外線照射といった消毒処理実験に使用されている．しかしながら，これら代替ウイルスとヒトノロウイルスとの間に，浄水処理における挙動の相関がどの程度あるかは明らかになっていない．

その一方で，ヒトノロウイルスRNAの構造タンパク質領域をバキュロウイルスに取り込み，昆虫細胞で発現させることによって，ヒトノロウイルスの外套タンパク粒子（rNV-VLPs: recombinant Norovirus-Virus Like Particles）を多量に得る技術が確立されている[13]．rNV-VLPsは，野生のヒトノロウイルスと構造的あるいは抗原的に等しいため[14]，抗原・抗体反応を利用したELISA（酵素免疫測定法）によるヒトノロウイルスの定量法も確立され[15]，培養法に頼らないヒトノロウイルス粒子の簡便かつ迅速な検出が可能となった．

本研究では，このrNV-VLPsを用いることで，これまでほとんど明らかにされてこなかったヒトノロウイルス粒子の物理的な浄水処理性について詳細に検討することを目的とし，凝集沈澱処理における処理性を評価した．また，病原性ウイルスの代替指標として広く用いられて

いる大腸菌ファージQβ，MS2との処理性比較も行い，これら大腸菌ファージのヒトノロウイルス粒子に対する代替指標としての可能性を検討した．なお，先にも記述したように，rNV-VLPsは，野生のヒトノロウイルスと構造的あるいは抗原的に等しいため，表面電位など浄水処理性に大きく寄与するであろう特性も同等と考えられる．また，内部にRNAを持たず感染性がないため，特別な施設を必要とせず安全にヒトノロウイルス粒子の浄水処理性について検討することが可能となる．

これまで，rNV-VLPsを土壌浸透の研究に使用した例[16]はあるが，水処理分野に応用した例はない．従って，本研究は，ヒトノロウイルスの浄水処理性評価にrNV-VLPsを使用した世界で初めての試みである．

2. 実験方法

(1) 使用したrNV-VLPs，大腸菌ファージ

a) rNV-VLPsの作成，精製，定量法

本研究では，我が国で分離されたヒトノロウイルス（Chiba virus，Genogroup I，AB042808）の外套タンパクを組換えバキュロウイルスとカイコを用いたタンパク質発現法によって作成し，実験に使用した．

Chiba virusは直径約38 nmの正20面体構造を有しており，一本鎖RNA（7,697 bases）を遺伝子として持つ[17]．rNV-VLPsの作成に先立ち，RNAの構造タンパク質領域であるORF（Open Reading Frame）2，ORF3および非翻訳領域である3'UTR（Untranslated Reasion）の5,346-7,697 bases部分（2,352 bases）の両末端に制限酵素認識部位（*Eco*RIおよび*Pst*I）を付加した2,422 basesからoligo DNAを合成し，連結，伸長させることで二本鎖DNA全長断片（人工合成遺伝子断片）を作成した．これをGateway BP反応によりドナーベクターpDONR221（Invitrogen）にクローニングした後，改めてトランスファーベクターpM0NHT4（片倉工業）に挿入した．得られたpM0NHT4とバキュロウイルスとをカイコ細胞（BmN細胞，片倉工業）に同時挿入することにより遺伝子組換えバキュロウイルスを作成し，新たに遺伝子組換えバキュロウイルスをカイコ（*Bombyx mori*，片倉工業）に感染させることでChiba virusのrNV-VLPsを発現させた．

rNV-VLPsを発現したカイコを磨砕処理した後，遠心分離し，上清をニッケルカラムを用いて精製した．その後，透析処理を行い，Milli-Q水にバッファー置換することでrNV-VLPsの高濃度保存液を得た．なお，ニッケルカラムによるrNV-VLPsの精製に先立ち，発現タンパク質にHisタグの導入を行った．精製後のHisタグの切り離しは行っていないが，導入したHisタグの分子量（約1 kDa）は，rNV-VLPsを構成する発現タンパク質（約58 kDa）に比べて非常に小さいため，HisタグがrNV-VLPの凝集沈澱処理性に影響する可能性は極めて小さいと考えられた．

rNV-VLPsの定量には，野生のヒトノロウイルス検出に用いられる抗原キット（NV-AD II，デンカ生研）を用い，ELISAにより行った．なお，マイクロプレートの吸光度（主波長450 nm，副波長630 nm）の測定には，マイクロプレートリーダー（MTP-300，コロナ電気）を用いた．

b) 大腸菌ファージの培養，精製，定量法

本研究では，（独）製品評価技術基盤機構（NITE）バイオテクノロジー分野 生物遺伝資源部門（NBRC）から分譲された大腸菌ファージQβ（NBRC 20012）およびMS2（NBRC 102619）を使用した．レビウイルス科に属する大腸菌ファージQβおよびMS2は，直径約24 nmの正20面体構造を有しており，一本鎖RNAを遺伝子として持つ[18]．この構造がA型肝炎ウイルスやポリオウイルスと類似しているため，水系感染症ウイルスの代替指標として広く用いられている．なお，大腸菌ファージQβの外套タンパクの疎水性は，MS2の外套タンパクに比べて高いことが知られている[19]．

大腸菌ファージは，F繊毛大腸菌（NBRC 13965）を用いて37℃のシェイキングバス内にて22-24時間振とう培養した後，2,000 × *g*にて10分間遠心分離し，上清をメンブレンフィルター（膜孔径 0.45 μm，酢酸セルロース，Advantec）にて滅菌ろ過することにより高濃度保存液を得た．得られた高濃度保存液中の有機物の持ち込みを低減させるため，実験に先立ち，遠心式フィルターユニットAmicon Ultra-15（分画分子量 100,000，再生セルロース，Millipore）を用いて，12 mLの高濃度保存液を5,000 × *g*にて20分間遠心濃縮し，得られた約100 μLの濃縮液に12 mLのMilli-Q水を加えることでバッファー置換した．

大腸菌ファージQβおよびMS2の定量には，プラック形成法およびリアルタイム定量RT-PCR法を用いた．なお，プラック形成法は，Adams[20]の方法に従って行った．一方，リアルタイム定量RT-PCR法においては，QIAamp MinElute Virus Spin Kit（Qiagen）を用いて大腸菌ファージのRNAを抽出し，これをHigh Capacity cDNA Reverse Transcription Kit with RNase Inhibitor（Applied Biosystems）を用いて逆転写させ，cDNAを合成した．このcDNAをTaqMan Universal PCR Master Mix with UNG（Applied Biosystems），プライマー（最終濃度 400 nM，タカラバイオ），プローブ（最終濃度 250 nM，Applied Biosystems），Distilled waterと混合した後，ABI 7300 Real-Time PCR System（Applied Biosystems）に供した．本研究

