Supplementary Information

Elimination of representative contaminant candidate list viruses, coxsackievirus, echovirus, hepatitis A virus, and norovirus, from water by coagulation processes

N. Shirasaki*, T. Matsushita, Y. Matsui, K. Murai, A. Aochi

Division of Environmental Engineering, Faculty of Engineering, Hokkaido University, N13W8, Sapporo 060-8628 Japan

*Corresponding author. Tel.: +81-11-706-7282; fax: +81-11-706-7282; e-mail address: nobutaka@eng.hokudai.ac.jp

Table S1. Oligonucleotide sequences of the primers and probes used in the present study.

Viruses		Oligonucleotide sequences (5' -> 3')[a]	Positions[b]	References
Coxsackievirus and Echovirus	Forward primer	CCT CCG GCC CCT GAA TG	449-465 (CV), 450-466 (EV)	Shieh *et al.* , 1995
	Reverse primer	ACC GGA TGG CCA ATC CAA	643-626 (CV), 645-628 (EV)	
	TaqMan probe	FAM-CCG ACT ACT TTG GGT GTC CGT GTT TC-TAMRA	542-567 (CV), 543-568 (EV)	Katayama *et al.* , 2002
Hepatitis A virus	Forward primer	GGT AGG CTA CGG GTG AAA C	393-411	Jothikumar *et al.* , 2005
	Reverse primer	GCG GAT ATT GGT GAG TTG TT	481-462	
	TaqMan probe	FAM-CTT AGG CTA ATA CTT CTA TGA AGA GAT GC-TAMRA	414-442	
Murine norovirus	Forward primer	CCG CAG GAA CGC TCA GCA G	5028-5046	Kitajima *et al.* , 2010
	Reverse primer	GGY TGA ATG GGG ACG GCC TG	5156-5137	
	TaqMan probe	FAM-ATG AGT GAT GGC GCA-TAMRA	5062-5076	

[a] FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

[b] GeneBank accession numbers for sequence positions are AF114383 for coxsackievirus, AJ577594 for echovirus, M14707 for hapatitis A virus and NC_008311 for murine norovirus.

Table S2. Analysis of covariance examining the differences between the removal ratios of CV or rNV-VLPs and that of MS2 and φX174.[a]

Data sets	*P*- value					
	CV and MS2	CV and MS2	rNV-VLPs and MS2	CV and φX174	CV and φX174	rNV-VLPs and φX174
	PFU	PCR	PCR	PFU	PCR	PFU
PACl (settling) vs. alum (settling)	0.92	0.09	0.14	0.12	0.97	0.43
PACl (settling) vs. $FeCl_3$ (settling)	0.68	0.97	0.98	0.40	0.90	1.00
alum (settling) vs. $FeCl_3$ (settling)	0.99	0.08	0.14	0.03	0.70	0.76
PACl (filtration) vs. alum (filtration)	1.00	0.07	0.79	0.69	0.71	1.00
PACl (filtration) vs. $FeCl_3$ (filtration)	0.05	0.54	ND	0.04	1.00	ND
alum (filtration) vs. $FeCl_3$ (filtration)	0.03	0.01	ND	0.07	0.94	ND
PACl (settling) vs. PACl (filtration)	0.00	0.46	0.00	0.05	0.50	0.02
alum (settling) vs. alum (filtration)	0.01	0.71	0.00	0.35	0.27	0.00
$FeCl_3$ (settling) vs. $FeCl_3$ (filtration)	0.00	0.42	ND	1.00	0.25	ND

[a] ND, not determined. Because the removal ratios of rNV-VLPs by means of coagulation with $FeCl_3$ followed by filtration were all below the detection limit, except in one case, we did not conduct an analysis of covariance using those datasets.

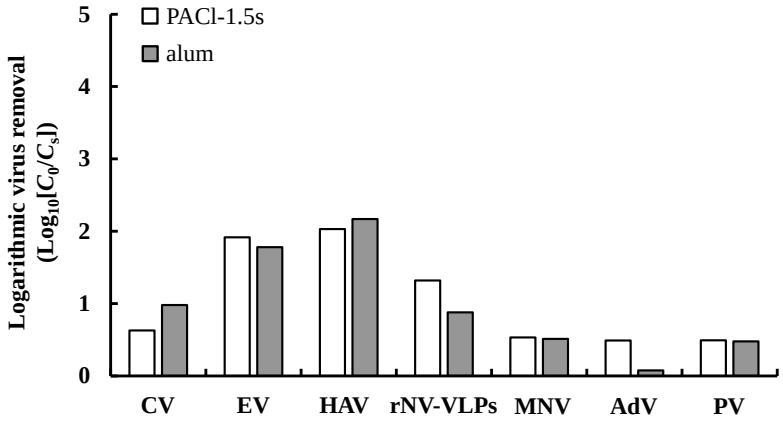

Figure S1. Effect of coagulant type on infectious virus removal, as evaluated after settling by using the PFU method (CV, EV, HAV, MNV, AdV, and PV) or an enzyme-linked immunosorbent assay (rNV-VLPs). Source water, D.

