Food and Environmental Virology (2019) 11:9–19
https://doi.org/10.1007/s12560-018-9361-5

ORIGINAL PAPER

Detection of Norovirus and Rotavirus Present in Suspended and Dissolved Forms in Drinking Water Sources

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Received: 21 September 2018 / Accepted: 10 December 2018 / Published online: 17 December 2018

Abstract

We investigated the present forms of genogroup II norovirus and group A rotavirus in surface water used for drinking water production. River water samples ($N = 15$) collected at a drinking water treatment plant (DWTP) monthly from June 2017 to August 2018 were fractioned by filtration through 10- and 0.45-µm-pore-size membranes, and viruses present in suspended and dissolved forms were quantitatively detected. Norovirus GII was present in > 10-µm- and 0.45–10-µm-suspended and dissolved forms with detection rates of 33%, 60%, and 87%, respectively. Rotavirus A was detected more frequently than norovirus GII in each form (> 10 µm suspended, 73%; 0.45–10 µm suspended, 93%; dissolved, 100%). We also analyzed surface water samples from 21 DWTPs all over Japan in non-epidemic and epidemic seasons of gastroenteritis. Norovirus GII was detected in 48% and 81% of samples with the concentrations of up to 4.1 and 5.3 $\log_{10}$ copies/L in dissolved form in non-epidemic and epidemic seasons, respectively, and GII.4 Sydney 2012 was predominant genotype followed by GII.2. Rotavirus A was detected in 95% and 86% of samples with the maximum concentrations of 5.5 and 6.3 $\log_{10}$ copies/L in dissolved form in respective seasons. Concentration of norovirus GII was similar in 0.45–10-µm suspended and dissolved forms, while there was a significant difference for rotavirus A ($P < 0.01$, pared *t* test), indicating that rotavirus A was less associated with suspended solids in the surface water samples compared to norovirus GII. Our observations provide important implications for understanding of viral behavior in environmental waters.

Keywords Norovirus · Rotavirus · Suspended solids · Adsorption · Surface water · Drinking water source

Introduction

Contamination of surface water or groundwater with human pathogenic viruses constitutes a potential risk of infection through drinking water or food (Moreira and Bondelind 2017; Mathijs et al. 2012). Enteric viruses have been detected in surface water and groundwater sources for drinking water production, as they are shed in stools and vomitus of infected individuals at high concentrations and thus contained in treated or untreated wastewater effluents discharging to water environments. For the safe drinking water supply, we need to further understand the occurrence and forms of enteric viruses present in source water, which is necessary in discussions to determine the water quality standards or water treatment requirements (i.e., log credit).

Gastroenteritis viruses are the most frequently detected enteric viruses in sewage and thus in surface water. Noroviruses are important etiological agents of acute viral gastroenteritis and have linear, positive-sense, single-stranded RNA genome in non-enveloped icosahedral capsids with a diameter of 27–40 nm. They are genetically diverse, and genogroups I (GI), GII, and GIV infect humans (de Graaf et al. 2016). Rotaviruses are the most important cause of severe diarrheal disease in infants and young children. They are non-enveloped with triple-layered icosahedral capsids that are approximately 100 nm diameter, and they have double-stranded segmented RNA genomes (Estes and Greenberg 2013). Group A rotavirus (rotavirus A) is associated with the epidemics of diarrheal diseases in human populations

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Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12560-018-9361-5) contains supplementary material, which is available to authorized users.

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and has been found in both humans and animals. Therefore, surface water environment is possibly contaminated with rotavirus A discharged from both point and diffuse fecal pollution sources of animal origin as well as humans.

Surface water samples are often concentrated with the adsorption-elution method using a membrane filter (Haramoto et al. 2012) or glass wool (Pérez-Sautu et al. 2012), and viruses have been detected without discriminating those present in suspended and dissolved forms. Some viruses may be associated with both organic and inorganic suspended materials, such as algae, protozoa, bacteria, sand, silt, and clay possibly contained in surface water, and others may be associated with the organic molecules smaller than 0.45 µm which is called dissolved organic matter or present as freely moving particles. Electrostatic charge and hydrophobicity of viral capsid proteins, the two major factors in viral adsorption (Michen and Graule 2010; Armanious et al. 2016) may be responsible for the different viral adsorptive behavior to suspended solids, resulting in different stability or transmissibility of viruses in surface water. Recent studies have investigated the effect of viral association with organic compounds or suspended solids on the stability of viral particles in water targeting enteric viruses or bacteriophages (Waldman et al. 2017; Fauvel et al. 2017). However, in situ observations of association of enteric viruses with suspended solids in surface water are extremely limited although there are some reports for wastewater samples (Sano et al. 2006; da Silva et al. 2008; Miura et al. 2015, 2018). Enteric viruses have different surface characteristics and may present virus type-specific distribution in suspended and dissolved fractions in surface water, which may affect their treatability in drinking water treatment processes.