表-1. プライマーとプローブの塩基配列

Coliphage		Oligonucleotide sequences	Positions	References
Qβ	Forward primer	5'-TCA AGC CGT GAT AGT CGT TCC TC-3'	49-71	21)
	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	22)
	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	

で使用したプライマーおよびプローブの塩基配列を表-1に示す．なお，PCR反応は，50℃で2分間および95℃で10分間の加熱を行った後，95℃で15秒間と60℃で1分間から成るサイクルを40回繰り返した．なお，本研究で行ったプラック形成法には，大腸菌ファージQβおよびMS2の宿主であるF繊毛大腸菌(NBRC 13965)を用いたため，後述する同時添加試料中のQβおよびMS2濃度を判別定量することができなかった．そのため，判別定量が可能なリアルタイムRT-PCR定量法にて同時添加試料中のQβおよびMS2濃度を定量した．

(2) 凝集沈澱処理実験

本研究では，回分式凝集沈澱処理によるrNV-VLPs，大腸菌ファージの処理性を評価した．精製したrNV-VLPsを10^{11} VLPs/mL，大腸菌ファージQβおよびMS2を10^{8} PFU/mLになるように同時添加した北海道札幌市豊平川河川水（札幌市水道局藻岩浄水場原水: pH 7.2，DOC 0.8 mg/L，OD260 0.019 cm^{-1}，濁度 0.6 NTU，アルカリ度 17.2 mg-$CaCO_3$/L）を原水とし，ビーカーに400 mL添加した．ここに，凝集剤としてポリ塩化アルミニウム（PACl，多木化学），硫酸バンド（Alum，多木化学），塩化第二鉄（$FeCl_3$，和光純薬）のいずれかを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），60 μM（1.62 mg-Al/L or 3.36 mg-Fe/L）になるように添加し，直ちにHClもしくはNaOHにてpHを5.8あるいは6.8に調整した．これを攪拌翼を用いてG値200 s^{-1}にて2分間急速攪拌，20 s^{-1}にて28分間緩速攪拌し，20分間静置した．なお，静置後のpH変化が最大で0.2程度であったため，凝集反応時のpH調整は行わなかった．試料は，原水，急速攪拌後，緩速攪拌後，静置後に採取し，rNV-VLPsあるいは大腸菌ファージの濃度を定量した．

(3) 電子顕微鏡観察

作成したrNV-VLPsの粒子形状を確認するために，ネガティブ染色法による透過型電子顕微鏡観察を行った．精製したrNV-VLPs溶液10 μLをコロジオン膜貼付銅製グリット（400メッシュ，日新EM）に添加し，1分間静置することでrNV-VLPsをグリットに吸着させた．余剰のrNV-VLPs溶液をろ紙を用いてグリット上から排除した後，染色液として2%リンタングステン酸（pH 5.5）10 μLをグリットに添加し，45秒間静置することでグリット上のrNV-VLPsを染色した．余剰の染色液をろ紙を用いてグリット上から排除した後，グリットを透過型電子顕微鏡（TEM，H-7650，日立ハイテク）に供し，rNV-VLPsの構造を観察した．

(4) 塩化セシウム平衡遠心法

作成したrNV-VLPsの比重を測定するために，塩化セシウム平衡遠心法を行った．滅菌済み塩化セシウム溶液（1.6 g/cm^3）をMilli-Q水にて段階希釈し，1.2-1.6 g/cm^3の塩化セシウム溶液を作成した．なお，rNV-VLPsは，1.2 g/cm^3の塩化セシウム溶液に10^{12} VLPs添加した．それぞれの塩化セシウム溶液を遠沈管に充填し，密度勾配を作成した後，冷却超遠心機（CP80MX，日立工機）に供し，100,000 × gにて18時間超遠心を行った．その後，試料をシリンジを用いて約1 mLずつに分画し，各画分のrNV-VLPs濃度を定量した．また，各画分の比重を電子天秤（AG245，メトラー・トレド）を用いて秤量した．

(5) 電気移動度測定

河川水中におけるrNV-VLPsおよび大腸菌ファージQβ，MS2の表面電位特性を把握するために，電気移動度の測定を行った．分画分子量100,000のUF膜（YM-100，再生セルロース，Millipore）にてろ過した豊平川河川水を，HClを用いてpH 6.8に調整した後，精製したrNV-VLPsを10^{10} VLPs/mL，あるいはQβ，MS2を10^{10} PFU/mLになるように添加した．これらの試料の電気移動度をレーザーゼータ電位計（Zetasizer Nano ZS，Malvern）にて測定した．

3. 結果と考察

(1) ELISAによるrNV-VLPsの定量

ELISAによるrNV-VLPsの定量範囲を把握するため，作成したrNV-VLPsの高濃度保存液（濃度既知）を豊平川河川水を用いて10^{6}-10^{11} VLPs/mLに希釈した後，ELISAにより定量した．結果を図-1に示す．図より，10^{75} VLPs/mL以下の濃度および10^{105} VLPs/mL以上の濃度にお

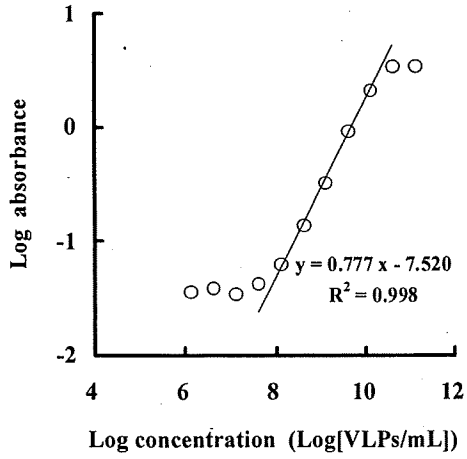

図-1. ELISAによるrNV-VLPsの検量線

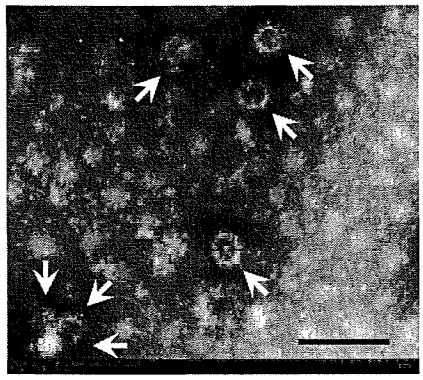

図-2. 作成したrNV-VLPsの電子顕微鏡写真: Scale bar 100 nm

いては，濃度依存性なく吸光度は同程度であった．従って，本研究で使用したヒトノロウイルス検出キットでは，10^8-10^{10} VLPs/mLの濃度範囲においてrNV-VLPsの定量が可能であると判断された．また，図に示すように，10^8-10^{10} VLPs/mLの濃度範囲においてrNV-VLPs濃度と吸光度の間に高い直線性が得られた．以上の結果から，作成したrNV-VLPsは，野生のヒトノロウイルスと同等の抗原性を有し，ELISAによって定量することが可能であると判断された．なお，10^{10} VLPs/mLを超える試料においては，豊平川河川水にて適宜希釈した後にELISAによって定量した．