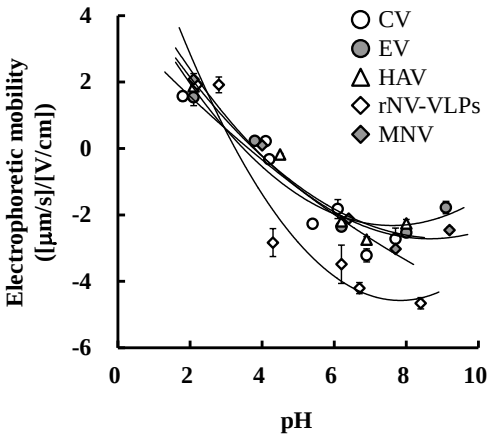

Figure S2. Electrophoretic mobilities of CV, EV, HAV, rNV-VLPs, and MNV in Milli-Q water at various pH values. Values are means and error bars indicate standard deviations (*n* = 8 or 9).

Characteristics of coagulants

Three commercially available aluminum-based coagulants were used in the batch experiments: PACl-1.5s, PACl-2.1s, and alum. PACl-1.5s (PACl 250A) has a basicity ($[OH^-]/[Al^{3+}]$) of 1.5, contains 10.1% (w/w) Al_2O_3 and 2.9% (w/w) sulfate, and has a relative density of 1.2 at 20 °C; the "1.5" in the name indicates the basicity, and the "s" indicates that the PACl is sulfated. PACl-2.1s (PACl 700A) is a high-basicity PACl (basicity, 2.1) that contains 10.2% Al_2O_3 and 2.0–2.1% sulfate and has a relative density of 1.2 at 20 °C. Alum has a basicity of 0, contains 8.1–8.2% Al_2O_3 and 22.6–22.7% sulfate, and has a relative density of 1.3 at 20 °C. All three coagulants were obtained from Taki Chemical Co. (Kakogawa, Japan).

The removal efficiencies of PACl-1.5s, PACl-2.1s, and alum were compared with that of PACl-2.1ns, a nonsulfated (ns), high-basicity (2.1) PACl that contains 10.4% Al_2O_3 and has a relative density of 1.2 at 20 °C (Taki Chemical Co.), and that of $FeCl_3$ solution prepared by dissolution of reagent-grade iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$, Wako Pure Chemical Industries, Osaka, Japan) in Milli-Q water (Milli-Q Advantage, Millipore Corp., Billerica, MA, USA). All coagulants were diluted with Milli-Q water immediately prior to the coagulation experiments.

Propagation and purification of CV, EV, HAV, and MNV

CV or EV stock solution, prepared as described below, was diluted 10-fold with Dulbecco's phosphate-buffered saline (PBS, without Ca and Mg ions: Nissui Pharmaceutical Co.). A monolayer of approximately 90% confluent buffalo green monkey kidney epithelial cells (BGM cells) in a 75-cm^2 flask was inoculated with 1 mL of the diluted stock solution, and the flask was incubated in a humidified incubator at 37 °C in 5% CO_2 for 30 min with rotation at 15 min. Then 20 mL of 1× Eagle's minimum essential medium (EMEM) was added to the flask, which was subsequently incubated at 37 °C in 5% CO_2 until cytopathic effects were confirmed in 100% of the BGM cells (2–3 days for CV, 3–4 days for EV), at which point the viruses were released from the infected cells by three cycles of freezing at –83 °C and thawing at 37 °C. The CV or EV culture solution was then centrifuged (2000 ×*g*, 10 min) and passed through a hydrophilic cellulose acetate membrane filter (nominal pore size, 0.2 μm; Dismic-25cs, Toyo Roshi Kaisha, Tokyo, Japan) to prepare the CV or EV stock solution. The filtrate was further purified by using a tangential-flow filtration cassette (nominal molecular weight cutoff, 1000 kDa; regenerated cellulose [RC] membrane; Pellicon XL, Millipore Corp.) to ensure that minimal amounts of dissolved organic carbon components from the culture medium were introduced into the virus-spiked source water. The concentration of CV or EV in the purified solution was approximately 10^7 and 10^6 PFU/mL, respectively.

