Quantification of bacteriophages

Viral RNA of the bacteriophages was quantified by real-time RT-PCR. 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 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.

Coagulation–ceramic MF process

Batch coagulation experiments were conducted with 400 mL of river water in glass beakers at 20°C. rNV-VLPs, Qβ, and MS2 were simultaneously added to the beakers at approximately 10^{11} VLPs/mL and 10^{8} PFU/mL, respectively, and mixed with an impeller stirrer. PACl or alum was injected into the water as the coagulant at a dose of 0.54 or 1.08 mg-Al/L. The pH of the water was immediately adjusted to, and maintained at, 6.8 using HCl or NaOH. The water was stirred rapidly for 2 min ($G = 200\,s^{-1}$) and then slowly for 28 min ($G = 20\,s^{-1}$). The water was then left at rest for 20 min to allow the generated aluminium floc particles to settle. The supernatant was fed through a ceramic MF membrane by a peristaltic pump at 83 L/(m^2·h). The raw water (C_0; before coagulant dosing) and the MF permeate (C; after 15, 30, 60, 90, and 120 min of filtration) was collected for quantification of the rNV-VLPs, Qβ, and MS2.

Electron microscopy

rNV-VLPs were observed with an electron microscope. Ten microlitres of rNV-VLP stock solution was put on a 400-mesh copper grid with a 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). Particle diameter of rNV-VLPs was expressed as the mean and standard deviation of any 10 particles on the electron micrograph.

RESULTS AND DISCUSSION

Characteristics of the rNV-VLPs produced

Figure 1 shows an electron micrograph of rNV-VLPs. The presence of particles was confirmed on the electron

Table 2 | Oligonucleotide sequences of the primers and the 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	

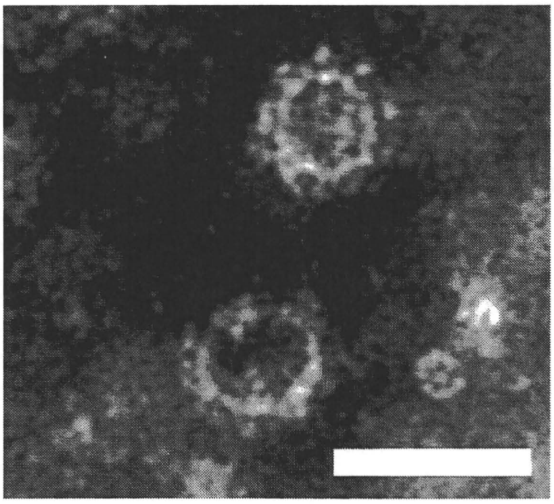

Figure 1 | Negatively stained electron micrograph of rNV-VLPs. Scale bar, 50 nm.

micrograph, and rNV capsid proteins spontaneously self-assembled into VLPs during expression. The rNV-VLP particle diameter was 35.7 ± 3.2 nm, measured by randomly choosing 10 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). This result suggests that rNV-VLPs were successfully produced by the baculovirus-silkworm expression system, and were morphologically similar to native NV.

Removal performance in the coagulation–ceramic MF process with PACl

Figure 2 shows the change in the removal ratio ($\log[C_0/C]$) of rNV-VLPs, Qβ, and MS2 with filtration time in the coagulation–ceramic MF process with PACl. Because the diameters of rNV-VLPs (35.7 ± 3.2 nm), Qβ, and MS2 (24–26 nm) are smaller than the nominal pore size of the ceramic MF membrane (0.1 μm), no removal ($<$0.2-log) of rNV-VLPs or either bacteriophage was observed without coagulation pretreatment (data not shown). In contrast, the coagulation–ceramic MF process effectively removed rNV-VLPs, Qβ, and MS2 at PACl doses of 0.54 (Figure 2a) and 1.08 mg-Al/L (Figure 2b). In addition, an effect of the coagulant dose (0.54 mg-Al/L vs. 1.08 mg-Al/L) on the removal of rNV-VLPs and both bacteriophages was observed: the filtration time-averaged removals of rNV-VLPs, Qβ, and MS2 were only 1.6-log, 0.5-log, and 0.9-log, respectively, at the coagulant dose of 0.54 mg-Al/L, whereas with the dose of 1.08 mg-Al/L, the removals were $>$ 2.9-log, 2.4-log, and 3.3-log for rNV-VLPs, Qβ, and MS2, respectively. This dose effect was most likely due to an increase in floc size with the increased coagulant dose. Increasing the coagulant dose from 0.54 to 1.08 mg-Al/L increased the size of the aluminium floc particles with adsorbed/entrapped rNV-VLPs or bacteriophages, and subsequently increased the amount of aluminium floc particles that settled out from the suspension during the

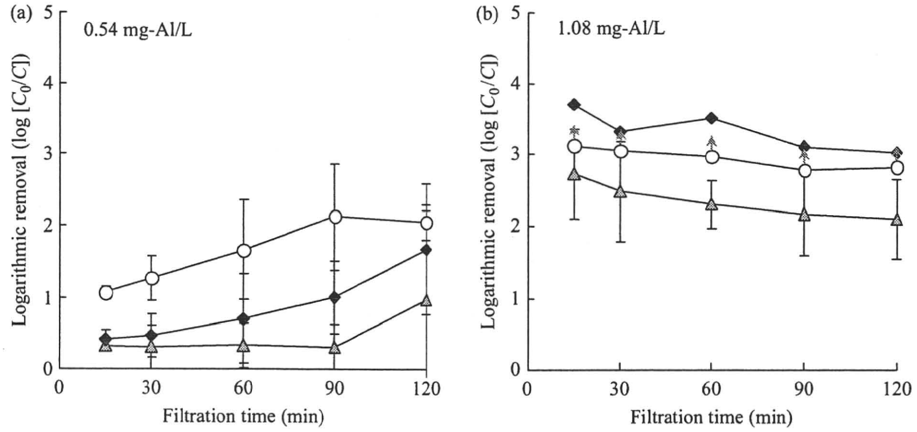

Figure 2 | rNV-VLPs, Qβ and MS2 removal by a coagulation–ceramic MF process with PACl coagulant at doses of (a) 0.54 mg-Al/L and (b) 1.08 mg-Al/L. Circles, triangles, and diamonds represent rNV-VLPs, Qβ and MS2, respectively. The arrows indicate values greater than those that could be estimated accurately by ELISA.

settling process. Consequently, the amount of aluminium floc particles that passed through a ceramic MF membrane was decreased, leading to a difference in the removal ratios of the rNV-VLPs and the two bacteriophages between PACl doses of 0.54 and 1.08 mg-Al/L.

The removal ratios of rNV-VLPs, Qβ, and MS2 gradually increased with filtration time at the PACl dose of 0.54 mg-Al/L. Ultimately, 2.0-log, 1.0-log, and 1.7-log removals were obtained for rNV-VLPs, Qβ, and MS2, respectively, at the end of the 2-h filtration. As described above, because the size and amount of aluminium floc particles that settled out from suspension were probably smaller with a PACl dose of 0.54 mg-Al/L than with one of 1.08 mg-Al/L, many of the aluminium floc particles were fed into the ceramic MF membrane and accumulated on the membrane surface as a cake layer during the MF process. This cake layer would act as a barrier to rNV-VLPs and both bacteriophages, causing the removal ratios of rNV-VLPs, Qβ, and MS2 to gradually increase with the growth of the cake layer at the PACl dose of 0.54 mg-Al/L.

In contrast, the removal ratios of rNV-VLPs, Qβ, and MS2 gradually decreased with filtration time at the PACl dose of 1.08 mg-Al/L. Our research group has demonstrated that the number of virus particles in the MF compartment increases with filtration time in the coagulation–ceramic MF system (Shirasaki *et al.* 2007). Although the experimental procedures of the present study and our previous study (Shirasaki *et al.* 2007) differed in terms of the coagulation process (batch coagulation vs. in-line coagulation), rNV-VLPs and both bacteriophages also probably accumulated in the MF compartment with filtration time in the present study. Accordingly, the concentrations of rNV-VLPs, Qβ, and MS2 in the MF permeate increased with the accumulation of rNV-VLPs and both bacteriophages in the MF compartment. In addition, because many of the aluminium floc particles had settled out from suspension during the settling process prior to the MF process with the 1.08 mg-Al/L PACl dose, the effect of the cake layer was probably smaller than with the 0.54 mg-Al/L PACl dose. The combination of these two phenomena caused the leakage of the rNV-VLPs and the two bacteriophages into the MF permeate, decreasing their removal ratios with filtration time.