(2) 発現したrNV-VLPsの基本特性

組換えバキュロウイルスとカイコを用いたタンパク質発現法によって作成したrNV-VLPsの電子顕微鏡写真を図-2に示す．図より，粒子状に自己組織化されたrNV-VLPs（矢印にて表示）が確認された．また，観察されたrNV-VLPsの中から10粒子を無作為に抽出し，写真上

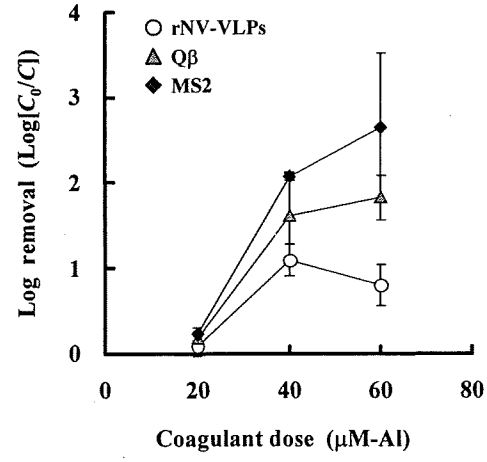

図-3. PACIを用いた凝集沈澱処理におけるrNV-VLPs，Qβ，MS2の除去率: pH 6.8，各値は2-3回の実験結果の平均値と標準偏差にて表示

で直径を測定したところ，35.7 nm ± 3.2 nmであった．この直径は，野生のヒトノロウイルスの直径（約38 nm）[17]と同程度であった．

加えて，塩化セシウム平衡遠心法を行った結果，作成したrNV-VLPsの比重は1.28 g/cm^{3}であった．この比重は，RNAを持たない分わずかに小さいものの，野生のヒトノロウイルスの比重（1.36-1.37 g/cm^3）[23]と同程度であると判断された．また，この値は，既往のrNV-VLPsの比重（1.27-1.31 g/cm^3）[13,24]と同程度であった．以上の結果から，作成したrNV-VLPsの構造は野生のヒトノロウイルスと同等であると判断された．

(3) PACIによる凝集沈澱処理

凝集剤としてPACIを用いた場合の凝集沈澱処理後（静置後）のrNV-VLPs，Qβ，MS2の除去率を図-3に示す．なお，図の縦軸はLog[C_0/C]（C_0: 原水のrNV-VLP濃度，あるいは大腸菌ファージ濃度，C: 処理水のrNV-VLPs濃度，あるいは大腸菌ファージ濃度）にて表記し，大腸菌ファージ濃度はリアルタイム定量RT-PCR法にて定量した．図より，20 μM-AlのPACI添加濃度において，rNV-VLPs，Qβ，MS2の除去率はそれぞれ0.2 log以下であったのに対し，PACI添加濃度を上げることにより，rNV-VLPs，Qβ，MS2共に除去率が向上し，40 μM-Al以上のPACI添加濃度では約1-2 logの除去率が得られた．また，rNV-VLPs，Qβ，MS2の除去率を比較すると，rNV-VLPsの除去率は，いずれのPACI添加濃度においてもQβ，MS2の除去率よりも低くなった．

凝集処理性に影響を与える因子の一つと考えられる表面電位特性を把握するために，電気移動度の測定を行ったところ，rNV-VLPs，Qβ，MS2の電気移動度はそれぞれ-0.630，-1.344，-1.160 (μm/s)/(V/cm)となり，rNV-VLPsの

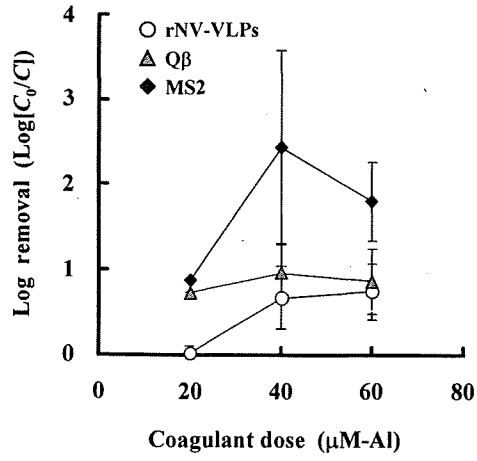

図-4. Alumを用いた凝集沈澱処理におけるrNV-VLPs, Qβ, MS2の除去率: pH 6.8, 各値は1-4回の実験結果の平均値と標準偏差にて表示

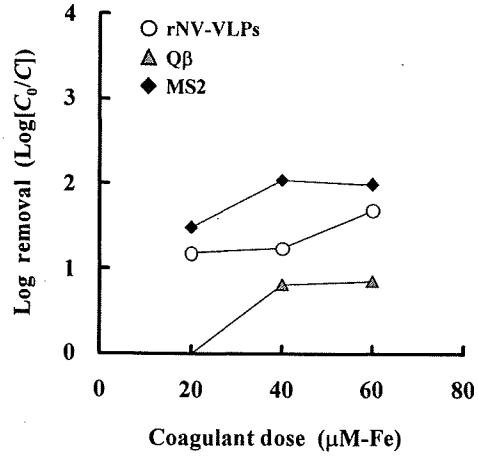

図-5. $FeCl_3$を用いた凝集沈澱処理におけるrNV-VLPs, Qβ, MS2の除去率: pH 5.8, 各値は1回の実験結果

電気移動度の絶対値はQβ, MS2に比べて小さかった. 電気移動度の絶対値が小さい粒子は, 粒子間の電気的反発力が小さいため, 凝集剤の添加により容易に凝集すると考えられるが, rNV-VLPsの除去率は, Qβ, MS2の除去率よりも低くなった. 従って, rNV-VLPsと大腸菌ファージの粒子径の違いや外套タンパクの疎水性の違いなど, 表面電位特性以外の因子も凝集処理性に寄与している可能性が考えられた.

(4) Alumによる凝集沈澱処理

凝集剤としてAlumを用いた場合の凝集沈澱処理後（静置後）のrNV-VLPs, Qβ, MS2の除去率を図-4に示す. 図より, 20 μM-AlのPACl添加濃度において, rNV-VLPs, Qβ, MS2の除去率はそれぞれ0.0, 0.7, 0.9 logであったのに対し, Alum添加濃度を上げることにより, rNV-VLPs, Qβ, MS2共に除去率が向上し, 40 μM-AlのAlum添加濃度ではそれぞれ0.7, 1.0, 2.4 logの除去率が得られた. また, rNV-VLPs, Qβ, MS2の除去率を比較すると, PAClを用いた場合と同様, rNV-VLPsの除去率は, いずれのAlum添加濃度においてもQβ, MS2の除去率よりも低くなった.