HAV stock solution, prepared as described below, was diluted 10-fold with 1× DMEM containing 100 U/mL penicillin and 100 μg/mL streptomycin. A monolayer of approximately 90% confluent FRhK-4 cells in a 75-cm^2 flask was inoculated with 1 mL of the diluted stock solution, and the flask was incubated in a humidified incubator at 37 °C in 5% CO_2 for 60 min with rotation every 15 min. After incubation, 20 mL of 1× DMEM supplemented with 10% heat-inactivated fetal

bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin was added to the flask, which was then incubated at 37 °C in 5% CO_2 until cytopathic effects were confirmed in 100% of the FRhK-4 cells (11–14 days). Fresh cell culture medium (20 mL) was added to the flask at 7 days of incubation. At the end of the incubation period, viruses were released from the infected FRhK-4 cells by three cycles of freezing at –83 °C and thawing at 37 °C. The HAV culture solution was centrifuged (2000 ×*g*, 10 min) and then passed through a hydrophilic cellulose acetate membrane filter (nominal pore size, 0.2 μm; Dismic-25cs, Toyo Roshi Kaisha) to prepare the HAV stock solution. The filtrate was further purified by using a tangential-flow filtration cassette (nominal molecular weight cutoff, 1000 kDa; RC membrane; Pellicon XL) to ensure that minimal amounts of dissolved organic carbon components from the culture medium were introduced into the virus-spiked source water. The concentration of HAV in the purified solution was $10^{5–6}$ PFU/mL.

MNV stock solution, prepared as described below, was diluted 10-fold with PBS. A monolayer of approximately 90% confluent RAW264.7 cells in a 75-cm^2 flask was inoculated with 1 mL of the diluted stock solution, and the flask was incubated in a humidified incubator at 37 °C in 5% CO_2 for 60 min with rotation every 15 min. Then 20 mL of 1× EMEM was added to the flask, which was subsequently incubated at 37 °C in 5% CO_2 until cytopathic effects were confirmed in 100% of the RAW264.7 cells (2–3 days), at which point viruses were released from the infected cells by three cycles of freezing at –83 °C and thawing at 37 °C. The MNV culture solution was centrifuged (2000 ×*g*, 10 min) and then passed through a hydrophilic cellulose acetate membrane filter (nominal pore size, 0.2 μm; Dismic-25cs, Toyo Roshi Kaisha) to prepare the MNV stock solution. The filtrate was further purified by using a tangential-flow filtration cassette (nominal molecular weight cutoff, 1000 kDa; RC membrane; Pellicon XL) to ensure that minimal amounts of dissolved organic carbon components from the culture medium were introduced into the virus-spiked source water. The concentration of MNV in the purified solution was approximately 10^6 PFU/mL.

Experimental procedure for the batch coagulation experiments

Purified solutions of the bacteriophages MS2 and φX174 were simultaneously added to a beaker at initial concentrations (C_0) of approximately 10^6 PFU/mL and $10^{4–5}$ PFU/mL, respectively; then purified CV, EV, HAV, MNV, or rNV-VLPs was added to the beaker at an initial concentration (C_0) of $10^{2–3}$ PFU/mL or $10^{10–11}$ VLPs/mL. After enough HCl or NaOH was added to the spiked water to bring the final pH to 7 for PACl and alum or to 6 for $FeCl_3$, one of the coagulants was injected into the water. The coagulant dosages were the same as the dosages used at the corresponding drinking water treatment plant on the day the source water was sampled (Table 1). The water was stirred rapidly for 1 min (G = 200 s^{-1}, 94 rpm) and then slowly for 10 min (G = 20 s^{-1}, 20 rpm) with an impeller stirrer. The water was then allowed to stand for 60 min to settle the generated aluminum or iron floc particles. After settling, approximately 100 mL of the supernatant was withdrawn from the beaker and the enteric virus or virus-like particle and bacteriophage concentration (C_s) were determined. Then, approximately 50 mL of the supernatant was withdrawn from the beaker, filtered through a polytetrafluoroethylene membrane filter (nominal pore size 0.45 μm; Dismic-25HP, Toyo

Roshi Kaisha), and again the enteric virus or virus-like particle and bacteriophage concentrations (C_f) were determined.