The removal performance for rNV-VLPs, Qβ, and MS2 with the 1.08 mg-Al/L PACl dose at the end of the filtration (2 h) was still higher than that with the 0.54 mg-Al/L PACl dose, although the removal ratios decreased with filtration time as described above. Therefore, a PACl dose of 1.08 mg-Al/L was more effective for the removal of rNV-VLPs and both bacteriophages than the dose of 0.54 mg-Al/L in the coagulation–ceramic MF process.

Removal performance in the coagulation–ceramic MF process with alum

Figure 3 shows the change in the removal ratio of rNV-VLPs, Qβ, and MS2 with filtration time in the coagulation–ceramic MF process with alum. The coagulation–ceramic MF process effectively removed rNV-VLPs, Qβ, and MS2 at alum doses of 0.54 (Figure 3a) and 1.08 mg-Al/L (Figure 3b). In addition, an effect of the coagulant dose (0.54 mg-Al/L vs. 1.08 mg-Al/L) on the removal of rNV-VLPs and of both bacteriophages was also observed with alum: the filtration time-averaged removals of rNV-VLPs, Qβ, and MS2 were only 1.9-log, 0.9-log, and 2.3-log, respectively, at the 0.54 mg-Al/L coagulant dose, whereas the 1.08 mg-Al/L dose achieved time-averaged removals of > 3.1-log, 1.3-log, and 3.1-log for rNV-VLPs, Qβ, and MS2, respectively.

The removal ratios of rNV-VLPs, Qβ, and MS2 gradually increased with filtration time at the 0.54 mg-Al/L alum dose, probably owing to the aluminium floc particles accumulating on the membrane surface as a cake layer during the MF process. Ultimately, removals of 2.3-log, 1.0-log, and 2.7-log were obtained for rNV-VLPs, Qβ, and MS2, respectively, at the end of the 2-h filtration. In contrast, the removal ratios of rNV-VLPs, Qβ, and MS2 gradually decreased with filtration time at the 1.08 mg-Al/L alum dose, for the same reason as in the PACl experiment, described above.

The removal performance for rNV-VLPs, Qβ, and MS2 with the 1.08 mg-Al/L alum dose at the end of the filtration (2 h) was also higher than that with the 0.54 mg-Al/L alum dose, as in the PACl experiments. Accordingly, the alum dose of 1.08 mg-Al/L was more effective for removal of rNV-VLPs and both bacteriophages than the 0.54 mg-Al/L alum dose in the coagulation–ceramic MF process.

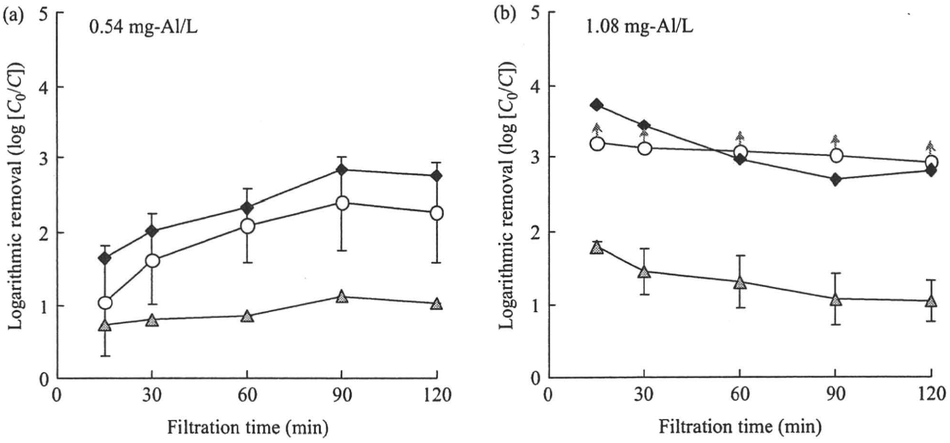

Figure 3 | rNV-VLPs, Qβ and MS2 removal by a coagulation–ceramic MF process with alum coagulant at doses of (a) 0.54 mg-Al/L and (b) 1.08 mg-Al/L. Circles, triangles, and diamonds represent rNV-VLPs, Qβ and MS2, respectively. The arrows indicate values greater than those that could be estimated accurately by ELISA.

Comparison of removal performances between PACl and alum

Because the concentration of rNV-VLPs in the MF permeate with the 1.08 mg-Al/L coagulant dose (Figures 2b and 3b) was less than the detection limit of the ELISA kit (approximately 10^8 VLPs/mL), further concentration using a centrifugal filter device (molecular weight cutoff 30,000, regenerated cellulose; Amicon Ultra-15, Millipore Corp.) was performed in the present study to evaluate 4-log removal, as regulated by the U.S. Environmental Protection Agency (USEPA) National Primary Drinking Water Standards (U.S. Environmental Protection Agency 2001). An approximately 10-fold concentration was obtained by the concentration method for rNV-VLPs in the MF permeate.

Figure 4 shows the effect of coagulant type (PACl vs. alum) on the removals of rNV-VLPs, Qβ, and MS2 in the coagulation–ceramic MF process with a 1.08 mg-Al/L coagulant dose. The experiment with PACl achieved >4-log removal of rNV-VLPs regardless of the filtration time, whereas with alum 3.3-log to >3.9-log removals were observed. This means that the experiment with PACl more effectively removed rNV-VLPs than that with alum, and that it satisfied the USEPA requirement of 4-log removal/inactivation. The difference in removal performance between PACl and alum was possibly due to differences in the characteristics of the aluminium floc particles generated during the coagulation process. Gregory & Dupont (2001) reported that aluminium floc particles formed with PACl are larger, stronger, and more readily separated by the settling process than those formed with alum. In the present study, because the rNV-VLPs were adsorbed to or entrapped in the aluminium floc particles, and settled out from suspension during the settling process or were separated during the MF process, the difference in the characteristics of the aluminium floc particles between PACl and alum may account for the difference in the removal performance for rNV-VLPs of the coagulation–ceramic MF process. Further investigation is needed.

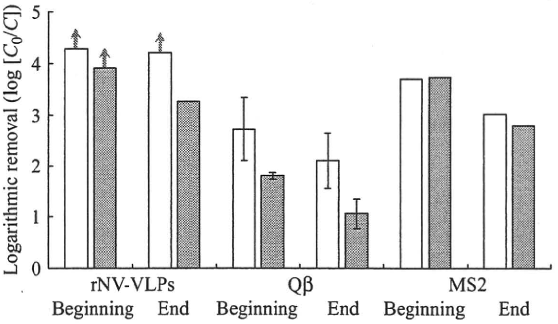

Figure 4 | Effect of coagulant type on the removal of rNV-VLPs Qβ and MS2 at the beginning and end of filtration in a coagulation–ceramic MF process. White and gray columns represent PACl and alum, respectively. The coagulant dose was 1.08 mg-Al/L. The arrows indicate values greater than those that could be estimated accurately by ELISA.

The removal ratios of Qβ and MS2 were smaller than those of rNV-VLPs in the coagulation–ceramic MF process with both aluminium coagulants: the ratio of Qβ was approximately 2-log smaller than that of rNV-VLPs, and the MS2 ratio was approximately 1-log smaller. Accordingly, both bacteriophages have the potential to become appropriate surrogates for native NV in the coagulation–ceramic MF process, with Qβ being the more conservative surrogate of the two. We imagine that the characteristics of rNV-VLPs, Qβ, and MS2 such as surface charge and hydrophobicity affect the removal performance in the coagulation–MF process. Further investigation of differences in surface properties between rNV-VLPs and the two bacteriophages is needed.

Our research group has shown that PACl exhibits virucidal activity during the coagulation process (Shirasaki *et al.* 2009*a*,*b*): an approximately 4-log difference between the total (infectious + inactivated) concentration measured by real-time RT-PCR and the infectious concentration measured by the PFU method was observed during the coagulation process, 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 removal (including inactivation) of native NV might be easily achieved during the coagulation–ceramic MF process.

CONCLUSIONS

- The removal performance of NV as particles was evaluated by using rNV-VLPs in a coagulation–ceramic MF process.
- Both coagulation type and coagulant dose affected the removal performance of rNV-VLPs: the experiment with a 1.08 mg-Al/L PACl dose achieved high ratios of rNV-VLP removal, >4-log, which satisfies the USEPA requirement of 4-log removal/inactivation.
- The removal ratios of rNV-VLPs were higher than those of Qβ and MS2 in the experiments with the 1.08 mg-Al/L coagulant dose: the Qβ ratio was approximately 2-log smaller than that of rNV-VLPs, and the MS2 ratio was approximately 1-log smaller. This means that both bacteriophages have the potential to become appropriate surrogates for native NV in the coagulation–ceramic MF process, with Qβ being the more conservative of the two.