凝集剤として約50 μM-AlのAlumを用いた既往の凝集沈澱処理研究[5]においては, A型肝炎ウイルスで0.9 log, ポリオウイルスで0.2 log, MS2で0.3 logの除去率が得られている. 既往研究において, MS2の除去率がA型肝炎ウイルスの除去率よりも低く, ポリオウイルスの除去率と同程度であったことを考慮すると, Alumを用いた凝集沈澱処理においては, rNV-VLPsはA型肝炎ウイルスやポリオウイルスに比べて除去し難い可能性が考えられた.

(5) $FeCl_3$による凝集沈澱処理

凝集剤として$FeCl_3$を用いた場合の凝集沈澱処理後（静置後）のrNV-VLPs, Qβ, MS2の除去率を図-5に示す. なお, 一般的な$FeCl_3$の最適凝集pHは, PAClやAlumに比べて低いことが知られているため[25], pH 5.8において凝集沈澱処理実験を行った. 図より, 20 μM-FeのPACl添加濃度において, Qβ, MS2の除去率はそれぞれ0.0, 1.5 logであったのに対し, $FeCl_3$添加濃度を上げることにより, QβおよびMS2の除去率は向上し, 40 μM-Feの$FeCl_3$添加濃度ではそれぞれ0.8, 2.0の除去率が得られた. 一方, rNV-VLPsの除去率は, 20 μM-Feの$FeCl_3$添加濃度において1.2 logであり, $FeCl_3$添加濃度を40 μM-Feに上げた場合であっても除去率は向上しなかったものの, 60 μM-Feの$FeCl_3$添加濃度では1.7 logの除去率が得られた. また, rNV-VLPs, Qβ, MS2の除去率を比較すると, PAClやAlumを用いた場合と異なり, rNV-VLPsの除去率は, いずれの$FeCl_3$添加濃度においてもQβの除去率よりも高くなり, MS2の除去率よりも低くなった.

本研究で用いた凝集剤添加濃度とは大きく異なるが, 凝集剤として約250 μM-Feの$FeCl_3$を用いた既往の凝集沈澱処理研究（pH 5-6）[26]においては, アデノウイルスで2.6 log, ネコカリシウイルスで2.5 log, MS2で2.3 logの除去率が得られている. 既往研究において, MS2の除去率がアデノウイルス, ネコカリシウイルスの除去率よりも低かったことを考慮すると, $FeCl_3$を用いた凝集沈澱処理においては, rNV-VLPsはアデノウイルスやネコカリシウイルスに比べて除去し難い可能性が考えられた.

(6) 各凝集剤における除去率の比較

凝集剤添加濃度40 μMにおける凝集沈澱処理後（静置

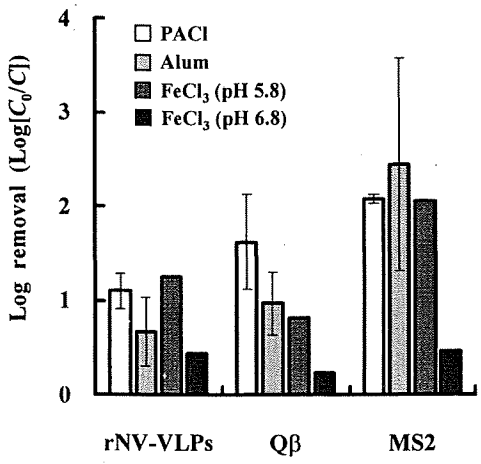

図-6. 各凝集剤におけるrNV-VLPs，Qβ，MS2の除去率比較: Coagulant dose 40 μM

後）のrNV-VLPs，Qβ，MS2の除去率を図-6に示す．比較のため，pH 6.8における$FeCl_3$を用いた実験結果についても示す．図より，rNV-VLPsにおいては，凝集剤としてPACIおよび$FeCl_3$（pH 5.8）を用いた場合に1 log以上の除去率が得られた．一方，Alum，$FeCl_3$（pH 6.8）を用いた場合の除去率はそれぞれ0.7，0.4 logであった．Qβにおいては，PACIを用いた場合の除去率が1.6 logと最も高く，また，MS2においてはPACI，Alum，$FeCl_3$（pH 5.8）のいずれにおいても2 log程度の除去率が得られた．この結果から，PACIが最も効果的にrNV-VLPs，Qβ，MS2を除去できることが分かった．

rNV-VLPs，Qβ，MS2の除去率を比較すると，$FeCl_3$（pH 6.8）を用いた場合を除き，rNV-VLPsとQβは全体として約1 log程度の除去率であったのに対し，MS2は約2 log程度の除去率となった．欧米諸国においては，MS2が病原性ウイルスの代替指標として幅広く用いられているが，本研究で行った凝集沈澱処理においては，rNV-VLPsの除去率はMS2の除去率よりも低く，Qβの除去率と同程度であったため，MS2に比べてQβがヒトノロウイルスの指標と成り得る可能性が考えられた．

我が国の水道水質基準には，微生物に関する項目として一般細菌，大腸菌が規定されているが，ウイルスに関する基準は現在のところない．一方，U.S.EPAは，浄水処理（ろ過および消毒）によって表流水，地下水の腸管系ウイルスを4 log除去あるいは不活化することを規定している[27]．本研究で行った凝集沈澱処理においては，rNV-VLPsの除去率は最大で1.7 log（$FeCl_3$，60 μM-Fe，pH 5.8）であったことから，ヒトノロウイルスを4 log除去あるいは不活化するためには，後段にろ過処理，消毒処理等を組み合わせる必要があると考えられる．

4. 結論

本研究では，細胞培養系が確立されていないヒトノロウイルスの凝集沈澱処理性を，粒子形状が保証されたヒトノロウイルスの外套タンパク粒子（rNV-VLPs）を用いることによって評価した．また，病原性ウイルスの代替指標として広く用いられている大腸菌ファージQβ，MS2との処理性比較を行った．

本研究で得られた知見を以下にまとめる．

(1) rNV-VLPsを用いることにより，ヒトノロウイルスの凝集沈澱処理性を評価することができた．

(2) 凝集沈澱処理におけるrNV-VLPsの除去率は，凝集剤添加濃度40 μMにおいて0.4-1.2 logであり，PACI，$FeCl_3$（pH 5.8）を用いた場合に1 log以上の除去率が得られた．

(3) 凝集沈澱処理におけるrNV-VLPsの除去率は，MS2の除去率よりも低く，また，Qβの除去率と同程度であった．従って，MS2に比べてQβがヒトノロウイルスの指標と成り得る可能性が考えられた．

謝辞：本研究は，科学研究費補助金　若手研究(B)「ウイルス外套タンパク粒子を用いたノロウイルスの浄水処理性の検討」（平成19-20年度，課題番号 19760368，代表　松下拓）ならびに厚生労働科学研究費補助金「飲料水の水質リスク管理に関する統合的研究」（平成19-21年度，課題番号 19310801，主任研究者　松井佳彦）により行われた．記して感謝の意を表す．

参考文献

1) Havelaar A.H., Olphen M.V. and Drost Y.C.: F-specific RNA bacteriophage are adequate model organisms for enteric viruses in fresh water, *Applied and Environmental Microbiology*, Vol. 59, No. 9, pp. 2956-2962, 1993.