In this study, we investigated the present forms of norovirus GII and rotavirus A in surface water used for drinking water production. To evaluate a fractionation approach, source water samples at a drinking water treatment plant (DWTP) were collected monthly and norovirus GII and rotavirus A were quantitatively detected with and without performing the fractionation by filtration using 10- and 0.45-µm-pore-size membranes. We collected surface water samples from 21 DWTPs all over Japan in non-epidemic and epidemic seasons of gastroenteritis and performed the fractionation to investigate the distribution of norovirus GII and rotavirus A in the suspended and dissolved forms. We also investigated the norovirus GII genotypes detected in the surface water samples collected in 2017–2018.

Materials and Methods

Sample Collection

In order to evaluate the effect of fractionation on the detection of viruses, a total of 15 river water samples were collected monthly from June 2017 to August 2018 at DWTP E located in the Kanto region and were analyzed with and without performing the fractionation. The sampling period includes an epidemic season of gastroenteritis in Japan (usually between November and March), and water quality parameters of collected samples are listed in Table 1.

To investigate the present form of viruses in different types of surface water, a total of 21 DWTPs (i.e., DWTP A–U including DWTP E investigated above) treating river, lake, and reservoir water were selected from six different regions in Japan considering their locations and the distribution of population (Table 2). At least one DWTP was selected in each region, and up to six DWTPs were done in the Kanto region with one-third of Japanese population.

Table 1 Water quality parameters of river water samples collected at DWTP E

Parameter	Units	Mean ± SD (range)	*N*	Method
Temperature	°C	17 ± 7.6 (5.2–27)	15	Thermometer (Pettenkofer type)
pH	–	7.5 ± 0.2 (7.2–7.8)	15	Glass electrode method
EC	µS/cm	196 ± 39 (125–244)	15	Electrode method
Turbidity	NTU	5.6 ± 2.6 (2.6–10, 290)[a]	15	Integrating sphere photoelectric photometry
Alkalinity	mg/L	32 ± 7.7 (18–46)	15	Titration with 0.01 M H_2SO_4
TOC	mg/L	1.2 ± 0.4 (0.8–2.6)	15	Combustion oxidation method
NO_2-N and NO_3-N	mg/L	1.8 ± 0.6 (0.9–2.8)	15	Ion chromatography
NH_4-N	mg/L	0.061 ± 0.036 (< 0.02–0.11)	15	Ion chromatography
NO_2-N	mg/L	0.028 ± 0.016 (0.012–0.058)	15	Ion chromatography
Cl^-	mg/L	15 ± 4.5 (7.0–23)	15	Ion chromatography
Standard plate count	$\log_{10}$ CFU/mL	3.6 ± 0.7 (2.8–5.1)	15	Standard plate count agar method
E. coli	$\log_{10}$ MPN/100 mL	1.7 ± 0.7 (0.8–3.5)	15	MMO-MUG test[b]

[a] The sample affected by a typhoon in August 2017, which recorded the turbidity of 290 NTU, was excluded to calculate the mean and SD

[b] Colilert®, IDEXX Laboratories, Inc., Tokyo, Japan

Table 2 DWTPs and types of surface water samples collected in this study

Region	Population (in thousands)	No. of DWTPs studied (ID)	Types of surface water	
			River	Lake and reservoir
Hokkaido-Tohoku	14,266	4 (A–D)	3	1
Kanto	43,398	6 (E–J)	5	1
Chubu	21,594	2 (K, L)	2	–
Kinki	22,610	5 (M–Q)	2	3
Chugoku-Shikoku	11,298	1 (R)	1	–
Kyushu-Okinawa	14,541	3 (S–U)	3	–
Total	127,707	21	16	5

Thus, the sampling sites covered major river systems in Japan. Most of DWTPs were located downstream of one or more wastewater treatment plants (WWTPs), and treated wastewater effluents could be the major source of human pathogen contamination. There was also possible fecal contamination from livestock (i.e., beef and dairy cattle, and pigs) or wild animals (i.e., deer and boar) in some of the drinking water sources studied. Source water samples were collected two times in each DWTP; September 2017 (i.e., non-epidemic season) and January 2018 (i.e., epidemic season).

All water samples (5–6 L each) were kindly collected by the operators in each DWTP and were transported to the laboratory of National Institute of Public Health within 2 days of collection on cool condition (0–10 °C). The samples were processed within 24 h of arrival.

Process Control Virus

A murine norovirus (MNV) strain S7-PP3, kindly provided by Dr. Yukinobu Tohya (Nihon University, Japan), was used as a process control virus in all water sample analyses. MNV was prepared using RAW 264.7 cells (ATCC TIB-71) according to the procedure described by Kitajima et al. (2010b).