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Feasibility of in-line coagulation as a pretreatment for ceramic microfiltration to remove viruses

Nobutaka Shirasaki, Taku Matsushita, Yoshihiko Matsui, Makoto Kobuke and Koichi Ohno

ABSTRACT

The feasibility of in-line coagulation as a pretreatment for ceramic microfiltration (MF) was verified by comparing its efficiency in the removal of viruses with that of the traditional mechanical mixing approach for coagulation, and by examining the effect of coagulant dose and coagulation time on virus removal. The in-line coagulation–ceramic MF system efficiently removed bacteriophage Qβ and MS2: removal ratios were >8.2 log for infectious viruses and >5.4 log for total (infectious + inactivated) virus particles. These values were similar to those of the mechanical coagulation–ceramic MF system. The in-line coagulation system has potential as a useful pretreatment for the removal of viruses as an alternative to the mechanical mixing system, because the former efficiently removes viruses and has a smaller footprint in treatment plants. For the in-line coagulation–ceramic MF system, a coagulant dose of 1.08 mg-Al/L and a coagulation time of 1 min were required to achieve a high level of virus removal. Infectious Qβ and MS2 were removed to similar levels by the two precoagulation methods tested, but the removal of total MS2 particles was higher than that of Qβ particles, possibly because of the selective interaction with the cake layer.

Key words | bacteriophages, ceramic microfiltration, in-line coagulation, virus inactivation, virus removal

Nobutaka Shirasaki (corresponding author)
Taku Matsushita
Yoshihiko Matsui
Makoto Kobuke
Koichi Ohno
Division of Built Environment,
Graduate School of Engineering,
Hokkaido University,
N13W8,
Sapporo 060-8628,
Japan
Tel.: +81-11-706-7281
Fax: +81-11-706-7279
E-mail: *nobutaka@eng.hokudai.ac.jp*

INTRODUCTION

Membrane filtration processes using microfiltration (MF) and ultrafiltration (UF) membranes have been widely used to produce drinking water. One of the primary reasons for the increasing use of membrane filtration is that it can be used to remove pathogenic microorganisms such as *Cryptosporidium* and *Giardia*. Complete removal of *Cryptosporidium parvum* oocysts and *Giardia muris* cysts was achieved by direct MF/UF membranes (Jacangelo *et al.* 1995). By comparison, because viruses are the smallest pathogenic microorganisms among the causative agents of waterborne disease, varying levels of virus removal have been reported for direct MF (<0.5 to >6 log)/UF (0.4 to >6 log) processes (Jacangelo *et al.* 1995; Urase *et al.* 1996; Otaki *et al.* 1998; Hu *et al.* 2003; Arkhangelsky & Gitis 2008; Langlet *et al.* 2009). The 4-log virus removal required by the US Environmental Protection Agency (USEPA 2001) is regularly not satisfied by MF/UF processes alone, particularly direct MF processes.

Recently, coagulation as a pretreatment for membrane filtration has become attractive because it is expected to improve removal of organic compounds (Yuasa 1998; Kim *et al.* 2006; Choi *et al.* 2008; Kimura *et al.* 2008) and to mitigate membrane fouling (improve flux decline) (Kunikane *et al.* 1995; Judd & Hillis 2001; Oh & Lee 2005; Cho *et al.* 2006; Kim *et al.* 2006; Choi *et al.* 2008; Kimura *et al.* 2008). The combination of coagulation and MF was shown to achieve higher levels of virus removal than that by MF alone (Zhu *et al.* 2005; Fiksdal & Leiknes 2006).

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Our group has also reported the usefulness of a coagulation-ceramic MF system for virus removal (Matsui *et al.* 2003*a*; Matsushita *et al.* 2005): a >6-log removal of viruses was achieved by this system with an aluminium coagulant.

In the coagulation-MF processes, coagulation conditions, including coagulant dose and coagulation time, affect membrane performance. Although many studies have focused mostly on the influence of membrane fouling (e.g. Lee *et al.* 2000; Judd & Hillis 2001; Oh & Lee 2005; Cho *et al.* 2006; Kimura *et al.* 2008), few researchers have reported the effect of coagulant dose (Matsui *et al.* 2003*a*; Matsushita *et al.* 2005; Zhu *et al.* 2005; Fiksdal & Leiknes 2006) and coagulation time (Matsushita *et al.* 2005) on virus removal. Zhu *et al.* (2005) combined polyvinylidene fluoride MF with jar coagulation (without settling) and reported a dramatic increase in virus removal with an increase in the dose of ferric chloride. Matsushita *et al.* (2005) also reported that coagulant dose strongly affects virus removal: only 2.8-log removal was achieved with 0.54 mg-Al/L polyaluminium chloride (PACl) in the in-line coagulation-ceramic MF process, whereas 6.4-log and 7.4-log removal was achieved with 1.08 and 1.62 mg-Al/L PACl, respectively. Matsushita *et al.* (2005) also revealed that a longer coagulation time provides a greater reduction in virus level.

Coagulation as a pretreatment for membrane filtration can be introduced by mechanical mixing or in-line mixing. Mechanical mixing is widely employed in the traditional treatment of drinking water consisting of coagulation, flocculation, sedimentation and rapid sand filtration processes. For these processes, rapid mixing and slow mixing tanks are installed in the system to allow colloids in water, following addition of the coagulant, to coagulate and flocculate. The MF process can be an alternative to the rapid sand filtration process as a physical barrier. Three applications are available for mechanical mixing in coagulation pretreatment. Raw water can be treated with the coagulant in the rapid mixing tank and then subjected to MF without slow mixing and sedimentation. Alternatively, raw water can be treated in the rapid mixing tank and then passed through a slow mixing tank only or through a slow mixing tank and then a sedimentation tank before MF. Treatment of the raw water with coagulant in the rapid mixing tank only is often applied to the ceramic MF process (Yonekawa *et al.* 2004), whereas the application of a slow mixing tank and a sedimentation tank is often used together with organic membranes (Bakersfield Water Treatment Plant, California, http://www.water-technology.net/projects/bakersfield/, accessed 4 March 2009; Columbia Heights Filtration Plant, Minneapolis, http://www.water-technology.net/projects/columbia/, accessed 4 March 2009).

As an alternative procedure, in-line coagulation as a pretreatment for MF has been tested in bench-scale and pilot-scale experiments (Judd & Hillis 2001; Matsui *et al.* 2003*a*; Matsushita *et al.* 2005; Oh & Lee 2005; Cho *et al.* 2006; Meyn *et al.* 2008) because of its advantages over mechanical mixing, including reduction of coagulant dose, coagulation time and energy consumption (Oh & Lee 2005; Meyn *et al.* 2008). Comparisons of the operation performance of these types of mechanical mixing are limited. The efficiency of dissolved organic carbon (DOC) removal has been compared among three different types of mechanical mixing for coagulation with 6 mg-Fe/L iron chloride dosing including in-line mixing (hydraulic retention time (HRT), 45 s), one-stage mechanical rapid mixing (HRT, 6.8 min), and two-stage flocculation with mechanical rapid mixing and slow mixing (total HRT, 20 min) for the ceramic MF process, and the efficiency was found to be almost the same, regardless of the mixing type (Meyn *et al.* 2008). The effect on virus removal has not been investigated, except in a previous study by our group (Matsui *et al.* 2003*a*).

The virus removal performance of membrane filtration processes, including the coagulation-MF process, is frequently evaluated by using bacteriophages (i.e. viruses that infect bacteria) as indicators of enteric viruses (Jacangelo *et al.* 1995; Urase *et al.* 1996; Otaki *et al.* 1998; Hu *et al.* 2003; Matsui *et al.* 2003*a*; Matsushita *et al.* 2005; Zhu *et al.* 2005; Fiksdal & Leiknes 2006; Arkhangelsky & Gitis 2008; Langlet *et al.* 2009; Shirasaki *et al.* 2009*a*). The plaque-forming unit (PFU) method is commonly used for quantification of bacteriophages, because it measures the concentration of infectious viruses. However, the removal of infectious viruses by the coagulation-MF process is due not only to physical removal during the membrane separation process but also to the virucidal activity of the aluminium coagulant (Matsui *et al.* 2003*b*; Matsushita *et al.* 2004; Shirasaki *et al.* 2009*b*) during the coagulation pretreatment.