2) U.S. Environmental Protection Agency: Drinking Water Contaminant Candidate List and Regulatory Determinations, Contaminant Candidate List 3 (CCL 3), U.S. Environmental Protection Agency, Washington DC., 2008.

3) Grabow W.O., Gauss-Müller V., Prozesky O.W. and Deinhardt F.: Inactivation of hepatitis A virus and indicator organisms in water by free chlorine residuals, *Applied and Environmental Microbiology*, Vol. 46, No. 3, pp. 619-624, 1983.

4) Herbold, K., Flehmig B. and Botzenhart K.,: Comparison of ozone inactivation, in flowing water, of hepatitis A virus, poliovirus 1, and indicator organisms, *Applied and Environmental Microbiology*, Vol. 55, No. 11, pp. 2949-2953, 1989.

5) Nasser A., Weinberg D., Dinoor N., Fattal B. and Adin A.: Removal of hepatitis A virus (HAV), poliovirus and MS2 coliphage by coagulation and high rate filtration, *Water Science and Technology*, Vol. 31, No. 5-6, pp. 63-68, 1995.

6) Zheng D.P., Ando T., Fankhauser R.L., Beard R.S., Glass R.I. and Monroe S.S.: Norovirus classification and proposed strain nomenclature, *Virology*, Vol. 346, No. 2, pp. 312-323, 2006.

7) Park G.W., Boston D.M., Kase J.A., Sampson M.N. and Sobsey M.D.: Evaluation of liquid- and fog-based application of sterilox hypochlorous acid solution for surface inactivation of human norovirus, *Applied and Environmental Microbiology*, Vol. 73, No. 14, pp. 4463-4468, 2007.

8) 北島正章，遠矢幸伸，松原康一，原本英司，宇田川悦子，片山浩之，大垣眞一郎：新たな代替指標としてマウス分離株を用いた水道水中のノロウイルスの塩素耐性の解明，環境工学研究論文集，Vol. 45, pp. 361-370, 2008.

9) Thurston-Enriquez J.A., Haas C.N., Jacangelo J., Riley K. and Gerba C.P.: Inactivation of feline calicivirus and adenovirus type 40 by UV radiation, *Applied and Environmental Microbiology*, Vol. 69, No. 1, pp. 577-582, 2003.

10) Thurston-Enriquez J.A., Haas C.N., Jacangelo J. and Gerba C.P.: Chlorine Inactivation of Adenovirus Type 40 and Feline Calicivirus, *Applied and Environmental Microbiology*, Vol. 69, No. 7, pp. 3979-3985, 2003.

11) Duizer E., Bijkerk P., Rockx B., Groot A., Twisk F. and Koopmans M.: Inactivation of Caliciviruses, *Applied and Environmental Microbiology*, Vol. 70, No. 8, pp. 4538-4543, 2004.

12) Thurston-Enriquez J.A., Haas C.N., Jacangelo J. and Gerba C.P.: Inactivation of enteric adenovirus and feline calicivirus by ozone, *Water Research*, Vol. 39, No. 15, pp. 3650-3656, 2005.

13) Jiang X., Wang M., Graham D.Y. and Estes M.K.: Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein, *Journal of Virology*, Vol. 66, No. 11, pp. 6527-6532, 1992.

14) White L.J., Ball J.M., Hardy M.E., Tanaka T.N., Kitamoto N. and Estes M.K.: Attachment and entry of recombinant Norwalk virus capsids to cultured human and animal cell lines, *Journal of Virology*, Vol. 70, No. 10, pp. 6589-6597, 1996.

15) Parker T.D., Kitamoto N., Tanaka T., Huston A.M. and Estes M.K.: Identification of genogroup I and genogroup II broadly reactive epitopes on the norovirus capsid, *Journal of Virology*, Vol. 79, No. 12, pp. 7402-7409, 2005.

16) Redman J.A., Grant S.B. and Olson T.M.: Filtration of recombinant Norwalk virus particles and bacteriophage MS2 in quartz sand: importance of electrostatic interactions, *Environmental Science and Technology*, Vol. 31, No. 12, pp. 3378-3383, 1997.

17) Someya, Y., Takeda N. and Miyamura T.: Complete nucleotide sequence of the chiba virus genome and functional expression of the 3C-like protease in *Escherichia coli*, *Virology*, Vol. 278, No. 2, pp. 490-500, 2000.

18) 植竹久雄: ウイルス学（第4版）, pp. 34, 理工学社, 1995.

19) Langlet J., Gaboriaud F., Duval J.F.L. and Gantzer C.: Aggregation and surface properties of F-specific RNA phages: implication for membrane filtration processes, *Water Research*, Vol. 42, No. 10-11, pp. 2769-2777, 2008.

20) Adams M.H.: *Bacteriophages*, Interscience Publishers, New York, NY, USA, pp. 450-454, 1959.

21) 片山浩之，嶋崎明寛，大垣眞一郎：陰電荷膜を用いた酸洗浄・アルカリ誘出によるウイルス濃縮法の開発，水環境学会誌，Vol. 25, No. 8, pp. 469-475, 2002.

22) O'Connell K.P., Bucher J.R., Anderson P.E., Cao C. ., Khan A.S., Gostomski M.V. and Valdes J.J.: Real time fluorogenic reverse transcription-PCR assays for detection of bacteriophage MS2, *Applied and Environmental Microbiology*, Vol. 72, No. 1, pp. 478-483, 2006.

23) Utagawa E.T., Takeda N., Inouye S., Kasuga K. and Yamazaki S.: 3'-Terminal sequence of a small round structured virus (SRSV) in Japan, *Archive of Virology*, Vol. 135, No. 1-2, pp. 185-192, 1994.

24) Katayama K., Hansman G.S., Oka T., Ogawa S. and Takeda N.: Investigation of norovirus replication in a human cell line, *Archive of Virology*, Vol. 151, No. 7, pp. 1291-1308, 2006.