Virus Concentration with Fractionation

We used two types of membranes with nominal pore sizes of 10 and 0.45 µm and (i) algae, protozoa, sand, and silt larger than 10 µm, (ii) bacteria, silt, and clay between 0.45–10 µm, (iii) dissolved organic matter smaller than 0.45 µm contained in surface water samples were fractionated. Briefly, 1 L of surface water sample was filtered first through a 10-µm hydrophilic polytetrafluoroethylene (PTFE) membrane (90 mm diameter; JCWP09025, Merck, Tokyo, Japan) and the filtrate (1L) was recovered in a sterile suction bottle. The 10-µm membrane was folded two times, made a cut in by sterile scissors, and recovered in a 50-mL tube. The filtrate was then passed through a 0.45-µm hydrophilic mixed cellulose esters (MCE) membrane (90 mm diameter; HAWP09000, Merck, Tokyo, Japan), and the filtrate (1L) was recovered in another sterile suction bottle. The 0.45-µm membrane was recovered in a 50-mL tube after the folding and cutting. Viruses in the filtrate were concentrated with the adsorption-elution method using a negatively charged membrane (Katayama et al. 2002). Magnesium chloride hexahydrate was added to the filtrate at a final concentration of 25 mM, and the solution was passed through another 0.45-µm MCE membrane. Subsequently, 200 mL of 0.5 mM H_2SO_4 (pH 3.0) was passed through the membrane, followed by 10 mL of 1 mM NaOH (pH 11.0) to elute viruses. The filtrate (10 mL) was recovered as viral concentrate in a 50-mL tube containing 100 µL of 50 mM H_2SO_4 and 100 µL of 100 × Tris-EDTA (TE) buffer for neutralization. The river water samples collected at DWTP E in August 2017 and July 2018 were affected by rain, and 0.2 L and 0.5 L were analyzed instead of 1 L.

Virus Concentration without Fractionation

Water samples were processed by the adsorption-elution method using a 0.45-µm MCE membrane as described above (Katayama et al. 2002), and 10 mL of viral concentrate was prepared.

Viral RNA Extraction

Viral RNA was extracted from 10- and 0.45-µm membranes and 1 mL of viral concentrate using the NucliSENS kit (bioMérieux, Lyon, France) according to the manufacturer's instructions with some modification for the membrane samples. Briefly, 15 mL of lysis buffer was added to the membrane recovered in a 50-mL tube, the tube was vortexed for 15 s and incubated for 10 min on a tube shaker followed by centrifugation at 2300×*g* for 5 min. The supernatant (15 mL) was recovered, and 140 µL of magnetic silica particles was added. All washing steps were performed using the NucliSENS miniMAG, and viral RNA was recovered in 100 µL of elution buffer.

Quantification of Viral Genomes

All viral RNA was amplified using previously reported primers and probes for MNV (Kitajima et al. 2010b), norovirus GII (Loisy et al. 2005; Kageyama et al. 2003), and rotavirus A (Pang et al. 2004, 2011; Miura et al. 2018) with an RNA ultrasense one-step quantitative RT-PCR system (Thermo Fisher Scientific, Tokyo, Japan). The cycle threshold (C_T) was defined as the cycle at which a significant increase in fluorescence occurred. Undiluted and tenfold diluted RNA extracts were tested, and absence of inhibition in real-time RT-PCR was verified for each sample. The number of genome copies in each reaction well was calculated by comparing the C_T value to a standard curve generated from a dilution series of plasmid containing each target region. Then the virus concentration in the sample was calculated based on the volume of viral RNA analyzed and was reported per liter. A C_T value less than 40 was regarded as positive (Kazama et al. 2017; Miura et al. 2018), and the detection limits were 110 and 85 copies/L for norovirus GII and rotavirus A in suspended forms, respectively (1100 and 850 copies/L for norovirus GII and rotavirus A in dissolved forms). Only samples with MNV recovery efficiencies above 1% in the dissolved form fraction (recovered as the virus concentrate) were considered for quantification. We used the MNV recovery efficiency as a quality assurance parameter only and did not use to adjust test results (Kazama et al. 2017; Miura et al. 2018).

Genotyping of Norovirus GII

Viral RNA samples from the dissolved fraction tested positive for norovirus GII in real-time RT-PCR were applied to RT reaction, and cDNA was synthesized using PrimeScript RT Master Mix (Perfect Real Time; RR036B, Takara Bio Inc., Kusatsu, Japan). Fifty microliters of the reaction mixture contained 25 μL of RNA, 10 μL of 5 × PrimeScript RT Master Mix (including RTase, RNase inhibitor, oligo dT primer, and random hexamers, dNTP, buffer, Mg^{2+}), and 15 μL of deionized distilled water (DDW). Thermal conditions consisted of RT at 37 °C for 15 min and 42 °C for 5 min, and inactivation of the enzyme at 85 °C for 5 s (Miura et al. 2015).