Plaque assay for quantification of CV, EV, HAV, and MNV

To quantify the concentration of infectious CV or EV, approximately 90% confluent BGM cells in a 75-cm^2 flask were seeded in 6-well (8.96 cm^2/well) tissue culture plates. A monolayer of 100% confluent BGM cells in a 6-well plate was inoculated with either 1 mL of a sample serially diluted 10-fold with 1× EMEM supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1.125 g/L sodium hydrogen carbonate or with 1 mL of a sample diluted 2-fold with 2× EMEM containing 4 mM L-glutamine, 200 U/mL penicillin, 200 μg/mL streptomycin, and 2.25 g/L sodium hydrogen carbonate, and then the plate was incubated in a humidified incubator at 37 °C in 5% CO_2 for 90 min. After incubation, the inoculum was removed by inverting the plate. Then, 3 mL of agar overlay, prepared by combining 2× EMEM (without phenol red; Nissui Pharmaceutical Co.) supplemented with 20% or 40% heat-inactivated fetal bovine serum (20% for CV, 40% for EV), 4 mM L-glutamine, 200 U/mL penicillin, 200 μg/mL streptomycin, and 2.25 g/L sodium hydrogen carbonate with an equal volume of 2.5% (w/v) agarose (Agar-EPI; Nacalai Tesque, Inc., Kyoto, Japan), was applied to the cell monolayer. After addition of the overlay, the plate was incubated at 37 °C in 5% CO_2 for 2 days for CV or 3 days for EV, the cell monolayer was stained with 1 mL of 0.15 g/L neutral red at 37 °C in 5% CO_2 for 3 h, and the excess stain was discarded by inverting the plate. The number of plaques in each well was counted each day until no new plaques appeared, which generally took 3 days for CV or 2 days for EV from the time that the neutral red was discarded. The average plaque count of triplicate wells or of 12 wells prepared from one sample was considered the infectious CV or EV concentration for that sample. The detection limit of the plaque assay for quantification of infectious CV or EV was 1/6 PFU/mL when 12 wells were prepared.

To quantify the concentration of infectious HAV, approximately 90% confluent FRhK-4 cells in a 75-cm^2 flask were seeded in 6-well (8.96 cm^2/well) tissue culture plates. One milliliter of a sample serially diluted 10-fold with 1× DMEM containing 100 U/mL penicillin and 100 μg/mL streptomycin or 1 mL of a sample diluted 2-fold with 2× DMEM (Life Technologies) containing 200 U/mL penicillin, 200 μg/mL streptomycin, and 7.4 g/L sodium hydrogen carbonate was inoculated onto a monolayer of 100% confluent FRhK-4 cells in a 6-well plate, and the plate was then incubated in a humidified incubator at 37 °C in 5% CO_2 for 90 min. After incubation, the inoculum was removed by inverting the plate, and 3 mL of agar overlay, prepared by combining 2× DMEM supplemented with 10% heat-inactivated fetal bovine serum, 200 U/mL penicillin, 200 μg/mL streptomycin, and 7.4 g/L sodium hydrogen carbonate with an equal volume of 2.4% (w/v) agarose (SeaKem ME; Lonza Group, Rockland, ME, USA) with 23.6 mM regent-grade magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$, Wako Pure Chemical Industries), was applied to the cell monolayer. After addition of the agar overlay, the plate was incubated at 37 °C in 5% CO_2 until plaques had formed on the monolayer (9–10 days). Fresh agar overlay (3 mL) was added to the

plate after 4 or 5 days of incubation. After incubation, cells were fixed by adding 6 mL of 5% (v/v) formalin solution and stored at 20 °C for 12 to 15 h. The formalin and agarose were removed and the cell monolayer was stained with 1 mL of 0.41 g/L crystal violet at 20 °C for 10 min. Excess stain was discarded by inverting the plate, and then the number of plaques on each plate was counted. The average plaque count of triplicate wells or of 12 wells prepared from one sample was considered the infectious HAV concentration for that sample. The detection limit of the plaque assay for quantification of infectious HAV was 1/6 PFU/mL when 12 wells were prepared.

To quantify the concentration of infectious MNV, approximately 90% confluent RAW264.7 cells in a 75-cm^2 flask were seeded in 6-well (8.96 cm^2/well) tissue culture plates. A monolayer of 100% confluent RAW264.7 cells in a 6-well plate was inoculated with 1 mL of a sample serially diluted 10-fold either with 1× EMEM supplemented with 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 1.125 g/L sodium hydrogen carbonate or with 1 mL of a sample diluted 2-fold with 2× EMEM containing 4 mM L-glutamine, 200 U/mL penicillin, 200 μg/mL streptomycin, and 2.25 g/L sodium hydrogen carbonate. The plate was then incubated in a humidified incubator at 37 °C in 5% CO_2 for 90 min. After incubation, the inoculum was removed by inverting the plate, and then 3 mL of agar overlay, prepared by combining 2× EMEM (without phenol red) supplemented with 20% heat-inactivated fetal bovine serum, 4 mM L-glutamine, 200 U/mL penicillin, 200 μg/mL streptomycin, and 2.25 g/L sodium hydrogen carbonate with an equal volume of 2.5% (w/v) agarose (Agar-EPI), was applied to the cell monolayer. After addition of the overlay, the plate was incubated at 37 °C in 5% CO_2 for 2 days, the cell monolayer was stained with 1 mL of 0.15 g/L neutral red at 37 °C in 5% CO_2 for 3 h, and excess stain was discarded by inverting the plate. The number of plaques in each well was counted each day until no new plaques appeared, which generally took 2 days from the time that the neutral red was discarded. The average of the plaque counts of triplicate wells or of 12 wells prepared from one sample was considered as the infectious MNV concentration for that sample. The detection limit of the plaque assay for quantification of infectious MNV was 1/6 PFU/mL when 12 wells were prepared.