25) American Water Works Association, in: F.W. Pontius (Ed.), Water Quality and Treatment, A Handbook of Community Water Supplies, 4th ed., McGraw-Hill, New York, pp. 300-305, 1990.

26) Abbaszadegan M., Mayer B.K., Ryu H. and Nwachuku N.: Efficacy of removal of CCL viruses under enhanced coagulation conditions, *Environmental Science and Technology*, Vol. 41, No. 3, pp. 971-977, 2007.

27) U.S. Environmental Protection Agency: National Primary Drinking Water Standards, EPA816-F-01-007, Office of Water, U.S. Environmental Protection Agency, Washington, DC, 2001.

(2009. 5. 22 受付)

Evaluation of Removal Performance of Human Norovirus during Drinking Water Treatment Process by using Recombinant Norovirus VLPs

Nobutaka SHIRASAKI[1], Taku MATSUSHITA[1], Yoshihiko MATSUI[1], Atsushi OSHIBA[1], Toshifumi URASAKI[1] and Koichi OHNO[1]

[1]Division of Built Environment, Graduate School of Engineering, Hokkaido University

Norovirus (NV) is the prototype strains of a group of human caliciviruses responsible for epidemic outbreaks of acute gastroenteritis in the worldwide. As a result of the lack of a mammalian cell culture model for this virus, the studies on drinking water treatment such as separation and disinfection processes are still hampered. In the present study, we tried to investigate the removal performance of NV as particles during the coagulation–sedimentation process by using recombinant NV virus-like particles (rNV-VLPs) that are morphologically and antigenically similar to native NV. In addition, the behaviors of bacteriophage Qβ and MS2 were also experimentally investigated to be compared with that of rNV-VLPs, and then to discuss the suitability of these bacteriophages as surrogates for NV. Approximately 1-log removal was observed for rNV-VLPs with 40 μM-Al of polyaluminum chloride dosage in the coagulation–sedimentation process. In addition, the removal ratios of MS2 were approximately 1-log larger than that of rNV-VLPs and Qβ. Accordingly, Qβ is more suitable surrogates for native NV compared to the MS2 in the coagulation–sedimentation process.

Author's personal copy

WATER RESEARCH 43 (2009) 605–612

Available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/watres

Comparison of behaviors of two surrogates for pathogenic waterborne viruses, bacteriophages Qβ and MS2, during the aluminum coagulation process

N. Shirasaki*, T. Matsushita, Y. Matsui, T. Urasaki, K. Ohno

Division of Built Environment, Graduate School of Engineering, Hokkaido University, N13W8, Sapporo 060-8628, Japan

ARTICLE INFO

Article history:
Received 9 May 2008
Received in revised form
2 October 2008
Accepted 6 November 2008
Published online 19 November 2008

Keywords:
Bacteriophages
Coagulation
Electrophoretic mobility
Particle size distribution
Real-time RT-PCR
Virus inactivation

ABSTRACT

Differences in the behaviors of two surrogates for pathogenic waterborne viruses, F-specific RNA bacteriophages Qβ and MS2, were investigated during the coagulation process by using river water spiked with these bacteriophages. The particle size and electrophoretic mobility of Qβ and MS2 were similar, but the removal performances of infectious Qβ and MS2, as measured by a plaque forming unit (PFU) method, differed markedly during the coagulation process. The removal ratio of the infectious Qβ concentration was approximately 2 log higher than that of the infectious MS2 concentration at all coagulant doses tested. The total Qβ and MS2 bacteriophage concentrations, which were measured by a real-time reverse transcription-polymerase chain reaction (RT-PCR) method and represented the total number of bacteriophages regardless of their infectivity, were similar after the coagulation process, suggesting that the behaviors of Qβ and MS2 as particles were similar during the coagulation process. The difference between total concentration and infectious concentration indicated that some of the bacteriophages were probably inactivated during the coagulation process. This difference was larger for Qβ than MS2, meaning that Qβ was more sensitive to the virucidal activity of the aluminum coagulant. Analysis of the PFU and real-time RT-PCR findings together suggested that the difference in removal performances of Qβ and MS2 during the coagulation process was probably caused by differences not in the extent of bacteriophage entrapment in the aluminum floc particles but in the sensitivity to virucidal activity of the aluminum coagulant.

© 2008 Elsevier Ltd. All rights reserved.

1. Introduction

During drinking water treatment, coagulation is an essential process for combining small particles into larger aggregates. Small particles in the drinking water source, such as viruses, that will not settle from suspension by gravity are destabilized and combined into larger aggregates during the coagulation process; this allows the small particles to be effectively removed by subsequent sedimentation and filtration processes. Several studies have reported the usefulness of the coagulation process for the removal of enteric viruses and bacteriophages, which are viruses that infect bacteria (Guy et al., 1977; Havelaar et al., 1995; Nasser et al., 1995).

Some bacteriophages have been evaluated as possible indicators for enteric viruses. For instance, F-specific RNA bacteriophage concentrations are highly correlated with those of enteric viruses in a wide range of water environments and water treatment processes (Havelaar et al., 1993). Because of their morphological similarity to hepatitis A viruses and polioviruses, F-specific RNA bacteriophage MS2 is widely used

* *Corresponding author.* Tel.: +81 11 706 7281; fax: +81 11 706 7279.
E-mail address: nobutaka@eng.hokudai.ac.jp (N. Shirasaki).
0043-1354/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.watres.2008.11.002

Author's personal copy

as a surrogate for pathogenic waterborne viruses in Europe and the United States (Jacangelo et al., 1995; Meng and Gerba, 1996; Redman et al., 1997; Shin and Sobsey, 1998, 2003; Sobsey et al., 1998; Meschke and Sobsey, 2003; Thurston-Enriquez et al., 2003; Zhu et al., 2005; Fiksdal and Leiknes, 2006), whereas the F-specific RNA bacteriophage Qβ tends to be used as a surrogate in Japan (Kamiko and Ohgaki, 1989; Urase et al., 1996; Otaki et al., 1998).

Previous studies have compared the behaviors of Qβ and MS2 in the physicochemical treatment process. Their behaviors are similar in the process of adsorption onto solid surfaces (such as cellulose, kaolin, carbon black and river sediment; Sakoda et al., 1997), the sandy aquifer treatment process (Dowd et al., 1998), and the direct microfiltration treatment process (Herath et al., 2000), whereas the sensitivities of Qβ and MS2 to ultraviolet radiation are significantly different (Blatchley et al., 2008). The behaviors of these two bacteriophages in the coagulation process may be different as well, although these differences have not been investigated except for previous studies of our research group (Matsui et al., 2003; Matsushita et al., 2004). If significant differences do exist, then Qβ and MS2 cannot be used as equivalent surrogates to evaluate the effectiveness of treatment processes for the removal of pathogenic waterborne viruses.