Semi-nested PCR was performed to amplify the capsid N/S region as described previously (Kazama et al. 2016). Briefly, in the first PCR, amplification was performed in a 50-μL reaction mixture containing 15 μL of cDNA, 25 μL of Premix Ex Taq Hot Start version (RR030A, Takara Bio Inc.), and 25 pmol of COG2F and G2SKR primers (Kojima et al. 2002; Kageyama et al. 2003). In the second PCR, the 50-μL reaction mixture consisted of 2 μL of the first PCR product, 25 μL of Premix Ex Taq Hot Start version (RR030A, Takara Bio Inc.), and 25 pmol of G2SKF and G2SKR primers (Kojima et al. 2002). The first and second amplification conditions were: 25 cycles at 98 °C for 10 s, 50 °C for 30 s, and 72 °C for 30 s. PCR products were visualized using agarose gel electrophoresis, and those with an expected length (344 bp) were then purified using QIAquick PCR Purification Kit (Qiagen, Tokyo, Japan) following the manufacturer's protocol. The purified PCR products were sent for direct sequencing by Eurofins Genomics K.K., Tokyo, Japan. Genotypes and variants of the obtained sequences were identified using the Norovirus Genotyping Tool version 1.0 (Kroneman et al. 2011).

Phylogenetic Analysis

To compare nucleotide sequences obtained from surface water samples, phylogenetic analysis was carried out for GII.4 genotype that was most frequently detected in the samples. The sequences of capsid N/S region (282 nucleotides) were aligned with ClustalW; bootstrapped phylogenetic trees were then constructed by the neighbor-joining method with 1000 bootstrap replications by using the MEGA7 software (Kumar et al. 2016). The genetic distances were calculated by the Kimura two-parameter method.

Quality Controls

Filter tips and dedicated rooms were used to prevent sample contamination, and sample and data analyses were carried out using the following quality controls.

(i) *Fractionation and virus concentration*. MNV was added to all samples as the whole process control virus, which is inoculated into samples before samples are processed (Haramoto et al. 2018), and its recovery efficiency was monitored. Recovery rate was calculated by dividing the copy number of the MNV detected in each fraction by the copy number of the MNV added to the sample. Although small portion of added MNV would be adsorbed to suspended solids, efficient recovery of the most portions in the dissolved fraction indicates that effect of filtration by the particles and macromolecules accumulated on the membrane surface (called as cake filtration) would be small. In order to evaluate the variability of the fractionation, the samples collected at DWTP E were analyzed in duplicate or triplicate.

(ii) *Viral RNA extraction from membrane*. As for the samples collected at DWTP E between February and August 2018 ($N = 7$), another 1 L (0.5 L for July 2018) of sample was processed without adding MNV and another sample set was prepared by fractionation and concentration. MNV was then added to the folded membrane in a 50-mL tube or to the 1 mL of dissolved fraction (virus concentrate) just before RNA extraction, and the RNA extraction efficiency was monitored.

(iii) *Quantification*. Absence of inhibition in real-time RT-PCR was verified for each sample as described above. After completion of all real-time RT-PCR runs, all standard curves for each virus were compared, and quality criteria were applied (AFNOR 2011). Only PCR runs in which standard curves with amplification efficiencies of 85 to 110% were kept for quantification (Le Mennec et al. 2017; Miura et al. 2018).

Statistical Analysis

To compare virus concentrations among the three fractions (i.e., > 10 µm suspended; 0.45–10 µm suspended; dissolved), the paired *t* test was performed using IBM SPSS Statistics Ver. 19.

Results

Recovery Efficiency of the MNV Process Control

MNV was added to the river water samples collected at DWTP E ($N = 15$) before performing the fractionation, and the most portions were recovered in the dissolved fraction (geometrical mean of MNV recovery rates, 28% [range, 7.4–68%]), while small portions were recovered in the suspended fractions (1.0% [0.09–3.2%] for 0.45–10 µm; 0.09% [0.005–1.8%] for > 10 µm) (MNV recovery rates in each sample are plotted in Fig. S1). MNV RNA extraction efficiencies were high enough for the dissolved fraction (89%) followed by 0.45–10-µm suspended (37%) and > 10-µm suspended fractions (8.5%). When the samples were processed without performing the fractionation (directly processed by the adsorption-elution method), the geometrical mean of MNV recovery rates was 25% (9.0–54%) except one sample (0.04%) affected by a typhoon in August 2017. Neither significant difference nor correlation was observed in the MNV recovery rates between the dissolved fraction and the unfractionated samples ($N = 15$, paired *t* test or Pearson's correlation).

As for the surface water samples from 21 DWTPs ($N = 42$), the geometrical means of MNV recovery rates were 27% (13–48%) for the dissolved fraction, 2.2% (0.2–7.9%) for the 0.45–10-µm suspended fraction, and 0.51% (0.013–2.4%) for the > 10-µm suspended fraction, respectively. The added MNV was stably recovered in the dissolved fraction, and some portions were adsorbed to the suspended solids as we observed for the monthly samples from DWTP E. (MNV recovery rates in each sample in epidemic and non-epidemic seasons are presented in Figs. S2, S3, respectively.) Based on our observing of no significant decrease in flow rate during the filtration together with the efficient MNV recovery rates in the dissolved fraction, we considered that the effect of cake filtration was negligible, giving confidence in the quantitative detection results.