In a previous study, our group applied the PFU method together with the polymerase chain reaction (PCR) method to measure the concentration of infectious viruses as well as inactivated viruses, to evaluate the performance of the coagulation-MF process in removing infectious viruses and inactivated viruses (Shirasaki *et al.* 2009*a*). We found a difference between total (infectious + inactivated) and infectious virus concentrations just before MF, indicating inactivation of viruses during coagulation. Although the mechanisms underlying the virucidal activity of the aluminium coagulant remain unclear, inactivated viruses in the MF permeate might recover their infectivity in the water distribution system. Virus removal performances evaluated by the PFU method might underestimate the risk of infection because this method cannot count the inactivated viruses that pass into the MF permeate.

The effects of coagulation conditions on the performance of infectious and inactivated virus removal have not been investigated. By comparison, the effects of the different mixing methods (in-line mixing vs. mechanical mixing) (Matsui *et al.* 2003*a*), coagulant dose (Matsui *et al.* 2003*a*; Matsushita *et al.* 2005; Zhu *et al.* 2005; Fiksdal & Leiknes 2006) and coagulation time (Matsushita *et al.* 2005) on the removal of infectious viruses have been widely investigated by using the PFU method alone.

Our objective in the present study was to verify the feasibility of in-line coagulation as a pretreatment for ceramic MF by comparing its efficiency for virus removal with that of the traditional mechanical mixing approach for coagulation, as well as by examining the effect of coagulant dose and coagulation time on virus removal. The efficiency of virus removal in these experiments was assessed by using the PFU method, to measure the concentration of infectious bacteriophage, and real-time reverse transcription-polymerase chain reaction (RT-PCR) method, to measure the concentration of total bacteriophage.

MATERIALS AND METHODS

Source water, coagulant and MF membranes

River water was sampled from the Toyohira River (Sapporo, Japan) on 12 June 2008 and was subjected to water quality analyses (Table 1). PACl (250A; 10.5% Al_2O_3, relative density 1.2 at 20°C; Taki Chemical Co., Ltd, Hyogo, Japan) was used for the coagulation process. The membrane used was a monolithic ceramic MF module installed in a stainless-steel casing (61-channel tubular; nominal pore size 0.1 μm, effective filtration area 0.048 m^2, membrane diameter 0.03 m, membrane length 0.1 m; NGK Insulators, Ltd, Nagoya, Japan).

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

Bacteriophages

The 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 (Urase *et al.* 1996; Otaki *et al.* 1998; Matsui *et al.* 2003*a*; Matsushita *et al.* 2005; Langlet *et al.* 2009; Shirasaki *et al.* 2009*a*) and MS2 (Jacangelo *et al.* 1995; Hu *et al.* 2003; Zhu *et al.* 2005; Fiksdal & Leiknes 2006; Arkhangelsky & Gitis 2008; Langlet *et al.* 2009; Shirasaki *et al.* 2009*a*) are widely used as surrogates for pathogenic waterborne viruses because of their morphological similarities to hepatitis A viruses and polioviruses, which need to be removed by the treatment of drinking water.

Qβ is the prototype member of the genus *Allolevivirus* in the family Leviviridae, and MS2 is the prototype member of the genus *Levivirus* in the family Leviviridae. The Qβ and MS2 genomes comprise a single molecule of linear positive-sense, single-stranded RNA 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, http://phene.cpmc.columbia.edu/index.htm, accessed 17 April 2008). 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 (2,000 × 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).

To prepare the bacteriophage stock solution, the filtrate was purified by using a centrifugal filter device comprising a regenerated cellulose membrane with a molecular weight cut off of 100,000 (Amicon Ultra-15, Millipore Corp., Billerica, Massachusetts).

In-line coagulation–ceramic MF experiments

The setup for the in-line coagulation–ceramic MF experiments is schematically depicted in Figure 1. The river water, placed in the raw water tank, was spiked with either Qβ or MS2 at approximately 10^8 PFU/mL. Throughout the experiments, the raw water was mixed constantly with an impeller stirrer. The raw water was fed into the system at a constant flow rate ($83.3\,L/(m^2\,h) = 2.0\,m/d$) by a peristaltic pump. To maintain the MF permeate at pH 6.8, hydrochloric acid or sodium hydroxide was added to the water before it reached the first in-line static mixer (HRT 1.8 s; 1/4-N40-172-0, Noritake Co., Ltd, Nagoya, Japan).

PACl was injected after the first in-line static mixer and before the second in-line static mixer at a constant dose rate (0.54, 1.08 or 1.62 mg-Al/L). To obtain the three different coagulation times, the in-line static mixer (G value 260/s, HRT 1.8 s), and a combination of the in-line static mixer and a subsequent Tygon® tube reactor (total HRT 1 or 5 min), were used as the second in-line static mixer. The total HRT was controlled by the length of the Tygon® tube reactor. After the PACl had been mixed in, the water was fed into the ceramic MF module in dead-end mode. Filtration was performed for 4 h without any backwashing. Bacteriophage concentrations in the raw water tank and in the MF permeate were measured every hour.

Mechanical coagulation–ceramic MF experiments

The setup for mechanical coagulation–ceramic MF experiments is schematically depicted in Figure 2. The river water, placed in the raw water tank, was spiked with either Qβ or MS2 at approximately 10^8 PFU/mL. Throughout the experiments, the raw water was mixed constantly with an impeller stirrer. The raw water was fed into the system at a constant flow rate (100 mL/min) by a peristaltic pump. To maintain the MF permeate at pH 6.8, hydrochloric acid or sodium hydroxide was added to the water before it reached the first in-line static mixer (HRT 1.2 s). pH-adjusted raw water was introduced and PACl was injected into the rapid mixing chamber with an impeller stirrer (G value 200/s, 109 rpm; hydraulic retention time 5 min) at a constant dose rate (1.08 mg-Al/L).

After the PACl had been mixed in, the water was passed through five chambers for slow mixing (G value 20/s, 23 rpm; HRT 5 min × 5 chambers) and then through a rectangular settler for settling of the aluminium floc particles (HRT 20 min). At the end of the rapid mixing or settling, the water was fed into the ceramic MF module in dead-end mode at a constant flow rate 67 mL/min, which

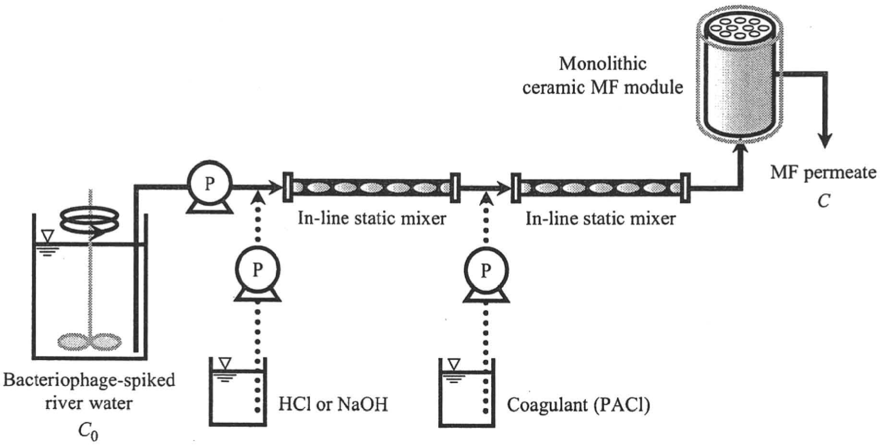

Figure 1 | In-line coagulation–ceramic MF system. C_0 and C are the bacteriophage concentrations (PFU or particles/mL) in the raw water tank and the MF permeate, respectively, at each sampling time.