Virucidal activity during the water treatment process has been widely investigated. Ultraviolet radiation (Kamiko and Ohgaki, 1989; Meng and Gerba, 1996; Sobsey et al., 1998; Thurston-Enriquez et al., 2003) as well as disinfectants such as free chlorine, chlorine dioxide (Sobsey et al., 1998), chloramines (Shin and Sobsey, 1998) and ozone (Shin and Sobsey, 2003) are well known to inactivate viruses. Our research group has reported the virucidal activity of aluminum coagulant (Matsui et al., 2003; Matsushita et al., 2004). In our previous studies, we used the plaque forming unit (PFU) method in an attempt to determine the mechanisms and kinetics of the virucidal activity of aluminum coagulant. During coagulation of the virus suspension, although the majority of viruses were entrapped in the aluminum floc particles, some of the viruses remained suspended in the liquid phase. Not all the suspended viruses were infectious; some were likely inactivated by the virucidal activity of aluminum coagulant. Thus, virus removal during the coagulation process includes two mechanisms: entrapment in floc particles and inactivation. The PFU method, however, can detect neither the viruses entrapped in the floc particles nor the inactivated viruses judging from its measurement principle. Therefore, even though the virus concentration measured by the PFU method decreases in the liquid phase during the coagulation process, it remains unclear whether the decrease results from the entrapment in floc particles, inactivation, or both.

The reverse transcription-polymerase chain reaction (RT-PCR) method is capable of amplifying small regions of viral nucleic acid. Thus, RT-PCR can detect infectious as well as inactivated viruses, unless the target nucleic acid is lost, but not all viruses entrapped in the floc particles can be detected by this method. Calculation of the concentration of entrapped virus particles simply entails subtracting the concentration of total viruses including infectious and inactivated viruses in the liquid phase, as determined by RT-PCR, from the total virus concentration as measured in the raw water. Likewise, subtracting the concentration of infectious viruses, as determined by the PFU method, from the total virus concentration, as determined by RT-PCR, gives the number of inactivated viruses. Therefore, combining the PFU and RT-PCR methods allows entrapped viruses and inactivated viruses in the coagulation process to be clearly distinguished.

Our objectives were to investigate and compare the behaviors of two bacteriophages, Qβ and MS2, during the coagulation process by using both PFU and real-time RT-PCR methods and to elucidate what caused the differences.

2. Materials and methods

2.1. Source water and coagulant

River water was sampled from the Toyohira River (Sapporo, Japan; water quality shown in Table 1) on 12 October 2007. Polyaluminum chloride (PACl) (250A; 10.5% Al_2O_3, relative density 1.2 at 20 °C; Taki Chemical Co., Ltd., Hyogo, Japan) was used for coagulation experiments.

2.2. Bacteriophages

The bacteriophages Qβ (NBRC 20012) and MS2 (NBRC 102619) were obtained from the NITE Biological Research Center (NBRC, Chiba, Japan). Qβ 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 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×*g*, 10 min) and then passed through a membrane filter (pore size 0.45 μm, 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; Centriplus-100, Millipore Corp., Billerica, MA, USA) to prepare the bacteriophage stock solution. The concentration of each bacteriophage stock solution was approximately 10^{12} PFU/mL.

2.3. Batch coagulation experiments

Batch coagulation experiments were conducted with 200 mL of bacteriophage-spiked river water in glass beakers at 20 °C. The bacteriophage stock solution was added to the beaker at

Table 1 – Water quality of the Toyohira River.

pH	7.5
DOC (mg/L)	0.90
OD260 (cm^{-1})	0.027
Turbidity (NTU)	0.50
Alkalinity (mg-$CaCO_3$/L)	19.1

Author's personal copy

approximately 10^6 or 10^8 PFU/mL and mixed with an impeller stirrer. PACl was injected into the water as a coagulant at dosages of 0.54, 1.08 or 1.62 mg-Al/L. The pH of the water was immediately adjusted to, and maintained at, 6.8 using HCl. The water was stirred rapidly for 2 min ($G = 200\ s^{-1}$, 61 rpm) and then slowly for 28 min ($G = 20\ s^{-1}$, 13 rpm). The water was then left at rest for 20 min to settle the aluminum floc particles generated. Samples were taken from the beaker before coagulant dosing and after rapid mixing, slow mixing, and settling for quantification of the bacteriophage concentrations.

To quantify the bacteriophage concentration in the liquid phase of the floc mixture, the suspended floc particles were separated from the mixture by centrifugation (2000×*g*, 10 min), and the bacteriophage concentration in the supernatant was measured by the PFU and the real-time RT-PCR methods (see Section 2.4).

In addition, to quantify the bacteriophage concentration in the floc particles, the particles were dissolved by raising the pH of the water to 9.5 with NaOH in 12% beef extract (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) solution and the water was vortexed for 5 h at 4 °C by using direct mixer (DM-301, As One Corp., Osaka, Japan) at 2000 rpm. Beef extract was used in an effort to prevent the inactivation of bacteriophages during floc dissolution (Matsui et al., 2003). Samples of raw water and rapid mixing were taken from the beaker directly for the floc dissolution, because these samples were considered to be mixed completely during the sampling. In contrast, the floc particles settled during the coagulation process were resuspended after the settling process by strong mixing with a magnetic stirrer at 1400 rpm for subsequent sampling and floc dissolution (floc dissolution procedure was described above). After floc dissolution, the bacteriophage concentration in the sample was measured by the PFU and real-time RT-PCR methods.

Because substances such as natural organic matter (Abbaszadegan et al., 1993; Kreader, 1996) and beef extract (Abbaszadegan et al., 1993; Sano et al., 2003) are known to inhibit the amplification of the viral genome by PCR, each sample was diluted 10-fold with Milli-Q water (Milli-Q Advantage, Millipore Corp.) before the real-time RT-PCR quantification.

2.4. *Bacteriophage assays*

2.4.1. *PFU method*

The infectious bacteriophages were enumerated according to the double-layer method (Adams, 1959) by using the bacterial host *E. coli* (NBRC 13965). The average of plaque counts of triplicate plates prepared from one sample was considered as the infectious bacteriophage concentration.

2.4.2. *Real-time RT-PCR method*

Viral RNA of bacteriophages was quantified by the real-time RT-PCR method, which detects viruses regardless of their infectivity. We defined concentration measured by the real-time RT-PCR method as total bacteriophage concentration. For quantification of bacteriophages in the raw water and liquid phase of the floc mixture, viral RNA was extracted from 140 μL of sample with a QIAamp Viral RNA Mini Kit (Qiagen K.K., Tokyo, Japan) to obtain a final volume of 60 μL. For quantification of bacteriophages in the floc dissolution sample containing beef extract, a 100-μL sample was heated at 90 °C for 10 min and then cooled to 4 °C for 1 min in a thermal cycler (Thermal Cycler Dice Model TP600, Takara Bio Inc., Shiga, Japan) to extract viral RNA by destroying the capsid. 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 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. 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 50 cycles of 95 °C for 15 s and 60 °C for 1 min in an ABI Prism 7000 Sequence Detection 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 PFU method and the number of cycles for amplification in the PCR process, which is based on the assumption that the freshly prepared stock solution did not contain any inactivated bacteriophages.