Inhibition was not detected in the dissolved fraction samples except for 5 out of 57 samples (8.8%), but inhibitory compounds persisted in undiluted (neat) RNA from 0.45- to 10-µm and > 10-µm suspended fraction samples and tenfold diluted RNA were analyzed. The C_T values of neat RNA were used for direct quantification in only two 0.45–10-µm suspended fraction samples, and those of diluted RNA were used in the other 112 samples. (The dilution factor was thus considered for quantification.)

Effect of Fractionation on the Detection of Norovirus GII and Rotavirus A

River water samples were collected at DWTP E monthly from June 2017 to August 2018 and analyzed with and without performing the fractionation. When the samples were fractionated, norovirus GII was detected in 13 out of 15 samples and the detection rate was the highest for the dissolved fraction (87%), followed by the 0.45–10-µm suspended fraction (60%) and the > 10-µm suspended fraction (33%) (Fig. 1). The two negative samples collected in July and October 2017 were positive in unfractionated samples, while five positive samples of June, August, September 2017, July, and August 2018 were negative in the unfractionated samples. Thus, discordant results were observed in the seven samples collected in non-epidemic seasons of gastroenteritis. Although the sample collected in August 2017 was affected by a typhoon with extremely high turbidity (290 NTU), norovirus GII was detected in the dissolved and 0.45–10-µm suspended fractions (Fig. S1). Norovirus GII concentrations in the dissolved fraction were significantly higher than those in the 0.45–10-µm suspended fraction ($P < 0.01$, paired *t* test), indicating that most of norovirus GII in the river water samples collected at DWTP E were associated with smaller molecules (< 0.45 µm) or present as freely moving particles. Geometric mean concentration was 4.3 $\log_{10}$ copies/L for the dissolved fraction, while that in the 0.45–10-µm suspended fraction was about 1-log lower (3.2 $\log_{10}$ copies/L).

Rotavirus A was detected more frequently than norovirus GII in each fraction, the dissolved fraction being the highest (100%) followed by the 0.45–10-µm suspended fraction (93%) and the > 10-µm suspended fraction (73%) (Fig. 1). A discordant result was observed in the typhoon-affected sample of August 2017, which was negative for rotavirus A in the unfractionated samples. Concentrations of rotavirus A were significantly higher than those of norovirus GII in the dissolved fraction, the 0.45–10-µm suspended fraction, and the unfractionated samples ($P < 0.01$, paired t test). Rotavirus A level in the dissolved fraction was significantly higher than that in the > 10-µm and 0.45–10-µm suspended fractions ($P < 0.01$, paired *t* test), indicating that most of rotavirus A in the river water samples were not efficiently associated with suspended solids.

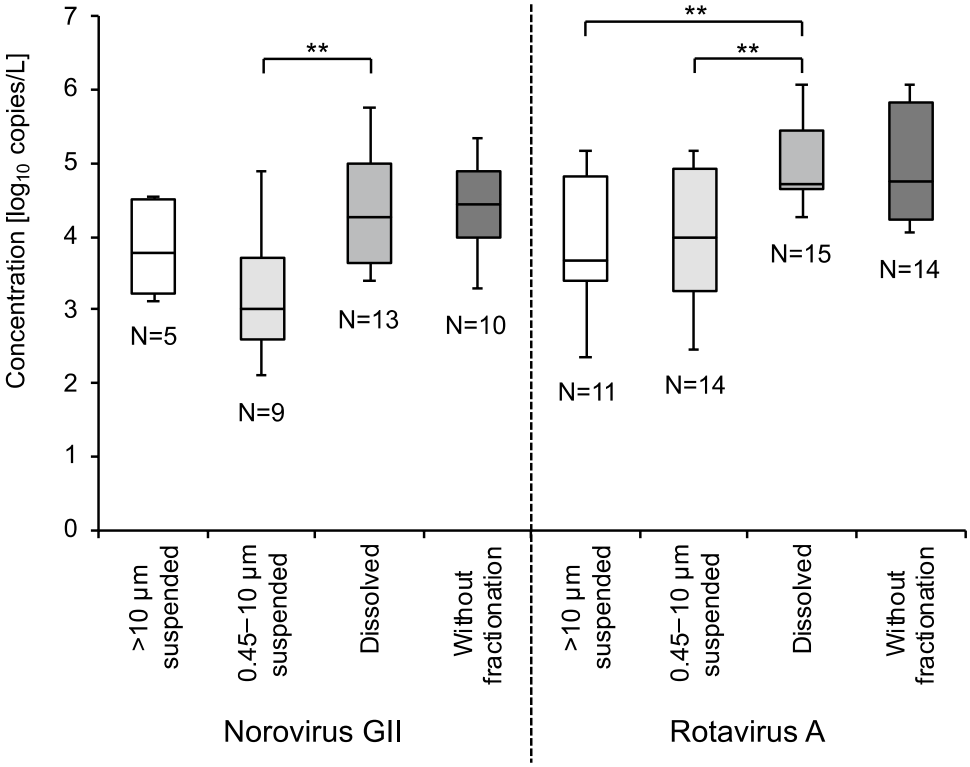

Fig. 1 Boxplots for norovirus GII and rotavirus A concentrations in each fraction of river water samples collected at DWTP E. The horizontal line within the box indicates median, box boundaries indicate 25th and 75th percentiles, whiskers indicate minimum and maximum values. *N* number of positive samples. $^{**}P < 0.01$ (paired *t* test)