Quantification of CV, EV, HAV, and MNV by real-time RT-PCR

Viral RNA was extracted from 200 μL of sample with a QIAamp MinElute Virus Spin Kit (Qiagen, Tokyo, Japan) to obtain 20 μL of RNA solution. The extracted RNA solution was added to a High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems Japan, Tokyo, Japan) for the reverse-transcription reaction, which was conducted at 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 s with subsequent cooling to 4 °C in a thermal cycler (Thermal Cycler Dice Model TP600, Takara Bio Inc., Otsu, Japan). The resulting cDNA solution was then amplified with a TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems Japan) with 400 nM primers (HQ-SEQ grade, Takara Bio Inc.) and 250 nM TaqMan probe (Applied Biosystems Japan). The oligonucleotide sequences of the primers and the probes used to quantify CV, EV, HAV, and MNV are shown in Table S1. Amplification was conducted at 50 °C for 2 min, 95 °C for 10 min, followed by 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). A standard curve for each virus was constructed based on the relationship between the concentration of infectious virus in a freshly prepared purified virus solution determined with the plaque assay and the number of PCR amplification cycles (the C_t value). The detection limit of the PCR assay for the quantification of virus concentration was 0.1 to 1 PFU/mL.

Measurement of electrophoretic mobility

The alkalinity of Milli-Q water was brought to 20 mg $CaCO_3$/L by the addition of 0.4 mM sodium hydrogen carbonate, and the pH was adjusted to 2 to 10 with HCl or NaOH. Source water samples were filtered through a stirred ultrafiltration cell (Model 8050, Millipore Corp.) with an ultrafiltration membrane (molecular weight cutoff, 100 kDa: RC membrane; Ultrafiltration Disks, PLHK, Millipore Corp.) to exclude large particles, and the pH of the filtrate was adjusted to 6 or 7 with HCl or NaOH. The Milli-Q water and the source water samples were allowed to stand for 1 day at 20 °C to stabilize the pH. Immediately prior to measurement of the electrophoretic mobility, purified virus solution was suspended in the water at approximately $10^{6–7}$ PFU/mL for CV, 10^6 PFU/mL for EV, $10^{5–6}$ PFU/mL for HAV, $10^{10–11}$ VLPs/mL for rNV-VLPs, and 10^6 PFU/mL for MNV, and then the water was filtered through a polytetrafluoroethylene membrane filter (nominal pore size, 0.2 μm; Dismic-25HP, Toyo Roshi Kaisha) to exclude dust. Electrophoretic mobilities of the enteric viruses or virus-like particles were measured with a Zetasizer Nano ZS (50-mW, 532-nm, green laser; Malvern Instruments, Malvern, Worcestershire, UK) at 25 °C and a 17° measurement angle.

Measurement of hydrophobicity

Three hydrocarbon solvents were used for the hydrophobicity measurements: *n*-hexadecane (log K_{ow} = 8.25), *n*-octane (5.18), and *p*-xylene (3.15). Virus was suspended at approximately $10^{6–7}$ PFU/mL for CV, 10^6 PFU/mL for EV, $10^{5–6}$ PFU/mL for HAV, $10^{10–11}$ VLPs/mL for rNV-VLPs, and 10^6 PFU/mL for MNV in 2 mL of PBS. Each solution was then supplemented with 2 mL of one of the three solvents. After 10 min of preincubation at 30 °C, each mixture was intensely vortexed for 2 min and then allowed to rest for 15 min at 20 °C to allow the water and solvent to separate. The concentrations of enteric virus or virus-like particles in the water phase were quantified by means of real-time RT-PCR or an enzyme-linked immunosorbent assay. The decrease in virus concentration in the water phase after mixing with the solvent was used as a measure of the surface hydrophobicity of the virus.

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