2.5. *Particle size distribution*

Particle size distribution of bacteriophages was measured in prepared Milli-Q water and filtered river water. To bring the alkalinity to 20 mg-$CaCO_3$/L, 0.4 mM $NaHCO_3$ was added to the Milli-Q water, and the pH of was adjusted to 6.8 with HCl. River water was filtered through a stirred ultrafiltration cell (Model 8400, Millipore Corp.) with 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 Milli-Q and river water samples were kept for 1 day at 20 °C to stabilize the pH. Just before the measurement of particle size distribution, each bacteriophage was suspended at approximately 10^{10} PFU/mL in the prepared Milli-Q water or filtered river water using the bacteriophage stock solution. The particle size distribution of the bacteriophages was measured with a fiber-optic dynamic light-scattering spectrophotometer (FDLS-3000, Otsuka Electronics Co., Ltd., Osaka, Japan) 200 or 400 times for each sample at 25 °C and at a 90° measurement angle.

2.6. *Electrophoretic mobility*

In Milli-Q water and river water prepared as described in Section 2.5, an electrophoretic light-scattering spectrophotometer (ELS-6000, Otsuka Electronics Co., Ltd.) was used to measure electrophoretic mobility. Just before the measurement, each bacteriophage was suspended at approximately 10^{10} PFU/mL in the prepared Milli-Q water or filtered river water using the bacteriophage stock solution. The electrophoretic mobility of the bacteriophages was measured 25 times for each sample at 25 °C and at a 15° measurement angle.

Author's personal copy

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

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

3. Results and discussion

3.1. *Particle size distribution*

Fig. 1 shows the particle size distributions of Qβ and MS2 in the prepared Milli-Q water and filtered river water at pH 6.8. In both the Milli-Q and river water, the particle sizes were distributed over the range of 20–30 nm. These values correspond with the particle diameters previously reported for Qβ and MS2 (The Universal Virus Database of the International Committee on Taxonomy of Viruses). Thus, we can assume that no virus–virus aggregate was generated and these two bacteriophages were stably monodispersed in the raw water used (without coagulant dosing).

Langlet et al. (2008) reported that Qβ had a tendency to aggregate in solutions with high ionic strength: Qβ aggregated in deionized water with 100 mM $NaNO_3$ at neutral pH (ionic strength, approximately 1×10^{-1}), whereas it did not aggregate in deionized water with 1 mM $NaNO_3$ (ionic strength, approximately 1×10^{-3}). The ionic strength of both our prepared Milli-Q water and the filtered river water was approximately 1×10^{-3}.

3.2. *Effect of coagulant dose on infectious bacteriophage removal*

Fig. 2 shows the effect of coagulant dose on infectious bacteriophage removal, as measured by the PFU method after settling without centrifugal separation. ' Bacteriophage removal was not observed without PACl dosing, and the removal ratio of infectious bacteriophages ($\log[C_0/C]$) increased with coagulant dose. At a PACl dose of more than 1.08 mg-Al/L, more than 2-log removal of infectious bacteriophage was achieved for both Qβ and MS2. Therefore, the coagulation process is effective for the removal of infectious bacteriophages. Other studies have reported the usefulness of the coagulation process for removing infectious enteric viruses and infectious bacteriophages (Guy et al., 1977; Havelaar et al., 1995; Nasser et al., 1995).

In our study, however, the removal performances of Qβ and MS2 were quite different. The removal ratio of infectious Qβ was approximately 2 log higher than that of infectious MS2 at each of the PACl doses tested. This difference is detailed in the following section.

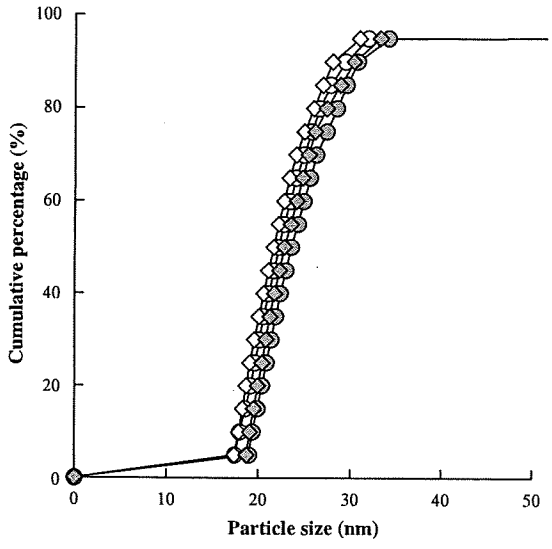

Fig. 1 – Particle size distribution of Qβ (white) and MS2 (gray) in the prepared Milli-Q water (circles) and filtered river water (diamonds), based on the number of particles. Values are the means of 200 or 400 measurements. Bacteriophage concentration in each sample was approximately 10^{10} PFU/mL.

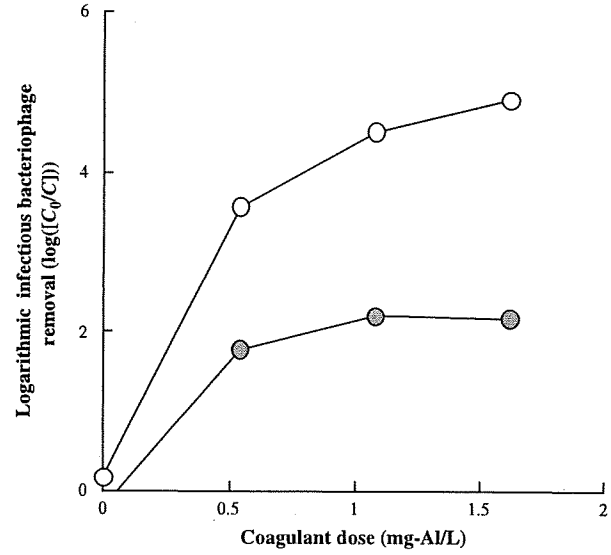

Fig. 2 – Effect of coagulant dose (0.54, 1.08 or 1.62 mg-Al/L) on infectious bacteriophage removal after settling without centrifugal separation. White and gray symbols represent Qβ and MS2, respectively. Initial bacteriophage concentrations in raw water were approximately 10^6 PFU/mL.