Norovirus GII and Rotavirus A Present in Surface Water in Japan

As the two viruses associated with suspended solids were successfully detected with performing the fractionation and the detection rate of norovirus GII in the dissolved fraction was higher than that in the unfractionated samples, we fractionated the surface water samples collected from 21 DWTPs in Japan (Table 3). In the non-epidemic season of gastroenteritis (September 2017), norovirus GII was detected in almost half (48%) of samples, and the geometric mean concentration in the positive samples was 3.2 ± 0.7 $\log_{10}$ copies/L. Six samples were positive only in the suspended fractions, while three samples were positive only in the dissolved fraction, resulting in the highest detection rate (29%) in the 0.45–10-µm suspended fraction. In the epidemic season (January 2018), 81% of samples were positive for norovirus GII with increased geometric mean concentration of 4.6 ± 0.6 $\log_{10}$ copies/L. Five samples were positive only in the suspended fractions and the 0.45–10-µm suspended fraction presented the highest detection rate (67%) as we observed in the non-epidemic season. In some samples, concentration of norovirus GII in the 0.45–10-µm suspended fraction was higher or similar compared to those

Table 3 Detection of norovirus GII and rotavirus A in surface water samples collected at 21 DWTPs in Japan

	Norovirus GII				Rotavirus A			
	> 10 µm suspended	0.45–10 µm suspended	Dissolved	Total[a]	> 10 µm suspended	0.45–10 µm suspended	Dissolved	Total[a]
September 2017								
GM ± GSD[$\log_{10}$ copies/L]	2.9 ± 0.3	2.6 ± 0.5	3.7 ± 0.3	3.2 ± 0.7	3.9 ± 0.7	3.7 ± 0.6	4.7 ± 0.4	4.8 ± 0.5
Detection rate[%]	9.5	29	14	48	76	76	90	95
January 2018								
GM ± GSD[$\log_{10}$ copies/L]	3.7 ± 0.5	4.0 ± 0.7	4.6 ± 0.4	4.6 ± 0.6	4.2 ± 0.8	4.2 ± 1.0	4.9 ± 0.7	5.1 ± 0.7
Detection rate[%]	43	67	57	81	62	81	86	86

A total of 42 samples were collected in non-epidemic and epidemic seasons of gastroenteritis. Geometric mean (GM) and geometric standard deviation (GSD) of all positive samples were calculated

[a] The sum of virus concentrations observed in the three fractions was calculated for each sample

in the dissolved fraction (Figs. S2, S3) and there was no significant difference between the two fractions (paired *t* test), indicating that norovirus GII was highly associated with 0.45–10-µm suspended solids in certain conditions.

Rotavirus A was detected in the surface water samples regardless of the seasons, and the detection rates were 95% and 86% in the non-epidemic and epidemic seasons, respectively (Table 3). Geometric mean concentrations in the positive samples were also similar in both seasons, which were 4.8 ± 0.7 and 5.1 ± 0.7 $\log_{10}$ copies/L, respectively. Unlike with norovirus GII, detection rate of rotavirus A in the dissolved fraction was the highest (90% and 86%) among the three fractions, and the concentration was significantly higher compared to the > 10-µm and 0.45–10-µm suspended fractions ($P < 0.01$, paired *t* test). The result of rotavirus A observed in the 21 DWTPs in the non-epidemic and epidemic seasons suggested that rotavirus A was less associated with suspended solids compared to norovirus GII.

Norovirus GII Genotypes Detected in 2017–2018

A total of seven sequences were obtained from the monthly samples collected in DWTP E ($N = 15$) and were belonged to GII.4 Sydney 2012 ($N = 4$) or GII.17 ($N = 3$). Only one sequence was obtained from 21 DWTPs in the non-epidemic season and was classified as GII.2. In the epidemic season, 10 sequences were obtained and GII.4 Sydney 2012 was predominant ($N = 7$). The other three strains belonged to GII.4 unknown sub-genotype, GII.2, or GII.5. As GII.4 Sydney 2012 was the major sub-genotype detected in the collected samples, the phylogenetic tree analysis was performed to compare the sequences. The GII.4 sequence of unknown sub-genotype was excluded in the analysis as the overlapping of chromatogram was observed. The GII.4 Sydney 2012 sequences obtained in this study clustered together with the strains isolated in Asia including Japan and China in 2015–2017 (Fig. 2). Genetically similar sequences were detected in the surface water samples collected in January 2018, indicating that GII.4 Sydney 2012 strains were circulating in the human populations in most parts of Japan.