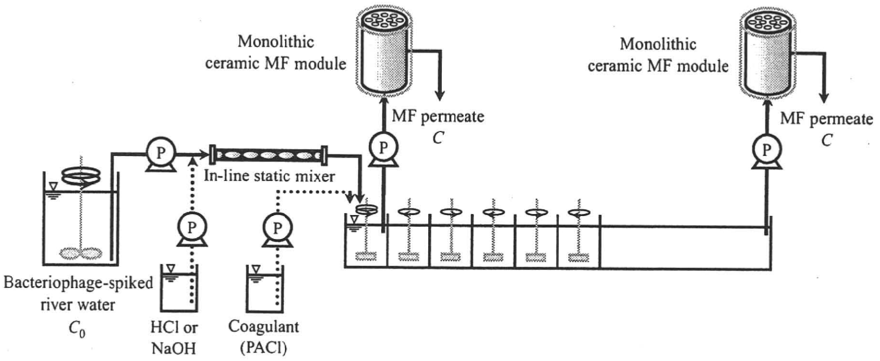

Figure 2 | Mechanical coagulation–ceramic MF system. C_0 and C are the bacteriophage concentrations (PFU or particles/mL) in the raw water tank and the MF permeate, respectively, at each sampling time.

was equivalent to 83.3 L/(m^2 h). Filtration was performed for 4 h without any backwashing. Bacteriophage concentrations in the raw water tank and in the MF permeate were measured every hour.

Bacteriophage assay

PFU method

The infectious bacteriophages were enumerated according to the double-layer method (Adams 1959) by using the bacterial host *E. coli* (NBRC 13965).

Serially diluted raw water or MF permeate (1 mL) was poured onto a solid bottom agar plate followed by 0.3 mL of host *E. coli* culture mixed with 3 mL of molten top agar. The plates were incubated for 16–24 h at 37°C. To measure the concentration of infectious bacteriophage in the water samples, we calculated the average plaque counts of triplicate plates prepared from one sample on plates with 30 to 300 PFU, which we considered a countable number of plaques, and determined the PFU/mL.

For quantification of low infectious bacteriophage concentrations (i.e. <30 PFU/mL) in the MF permeate, 50 mL of MF permeate was mixed with 5 mL of bacterial host *E. coli* culture and 50 mL molten agar, and the mixture was then poured into 10 plates (without bottom agar). The plates were incubated for 16–24 h at 37°C. We calculated the PFU/mL by dividing the total plaque counts for the 10 plates by the sample volume (50 mL).

Real-time RT-PCR method

Viral RNA of bacteriophages was quantified by real-time RT-PCR method. Real-time RT-PCR method detects the virus genome and therefore detects viruses regardless of their infectivity. We defined the concentration measured by real-time RT-PCR method as the total bacteriophage concentration. For quantification of bacteriophages in the raw water and MF permeate, viral RNA was extracted from 200 μL samples with a QIAamp MinElute Virus Spin Kit (Qiagen K.K., Tokyo, Japan) to obtain a final volume of 20 μL. The viral RNA was then subjected to RT reaction by using the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems Japan Ltd, Tokyo, Japan). RT reaction 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 a thermal cycler (Thermal Cycler Dice Model TP600, Takara Bio Inc., Shiga, Japan). The resultant cDNA was then amplified by using 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 listed in Table 2. Amplification was conducted at 50°C for 2 min, 95°C for 10 min, and then for 40 cycles at 95°C for 15 s and 60°C for 1 min in an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems Japan Ltd).

We constructed a standard curve for real-time RT-PCR method based on the relationship between the infectious

Table 2 | Oligonucleotide sequences of the primers and the 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	

bacteriophage concentration (PFU/mL) of freshly prepared bacteriophage stock solution, assumed not to contain inactivated bacteriophages, and the number of PCR amplification cycles.

RESULTS AND DISCUSSION

Infectious bacteriophage removal by the in-line coagulation–ceramic MF system

Figure 3 shows the effect of coagulant dose and coagulation time on the removal of infectious bacteriophage, assessed by the PFU method, by the in-line coagulation–ceramic MF system. Because the diameters of Qβ and MS2 were smaller than the nominal pore size of the ceramic MF membrane (0.1 μm), there was no removal of these infectious bacteriophages in the absence of coagulation pretreatment (data not shown). Other researchers also have reported an insufficient removal ratio for infectious Qβ and MS2 by MF in the absence of coagulation pretreatment: 1–2 log for Qβ (Urase *et al.* 1996) and <1 log for MS2 (Jacangelo *et al.* 1995).

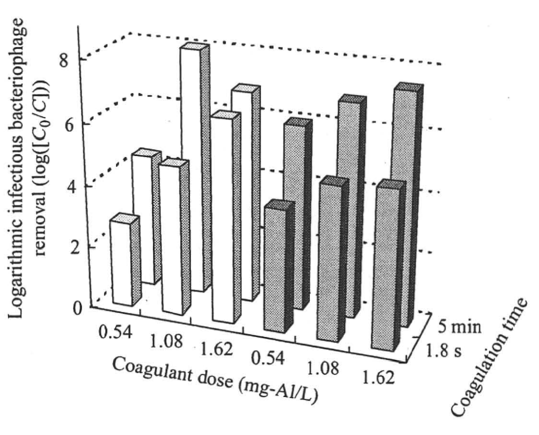

Figure 3 | Effect of coagulant dose and coagulation time on the filtration time-averaged infectious bacteriophage removal in the in-line coagulation–ceramic MF system. White and shaded columns represent Qβ and MS2, respectively.

By comparison, in-line coagulation pretreatment improved the removal ratios (log[C_0/C]) for infectious Qβ and MS2 by MF (Figure 3) because these phages were negatively charged at pH 6.8 (Shirasaki *et al.* 2009*a*) and became adsorbed to/entrapped by the positively charged aluminium floc particles (amorphous aluminium hydroxide, $Al[OH]_3$) generated during coagulation pretreatment. The aluminium floc particles exceeded the pore size of the ceramic MF membrane and were removed during the filtration process. More than 4-log removal was achieved for both bacteriophages, except at a coagulant dose of 0.54 mg-Al/L and a coagulation time of 1.8 s.

According to the USEPA National Primary Drinking Water Standards, 4-log removal or inactivation of enteric viruses from source water is required by filtration, disinfection or a combination of these technologies (USEPA 2001). Our hybrid system successfully met this requirement with a sufficient coagulant dose (≥1.08 mg-Al/L). Some researchers have also reported the usefulness of precoagulation to improve the removal of viruses by MF (Matsui *et al.* 2003*a*; Matsushita *et al.* 2005; Zhu *et al.* 2005; Fiksdal & Leiknes 2006). MF alone does not remove viruses, but the coagulation–MF system is effective in removing infectious viruses: 6–7-log for Qβ (Matsui *et al.* 2003*a*) and 6.7 to >7.5-log for MS2 (Fiksdal & Leiknes 2006).

An increase in the coagulant dose reduced the number of infectious bacteriophages (Figure 3). The time-averaged reduction in infectious Qβ and MS2 increased from 2.7 to 6.5-log and from 3.9 to 5.0-log, respectively, with an increase in the coagulant dose (0.54 to 1.62 mg-Al/L) at a coagulation time of 1.8 s. The same positive effect of coagulation has been reported in other studies (Matsui *et al.* 2003*a*; Matsushita *et al.* 2005). This effect was most likely due to an increase in floc size with increasing coagulant dose (Judd & Hillis 2001). Increasing the coagulant dose from 0.54 to 1.08 mg-Al/L increased the size of aluminium floc particles with adsorbed/entrapped bacteriophages, and subsequently reduced the number of aluminium floc particles that passed through a ceramic MF membrane.

An increase in coagulation time also reduced the number of infectious Qβ and MS2. An extension of the coagulation time from 1.8 s to 5 min increased the removal ratios for Qβ and MS2 at any coagulant dose, most likely because the floc size during the coagulation process increased with time. Our results indicated that a coagulation time of 1.8 s was insufficient to achieve a high removal ratio for infectious phages.

Overall, there was no difference in the removal ratio between infectious Qβ and MS2 when the system was operated with a sufficient coagulant dose of ≥1.08 mg-Al/L and at a coagulation time of 5 min. This result was consistent with the findings of our previous study (Shirasaki *et al.* 2009*a*). Under these conditions, a removal ratio of more than 6.9-log, based on time-averaged reduction, was achieved for Qβ and MS2 by the in-line coagulation–ceramic MF system. This removal ratio was almost the same as or higher than the removal ratios obtained with direct UF (0.4 to >6 log) (Jacangelo *et al.* 1995; Urase *et al.* 1996; Otaki *et al.* 1998; Hu *et al.* 2003; Arkhangelsky & Gitis 2008) and direct nanofiltration (NF) (1.9 to >6 log) (Urase *et al.* 1996; Otaki *et al.* 1998; Hu *et al.* 2003) processes. Thus, the in-line coagulation–ceramic MF system is a potential alternative to UF or NF processes for the removal of infectious viruses. Our results also demonstrated that a high removal ratio could be achieved with effective control of coagulant dose and coagulation time. We propose that a PACl dose of 1.08 mg-Al/L is required to achieve a high removal ratio of infectious viruses in the present in-line coagulation–ceramic MF system.