Discussion

Surface water often receives discharge from one or more wastewater treatment plants, and thus, the water quality is of great concern both for drinking water and fresh food production. Non-enveloped viruses are quite stable in the environment including water and fomites (Liu et al. 2009; Seitz et al. 2011), which may contribute to viral transmission

Fig. 2 Phylogenetic tree of the N/S region (282 nucleotides) of GII.4 strains obtained from surface water samples. Trees were built with the neighbor-joining method and bootstrapped with 1000 repetitions. The name of obtained sequence consists of the sampling date (year-month-day), followed by the ID of DWTP. The name of each reference sequence consists of the strain name or GII.4 variant name followed by the year, country, and accession number. The values at the nodes represent bootstrap rates of > 70%

(Geoghegan et al. 2016). Indeed, human noroviruses have been shown to cause foodborne outbreaks due to pre-harvest contamination from polluted irrigation water (Mathijs et al. 2012; Prez et al. 2018), as well as waterborne outbreaks originated from source water contamination (Larsson et al. 2014; Moreira and Bondelind 2017). Occurrence of enteric viruses in surface water has been investigated in recent studies (La Rosa et al. 2017; Masachessi et al. 2018; Mackowiak et al. 2018); however, knowledge on how viruses are present is still limited. We quantified norovirus GII and rotavirus A present in suspended and dissolved forms separately by applying several quality controls for the reliable real-time RT-PCR-based quantification (Le Mennec et al. 2017; Miura et al. 2018). Distribution of two viruses present in suspended and dissolved forms in the surface water samples was different, which may be linking to their different persistence and transmissibility through environmental waters.

In the present study, a first approach of fractionation of viruses in surface water was evaluated using the monthly collected river water samples from DWTP E targeting norovirus GII and rotavirus A. We succeeded in the quantitative detection of two viruses associated with suspended solids, and the detection rates of norovirus GII and rotavirus A were both higher in the 0.45–10-µm fraction compared to the > 10-µm fraction. This results suggest that norovirus GII and rotavirus A may be adsorbed to bacteria, silt, or clay between 0.45 and 10 µm rather than algae, protozoa, sand, and silt larger than 10 µm, although the evidence such as electron microscope images is needed but it is difficult to obtain due to the extremely low concentration of viruses in surface water samples. We were also able to quantitatively detect norovirus GII and rotavirus A present in the dissolved form, and their detection rates and concentrations were higher than those in the 0.45–10-µm suspended fraction. However, in the surface water samples collected at 21 DWTPs in Japan, the two viruses showed different distribution in the three fractions; norovirus GII were highly associated with the 0.45–10-µm suspended solids and rotavirus A were contained in the dissolved fraction. This discrepancy shows that several factors contribute to the present form of viruses, which may be impacted by amount and composition of dissolved and particulate organic matter and clay and silt particles depending on the watershed characteristics such as types of soil, land use, population of humans and animals, and hydrological condition. The consistent finding of abundant rotavirus A in the dissolved fraction indicates that rotavirus A is less associated with suspended solids in surface water compared to norovirus GII.

Rotavirus A has different surface characteristics compared to norovirus GII regarding electrostatic charge and hydrophobicity as well as viral particle size. Isoelectric points (pI) of rotavirus SA11 is 8.0 (Michen and Graule 2010) while norovirus GII.4, the major genotype observed in this study, has pI of 4.2–6.9 (Michen and Graule 2010; Samandoulgou et al. 2015). Rotavirus A and norovirus GII presented different adsorption characteristics to mixed liquor suspended solids in wastewater treatment processes, and their binding capacity depended on pH (Miura et al. 2018).Therefore, we speculated that the surface charge may be the key factor modulating the different behaviors observed in the surface water samples, although the net surface charges of rotavirus A and norovirus GII in the collected samples (pH 6.9–7.8) were not measured in this study. Hydrophobicity of the surface capsid protein also plays a significant role in viral adsorption, and it is different among viruses (Chaudhry et al. 2015; Armanious et al. 2016). Hydrophobic interaction between rotavirus A and suspended solids may be weaker compared to norovirus GII. As for rotavirus, the hydrophobic part of VP5* may not be exposed in the environment prior to the proteolytic cleavage that is required for the cell attachment (Denisova et al. 1999; Kim et al. 2010), while hydrophobic interaction between the norovirus protruding domain and histo-blood group antigens (HBGAs), the cell attachment factor, has been reported (Hansman et al. 2011). Norovirus GII was highly associated with the 0.45–10-µm suspended solid fraction that contains bacteria possibly expressing HBGA-like extracellular polymeric substances (EPS) (Miura et al. 2013). The hydrophobic interaction between viral particles and EPS in surface water must be investigated in the future studies. Interaction with bacterial compounds protects viral particles from inactivation stresses such as chlorine, heat, and UV (Li et al. 2015; Waldman et al. 2017). Sunlight radiation rather than temperature is critical to hepatitis A virus and MNV (Polo et al. 2015), and different viral adsorptive behavior to suspended solids may explain differences in stability and transport of viruses in surface waters.