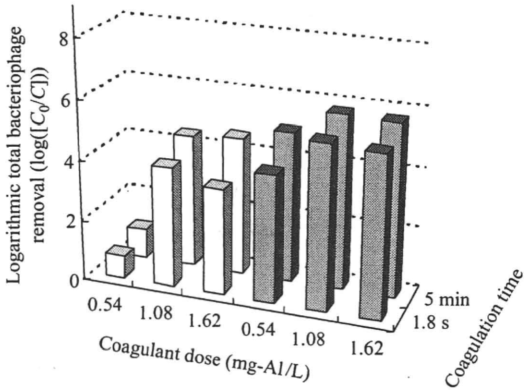

Figure 4 | Effect of coagulant dose and coagulation time on the filtration time-averaged total bacteriophage removal in the in-line coagulation–ceramic MF system. White and shaded columns represent Qβ and MS2, respectively.

Total bacteriophage removal by the in-line coagulation–ceramic MF system

Figure 4 shows the effect of coagulant dose and coagulation time on total bacteriophage removal, assessed by real-time RT-PCR method, by the in-line coagulation–ceramic MF system. A low removal ratio (<1.0-log) was observed for total Qβ with a coagulant dose of 0.54 mg-Al/L, but a high removal ratio of more than 4-log was achieved by increasing the coagulant dose from 0.54 to 1.08 mg-Al/L. In addition, extension of the coagulation time from 1.8 s to 5 min increased the removal ratio for total Qβ from 4.0-log to 4.4-log at a coagulant dose of 1.08 mg-Al/L. Thus, both coagulant dose and coagulation time were important factors dominating the removal performance not only of infectious Qβ but also of total Qβ in the in-line coagulation–ceramic MF system.

By comparison, our in-line coagulation–ceramic MF system achieved a high removal ratio of >4-log for total MS2 at all coagulation conditions. Although particle diameters and electrophoretic mobilities were almost the same for Qβ and MS2 (Shirasaki *et al.* 2009*a*), a marked difference in removal ratio was observed between total Qβ and MS2. The removal ratio for total Qβ was lower than that of total MS2 under all coagulation conditions. This difference was most likely the result of differences between the interactions of Qβ and MS2 with the aluminium floc particles accumulated on the membrane surface as a cake layer. It is possible that MS2 has a higher affinity for the cake layer than does Qβ (Shirasaki *et al.* 2009*a*).

Abbaszadegan *et al.* (2007) and Mayer *et al.* (2008) reported that the removal ratio for MS2 was lower than that of adenoviruses, feline caliciviruses, coxsackieviruses, echoviruses and polioviruses by an enhanced coagulation process using ferric chloride, and these researchers concluded that MS2 was an appropriate surrogate for enteric viruses. In contrast, our results suggest that Qβ may be a more appropriate surrogate than MS2, because we found that Qβ was more difficult to remove than MS2 by our in-line coagulation–ceramic MF system. Langlet *et al.* (2009) evaluated the efficiency of virus removal by direct MF and UF processes, also by using real-time RT-PCR method, and, consistent with our findings, their results demonstrated that the removal ratio for Qβ was lower than that of MS2; they concluded that Qβ was a better candidate than MS2 for characterizing membrane virus removal. We believe that Qβ, rather than MS2, has the potential to become a conservative surrogate for the evaluation of virus removal performance by drinking water treatment processes, including coagulation–MF processes.

The removal ratios of infectious Qβ and MS2 (Figure 3) were higher than those for total Qβ and MS2 (Figure 4). This result indicates that a large proportion of the bacteriophage population was inactivated by the coagulation–MF process. Our group previously reported that Qβ and MS2 were inactivated during the coagulation process and that Qβ was more sensitive to the virucidal activity of PACl than MS2 was (Matsui *et al.* 2003*b*; Matsushita *et al.* 2004; Shirasaki *et al.* 2009*b*). The removal efficiency of total Qβ is greater than that of MS2, but because of differences between these two bacteriophages in their sensitivity to the virucidal activity of PACl, the removal ratio of infectious Qβ and MS2 becomes almost the same.

In summary, the time-averaged removal ratios for total Qβ and MS2 were 4.4-log and 5.8-log, respectively, at a coagulant dose of 1.08 mg-Al/L and at a coagulation time of 5 min. These values were almost the same as or higher than the removal ratios by the direct UF process (1.5 to >6 log) (Langlet *et al.* 2009). Thus, the in-line coagulation–ceramic MF system is a potential alternative to the UF process for the removal of total viruses as well. From our observations, we propose that a PACl dose of 1.08 mg-Al/L is required to achieve a high removal ratio not only of infectious viruses but also of total viruses in the present in-line coagulation–ceramic MF system.

Comparison of removal performance between the in-line and mechanical coagulation processes

As described above, a PACl dose of 1.08 mg-Al/L was required to achieve high removal ratios for both infectious and total bacteriophages in the present in-line coagulation–ceramic MF system. To verify the feasibility of in-line coagulation as a pretreatment for the ceramic MF process, we compared the removal performances of Qβ and MS2 by in-line mixing and the traditional mechanical mixing approach at the same PACl dose (1.08 mg-Al/L).

Figure 5 shows a comparison of virus removal performance between the in-line and the mechanical coagulation–ceramic MF systems. Mechanical rapid mixing followed by MF achieved high removal ratios of >8.2-log for infectious and >4.3-log for total phage for both Qβ and MS2.

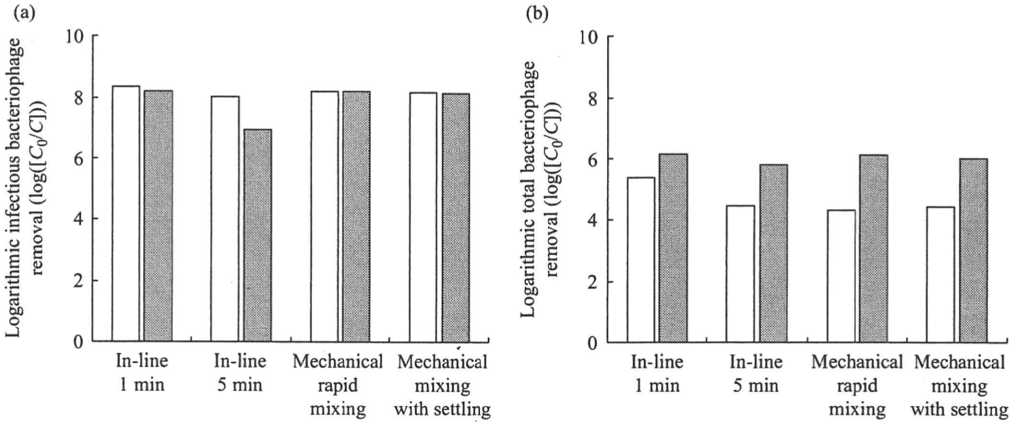

Figure 5 | Effect of mixing type of coagulation pretreatment on the removal of infectious bacteriophage (a) and total bacteriophage (b). White and shaded columns represent Qβ and MS2, respectively. PACl dosage was 1.08 mg-Al/L.

Our result for infectious Qβ removal agreed with that from the previous study (approximately 7.5-log) (Matsui *et al.* 2003*a*). Removal ratios did not improve even when slow mixing and settling processes were incorporated between the rapid mixing and MF processes. This result indicates that 5 min of rapid mixing was sufficient to achieve high removal ratios for infectious and total bacteriophage in the coagulation–ceramic MF system with mechanical mixing. Additional slow mixing and settling processes are not necessary for virus removal, enabling the actual treatment plant to be compact.

The high removal ratio for Qβ and MS2 achieved with the mechanical mixing system was similar to that achieved with equivalent coagulation time of 5 min in the in-line mixing system, demonstrating that in-line coagulation has potential as a pretreatment for MF giving high virus removal as an alternative to the mechanical mixing system. In addition, even when the coagulation time in the in-line mixing system was reduced to one-fifth (i.e. 1 min), high virus removal ratios of >8.2-log for infectious and >5.4-log for total bacteriophage, were still achieved (Figure 5). This means that 1 min of coagulation time is enough to achieve a high level of virus removal in the present in-line coagulation–ceramic MF system, leading to a reduction in the footprint required for the HRT in the actual treatment plant.

The removal performances for the two bacteriophages were similar for all tested mixing procedures, although the characteristics of aluminium floc particles (i.e. size, fractal dimension, and so on) fed into the ceramic MF module most likely differed depending on the mixing type. One possible explanation is that, during rapid mixing, regardless of mixing type, most of the bacteriophage population was adsorbed to/entrapped by aluminium floc particles that were larger than the pore size of the MF membrane and would thus be removed by the MF process. However, the small proportion of the bacteriophage population adsorbed to/entrapped by aluminium floc particles smaller than the MF pore size would pass through the system. It is possible that some of the aluminium floc particles would not be larger than the MF pore size, even after slow mixing and settling processes, and would pass through the MF membrane. Note that although a fraction of the bacteriophage population was not removed by the MF process, the removal ratio was very high. Enlargement of the floc size by traditional treatment with coagulation and sedimentation processes is not necessary for the filtration with a ceramic MF (Lerch *et al.* 2005). In-line coagulation for 1 min was enough to meet the requirement for bacteriophage removal prior to the MF process.

Another possibility is that the contribution to virus removal by the cake layer or foulant may be the same in the three mixing types tested. Formation of the cake layer on the surface of the membrane and the deposition of foulant on the internal wall of the pore of the membrane over time plays an important role in the performance of virus removal not only during the direct MF process (Jacangelo *et al.* 1995; Madaeni *et al.* 1995) but also during the coagulation–MF process (Shirasaki *et al.* 2008). The amount of aluminium floc particles introduced into the MF module for both the in-line mixing system and the rapid mechanical mixing system was approximately three times as high as that for the mechanical mixing system followed by settling, because approximately 75% of the total amount of aluminium floc particles had settled before the water was fed into the MF module. However, the cake layer and foulant do not always contribute to virus removal (Shirasaki *et al.* 2008). It is possible that the extent of cake layer formation and foulant deposition contributing to virus removal is similar in all three types of mixing system.

Here, we propose that a PACl dose of 1.08 mg-Al/L and a coagulation time of 1 min are required to achieve high removal ratios of infectious and total viruses in the present in-line coagulation–ceramic MF system. Virus removal by the in-line coagulation–ceramic MF system is similar to that by the coagulation–ceramic MF system incorporating mechanical mixing. However, virus removal may be affected by the quality of the source water. To elucidate whether the coagulation conditions employed in the present study are sufficient for all water sources, further investigations using a wide variety of water sources are needed.

CONCLUSIONS

1. The performance of the in-line coagulation–ceramic MF system in removing the bacteriophages Qβ and MS was efficient at a coagulant dose of 1.08 mg-Al/L, and its

performance was similar to that of the mechanical coagulation–ceramic MF system. The in-line mixing system has potential as a useful pretreatment for viruses as an alternative to the mechanical mixing system for coagulation. Replacement of the mechanical mixing system with the in-line mixing system will reduce footprint, because there is no requirement for mixing/settling tanks for coagulation.

2. Coagulant dose and coagulation time were important factors controlling the virus removal performance of the in-line coagulation–ceramic MF system: a coagulant dose of 1.08 mg-Al/L and a coagulation time of 1 min were required to achieve high ratios of virus removal, >8.2-log for infectious and >5.4-log for total viruses, which satisfy the USEPA requirement of a 4-log removal/inactivation.
3. The removal performances of the coagulation–ceramic MF process for total Qβ and MS2 were different: the removal ratio for total MS2 was higher than that for total Qβ under all tested coagulation conditions, which was most likely the result of differences between Qβ and MS2 in their interaction with the cake layer.

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環境水中のクリプトスポリジウム検出を目的としたRT-LAMP改善法の実用性評価

Validation for an Improved Reverse Transcription-loop-mediated Isothermal Amplification Assay for Detection of *Cryptosporidium* in Water Samples

猪又明子[1]，百田隆祥[2]，泉山信司[3]，勝山志乃[4]，岸田直裕[5]，秋葉道宏[5]，遠藤卓郎[6]

[1]東京都健康安全研究センター環境保健部／〒169-0073 東京都新宿区百人町3-24-1
[2]栄研化学株式会社生物化学研究所／〒329-0114 栃木県下都賀郡野木町大字野木143
[3]国立感染症研究所寄生動物部／〒162-8640 東京都新宿区戸山1-23-1
[4]神奈川県内広域水道企業団水質管理センター／〒243-0424 海老名市社家4587
[5]国立保健医療科学院水道工学部／〒351-0197 和光市南2-3-6
[6]国立感染症研究所細菌第一部／〒162-8640 東京都新宿区戸山1-23-1

AKIKO INOMATA[1], TAKAYOSHI MOMODA[2], SHINJI IZUMIYAMA[3],
SHINO KATSUYAMA[4], NAOHIRO KISHIDA[5], MICHIHIRO AKIBA[5],
and TAKURO ENDO[6]
[1]Department of Environmental Health and Toxicology, Tokyo Metropolitan Institute
of Public Health / 3-24-1 Hyakunin-cho, Shinjuku, Tokyo 169-0073, Japan
[2]Biochemical Research Laboratory, Eiken Chemical Co.,LTD.
/ 143 Nogi, Nogimachi, Shimotsugagun, Tochigi 329-0114, Japan
[3]Department of Parasitology, National Institute of Infectious Diseases
/ 1-23-1 Toyama, Shinjuku, Tokyo 162-8640, Japan
[4]Water Quality Management Center, Kanagawa Water Supply Authority
/ 4587 Shake, Ebina, 243-0424, Japan
[5]Department of Water Supply Engineering, National Institute
of Public Health / 2-3-6 Minami, Wako, 351-0197, Japan
[6]Department of Microbiology I, National Institute of Infectious Diseases
/ 1-23-1 Toyama, Shinjuku, Tokyo 162-8640, Japan

Abstract

An improved reverse transcription-loop-mediated isothermal amplification (RT-LAMP) assay which uses 1μL of RNA extract was evaluated. The original RT-LAMP assay using 5μL of RNA extract showed generally high-sensitive detection of *Cryptosporidium* in water samples; but in some types of water such as pigsty effluents and it's downstream river water, this assay failed to detect *Cryptosporidium*. This failure may be caused by substances in those water samples which block gene amplification. To eliminate the effect of gene amplification-blocking substances, we decreased the quantity of the RNA extract used for the RT-LAMP assay from 5μL to 1μL. The detection limit of the improved RT-LAMP assay was as low as 6×10^{-3} oocysts/LAMP test tube, which theoretically enables us to detect even one *Cryptosporidium* oocyst in a water sample. This improved RT-LAMP assay succeeded in detecting *Cryptosporidium* oocysts much more sensitively not only in pigsty effluents but also in other water samples as well.

1. はじめに

クリプトスポリジウムはヒトに感染すると下痢を主訴とするクリプトスポリジウム症を引き起こす原虫である。クリプトスポリジウム症に効果的な治療法はまだ知られておらず、免疫による自然治癒に依存するため、免疫不全者ではしばしば致死的となる[1,2]。

クリプトスポリジウムの汚染源は主に下水処理場や畜舎であり、これらの放流水が流入した水道原水中よりクリプトスポリジウムが適切な浄水処理で除去されないと、水道水に混入する恐れがある。水道水を介したクリプトスポリジウムの集団感染は1980年代から米国や英国で報告され始め、それ以降毎年報告されている[1,3,4]。わが国では1996年に埼玉県越生町で水道水を介した初めての集団感染が発生し、8,812人が感染する大事故となったことが知られている[5]。

水試料からのクリプトスポリジウムオーシストの主な検出方法は、厚生労働省の「水道における指標菌及びクリプトスポリジウム等の検査方法について[6]」に示されている蛍光抗体染色と微分干渉観察による検鏡法である。しかし、水試料中に存在する夾雑物の中から約5 μmと極めて微小なオーシストを顕微鏡で検出、同定するには、熟練した技術が必要である。また、検査に長時間を要し、多数の検体を迅速に検査することが困難であることから、正確で再現性が高く簡便な代替検査方法が求められている。このような条件を満たす検査方法として多数の遺伝子検査法が検討されているが、未だ実用化には至っていない[7]。