Norovirus GII concentrations in the river water samples collected at DWTP E increased in the epidemic season of gastroenteritis up to 5.0 $\log_{10}$ copies/L, which was 1- or 2-log higher compared to the previous studies reporting up to 10^3 to 10^4 copies/L of norovirus GI, GII, or GIV in river water in Japan (Kitajima et al. 2009; Kishida et al. 2012). In the surface water samples from 21 DWTPs, norovirus GII was detected in 81% of samples in the epidemic season and the level ranged between 3.7 and 5.3 $\log_{10}$ copies/L. Haramoto et al. analyzed surface water samples collected from 16 DWTPs in Japan in the non-epidemic and epidemic seasons of 2008 and 2009, and norovirus GII was detected in only one out of 64 samples (2%) (Haramoto et al. 2012). Although the DWTPs studied, year, sample preparation, detection methods, and major norovirus genotypes were not exactly the same, the difference in detection rate was significant and we need to keep a watch on the behavior of noroviruses.

In our study, a major norovirus genotype in each sample was investigated by the direct sequencing approach and GII.4 Sydney 2012 was predominant in 2017–2018. In one sample collected in the epidemic season, GII.4 sub-genotype could not be determined due to the overlapping of chromatogram. As surface water may be contaminated with multiple norovirus genotypes, cloning-sequencing or massive parallel sequencing (also called next-generation sequencing [NGS]) will be an appropriate approach to study the genetic diversity of norovirus more accurately (Kitajima et al. 2010a; Boonchan et al. 2017).

Although rotavirus A contamination in surface water has been investigated (Ishii et al. 2014; Prevost et al. 2015; La Rosa et al. 2017; Mackowiak et al. 2018), the level and seasonality of contamination is still unknown. We found high prevalence of rotavirus A in surface water samples (> 86%) regardless of seasons, and the concentrations were also high (3.3–6.4 $\log_{10}$ copies/L). In Japan, Rotarix® and RotaTeq® have been commercially available for voluntary vaccination since November 2011 and July 2012, respectively, and the number of gastroenteritis cases caused by rotavirus has been decreasing (Morioka et al. 2017). Therefore, there are two possible explanations for this observation. One is fecal contamination from livestock animals, such as cows and pigs that can be infected with rotavirus A, and the other is contamination of vaccine strains. Although vaccine strains have not been detected in sewage samples (Fumian et al. 2011; Tort et al. 2015), we cannot deny the possibility that feces of 5- or 6-month-old vaccinated babies, who start eating solid baby foods, are thrown into the toilet. Rotavirus vaccine strains (RotaTeq®) are stable in surface water (almost 2 months for one-$\log_{10}$ reduction) (Moresco et al. 2016), and environmental surveillance for the vaccine strains should be considered.

Population in Japan reached its peak of 128 millions in 2008 and has been decreasing over the following 10 years. Nevertheless, the population in Tokyo, Kanagawa, Saitama, and Chiba Prefectures belonging to the Tokyo metropolitan area in the Kanto region is still increasing, and the water-use efficiency will be enhanced further as observed in other large cities of the world. Microbiological water quality, especially the level of enteric viruses, is becoming a concerning issue for human health. In that context, information on human pathogenic viruses that are most stable in environmental waters or that are most difficult to remove in wastewater or drinking water treatment processes will be necessary in discussions to determine the target indicator in monitoring. We found that norovirus GII was associated with 0.45–10-µm suspended solids in surface water, and most of rotavirus A was present in the dissolve form. Our results provide important implications that can explain norovirus GII are so stable and transmitted through environmental waters. Our data also will be applicable to the hydrodynamic modeling of virus transport in surface water (Sokolova et al. 2015; Bhattarai et al. 2017), where the present form of viruses will be one of the key parameters. Enteric viruses detected in environmental waters are highly diverse, and knowledge of the present form of other human pathogenic viruses should be investigated in the future studies. There are also variations in the amount and composition of suspended solids in surface water depending on locations and weather conditions, and we need to obtain more observations from different area of the world.

Acknowledgements We are very grateful to people in the 21 waterworks in Japan for their great effort in providing the source water samples. We also thank Nobuko Maeda and Nobuyo Yoshida (National Institute of Public Health), Naoko Arakawa (Kushiro City), Nariko Shinohara (Chiba Prefecture), and Satoshi Matsumura (Suita City) for their technical assistance. This study was supported in part by the Japan Society for the Promotion of Science through KAKENHI (17K14752) and by the Ministry of Health, Labor, and Welfare, Japan through a Health and Labor Sciences Research Grant (H28-Kenki-Ippan-005).

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