そうした中で、著者らはクリプトスポリジウムのrRNAを標的とした逆転写Loop-Mediated Isothermal Amplification（RT-LAMP）法を開発し、環境水からのクリプトスポリジウム検出において検鏡法と同様か、あるいはより高感度な検出が可能であることを報告した[8]。しかし、その後様々な環境水についてRT-LAMP法検査を行ったところ、養豚場排水放流水及びその下流の河川水において、検鏡法でクリプトスポリジウムが検出されるにもかかわらずRT-LAMP法で陰性となることを経験した。この原因として、養豚場排水に含まれる強い遺伝子増幅阻害物質、すなわち糞便等の影響が考えられた。

糞便等は強力な遺伝子増幅阻害物質として知られており[9-11]、クリプトスポリジウム等は糞便中に排出されることから、糞便による阻害への対策が必須となる。その一方で、複雑な精製操作は試料のロスと煩雑な作業の増加を招く。そこで、RT-LAMP法の検出感度が6×10^{-3}オーシスト相当/反応チューブと極めて高い[8]ことから、阻害物質の影響を回避するために、RT-LAMP反応に用いるRNA抽出液量を従来の5 μlから1 μlに低減するという、極めて単純だが効果的と考えられる改善策を試行し、その結果を評価した。

前提として、RNA抽出液量を従来の1/5量に減らしても十分に検出可能であることが求められるため、本研究で改めてRT-LAMP法の検出感度を確認することとした。次いで、問題の養豚場排水放流水及びその下流の河川水検体、その他の環境水（表流水、湧水、浅井戸水、伏流水）検体、浄水検体について、検鏡法、RT-LAMP従来法（RNA抽出液量5 μl）及びRT-LAMP改善法（RNA抽出液量1 μl）によりクリプトスポリジウム検出を行った。各検査法の結果を比較することでRT-LAMP改善法の実用性を評価したので報告する。

2. 材料と方法

2.1 RNA抽出方法

クリプトスポリジウムオーシストのRNA抽出は先の報告と同様に行った[8]。すなわち、*Cryptosporidium parvum*オーシストを-80℃のドライバス（Micro Cool MC-100, TOMY）と37℃のヒートブロック（Dry Bath Incubator, Fast Gene）で1分間ずつ5回の凍結融解を行い、次に溶解液（10mM Tris-EDTA（pH7.6）, 20mM NaCl, 0.1% TritonX-100, 2 mM 1,4-Dithiothreitol, 1.5mAnson-U/ml Proteinase K）20μlを添加し、60℃で30分間溶解反応を行った。その後2分間の超音波処理を行い、さらに75℃で10分間の追加反応を行った。このRNA抽出液を95℃で5分間加熱してProteinase Kを失活させた後、氷中で急冷した。

2.2 RT-LAMP法

RT-LAMP法の試薬にはLoopampクリプトスポリジウム検出試薬キット（栄研化学）を使用した[8]。試薬キットにはアウタープライマーCryF3及びCryB3各5 pmol、ループプライマーCryLF及びCryLB各20pmol、インナープライマーCryFIP及びCryBIP各40pmol、*Bst* polymerase 8Uが含まれている。これらのプライマーは*Cryptosporidium parvum*, *Cryptosporidium serpentis*, *Cryptosporidium andersoni*, *Cryptosporidium saurophilum*及び*Cryptosporidium hominis*の遺伝子を全て増幅し、一方*Giardia intestinalis*, *Giardia muris*及び細菌10菌種には反応し

ないことを確認している[12]。さらにReverse Transcriptase (Roche) 1.5Uを添加し、1本のチューブ内で逆転写反応とLAMP反応を実施した。RT-LAMP従来法ではRNA抽出液5 μl、RT-LAMP改善法ではRNA抽出液1 μlと蒸留水4 μlを20μlの増幅反応試薬に加えて計25μlとし、63℃で60分間RT-LAMP反応を行った。濁度の連続測定にはLoopampリアルタイム濁度測定装置（LA-320C,栄研化学）を用いた。陽性対照として試薬キットに付属のものを用いた。これは増幅産物のアガロースゲル電気泳動パターンがクリプトスポリジウムとは異なるように配列が設計されており、陽性対照のコンタミネーションを判別できるものとなっている。陰性対照にはキットに付属の純水を用いた。

2.3　RT-LAMP法の検出感度試験

*Cryptosporidium parvum*オーシスト（H8株）は、感染マウスの糞便からショ糖浮遊法及び塩化セシウム浮遊法により精製した[13]。血球計算盤で計数して所定濃度のオーシスト液を調製した後、前述の方法でオーシストからRNAを抽出した。この抽出RNAを感度試験用の鋳型として6×10^{-1}オーシスト相当/5 μlから6×10^{-4}オーシスト相当/5 μlまで10倍毎の連続希釈濃度に調製し、各濃度段階につき8連でRT-LAMP反応を行った。

2.4　検鏡法、RT-LAMP従来法、RT-LAMP改善法による水試料からのクリプトスポリジウム検出

養豚場排水放流水及びその下流の河川水10検体、その他の環境水（表流水、湧水、浅井戸水、伏流水）57検体、浄水13検体についてクリプトスポリジウム検査を行った。なお、これらの検体の一部には既に報告したものが含まれている[8]。養豚場排水放流水は10ml、養豚場下流の河川水は10l又は20l、その他の環境水は20l、浄水は40lを検水量とした。養豚場排水放流水は検水量が10mlと少ないことから、5連で試験を行うこととし、他は1試料ずつ行った。浄水は孔経1 μmのポリカーボネートフィルター、その他の試料は孔径5 μmのPTFEフィルターを用いてろ過濃縮を行った後、フィルター上に捕捉された懸濁物を剥離させて5 mlの濃縮液を得た。濃縮液の半量2.5mlをそれぞれ検鏡法とRNA抽出とに用いた。各検体は採水後冷蔵し、概ね3日以内に検鏡法による検査を行った。多くは検鏡法と同時に遺伝子検査用試料の精製を行ったが、養豚場下流河川水の一部の試料については冷蔵で1カ月保存した後に精製操作を行い、参考試料として使用した。

検鏡法は厚生労働省「水道における指標菌及びクリプトスポリジウム等の検査方法について」[6]に準拠し、一部改良を加えた方法で行った。すなわち、水試料濃縮液から免疫磁気ビーズ（Dynabeads GC Combo, Invitrogen）を用いてクリプトスポリジウムオーシストを捕捉、精製した後、塩酸により免疫磁気ビーズからクリプトスポリジウムを解離した。濃縮液の沈殿物容量が0.5mlを超える場合は、沈殿物量が0.5ml以下になるように試料を分けてから免疫磁気ビーズ法を行った。塩酸解離後のクリプトスポリジウム試料を水酸化ナトリウムで中和し、100℃で5分間加熱処理した。これを観察用PTFEフィルターでろ過し、直接蛍光抗体染色（EasyStain, BTF）及びDAPI（4',6-diamidino-2-phenylindole）染色を行った。染色後のフィルターを封入してプレパラートを作成し、落射蛍光及び微分干渉観察により、クリプトスポリジウムの同定及び計数を行った。

遺伝子検査用試料として各濃縮液の残り半量2.5mlを用い、免疫磁気ビーズ法によりクリプトスポリジウムの精製を行った。上澄液を除去し、オーシストと免疫磁気ビーズとを結合させたまま前述の方法で核酸抽出した。直ちに抽出操作をしない場合は－20℃で保存した。

LAMP法で陽性となった増幅産物各1 μlについて、2％アガロースゲルによる電気泳動を行い、*Cryptosporidium parvum*オーシストのバンドパターンとの比較により、クリプトスポリジウム由来の増幅であることを確認した。

3. 結果及び考察

3.1　RT-LAMP法の検出感度

鋳型RNAを1反応あたり6×10^{-1}オーシストから6×10^{-4}オーシスト相当まで10倍毎に連続希釈し、8連でRT-LAMP法を行った結果をTable 1に示した。オーシスト濃度が低下するに従い、LAMP反応の陽性判定値（移動平均の微分値が0.07）を越えるのに必要な時間である*Tt*値は大きくなった。鋳型RNA濃度6×10^{-4}オーシストでは8回の*Tt*値の変動係数（CV値）が16.8%と大きく，6×10^{-3}オーシスト以上の濃度ではCV値0.5～4.4%であったことから、再現性のある検出感度は6×10^{-3}オーシスト/LAMP test tubeと判断した。この検出感度は著者らが以前に各濃度段階2連で行った結果[8]と同じであった。この検出感度から、RT-LAMP改善法に使用する鋳型RNA量を20μl中の1 μlに減らした場合でも、検水中1オーシストを確実に検出できるものと判断した（1オーシスト/20μl＝5×10^{-2}オーシスト相当/1 μl）。