Seasonal profiles of human noroviruses and indicator bacteria in a wastewater treatment plant in Tokyo, Japan

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Abstract The seasonal profiles of microorganisms in raw sewage, secondary-treated sewage, and final effluent at a wastewater treatment plant in Tokyo, Japan, were quantitatively determined each month for one year, from July 2003 to June 2004. Human noroviruses, which were determined by real-time PCR, in raw sewage varied from 0.17–260 copies/mL for genotype 1 and from 2.4–1900 copies/mL for genotype 2, showing much higher values in winter, the epidemic season. The concentration of total coliforms, *Escherichia coli*, or F-specific phages in raw sewage was almost constant throughout the year. Human noroviruses of genotype 2 were removed most effectively (3.69 $\log_{10}$ on average) at the wastewater treatment plant, followed by *E. coli* (3.37 $\log_{10}$), total coliforms (3.05 $\log_{10}$), F-specific phages (2.81 $\log_{10}$), and human noroviruses of genotype 1 (2.27 $\log_{10}$). The removal ratio of human noroviruses was almost constant, independent of the initial concentration of the viruses in raw sewage, which led to the increasing concentration of human noroviruses in final effluent in winter. None of the tested bacteria was judged to be a reliable indicator of human noroviruses in final effluent.

Keywords Human norovirus; indicator bacteria; real-time PCR; wastewater treatment plant

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Introduction

Human noroviruses are the major agents of acute nonbacterial gastroenteritis in patients of all age groups in both developed and developing countries (Green *et al.*, 2001), and they are estimated to be associated with over 90% of acute viral gastroenteritis cases worldwide (Green, 1997; Fankhauser *et al.*, 1998). In Japan, according to the report from the Ministry of Health, Labour and Welfare, Tokyo, 44.5% of the patients of foodborne illness in 2004 were infected with human noroviruses.

Human noroviruses are excreted in the faeces of infected patients at a high concentration, thus the faecal-oral route via contaminated food or water is a predominant mode of its transmission. They have been detected in various kinds of water samples, such as wastewater (Lodder *et al.*, 1999; Lodder and de Roda Husman, 2005; Pusch *et al.*, 2005; van den Berg *et al.*, 2005), river water (Hörman *et al.*, 2004; Haramoto *et al.*, 2005), seawater (Griffin *et al.*, 1999; Katayama *et al.*, 2002), and even tap water (Haramoto *et al.*, 2004), by using polymerase chain reaction (PCR). Human noroviruses were frequently detected in water samples in winter, while many of samples in summer were negative for the viruses, partly because of the low sensitivity of detection methods.

In addition to two known human-genotypes, several strains of noroviruses have been recently isolated from cattle or mice and classified as genotypes different from human-genotypes (van der Poel *et al.*, 2000; Karst *et al.*, 2003; Oliver *et al.*, 2003). There has been no evidence to date of zoonotic infection of noroviruses (Oliver *et al.*, 2003), which

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indicates that human noroviruses survive and circulate in human society in summer as well as in winter. However, the prevalence of human noroviruses in summer remains unclear.

Monitoring of raw sewage to a wastewater treatment plant (WWTP) can be one of the most appropriate approaches to understand the actual incidence of human enteric viruses in its service area, especially from the viewpoint that the raw sewage contains the viruses excreted from the patients of sporadic or asymptomatic cases.

Methods

Collection of wastewater samples

Raw sewage, secondary-treated sewage before chlorination, and final effluent after chlorination were collected from a WWTP in Tokyo, Japan, once a month for one year, from July 2003 to June 2004. This WWTP serves a population of approximately 63,000 and treats 28,000 m^3 of sewage per day, showing a coverage ratio of 100% in its service area. At the WWTP, a conventional activated sludge process is installed, followed by chlorination for disinfection of microorganisms. Final effluent is discharged into a nearby river.

All samples were collected and stored in plastic bottles on ice. Sodium thiosulfates were added to the final effluent sample within 15 minutes after sampling for dechlorination. The samples were delivered to the laboratory within one day after collection.

Quantification of human noroviruses

Concentration of wastewater samples. The method used for the concentration of wastewater samples was described previously (Katayama *et al.*, 2002). First, 2.5 M $MgCl_2$ was inoculated into the sample to obtain a concentration of 25 mM. The sample was then passed through an HA filter (0.45 μm pore size and 90 mm diameter; Millipore, Tokyo, Japan) attached to a glass filter holder (Advantec, Tokyo, Japan). The volumes filtered were 100 mL for raw sewage and 1000 mL for secondary-treated sewage or final effluent. The filter was rinsed with 200 mL of 0.5 mM H_2SO_4 (pH 3.0), followed by elution of viruses with 10 mL of 1 mM NaOH (pH 10.8). The filtrate was recovered in a tube containing 50 μL of 100 mM H_2SO_4 (pH 1.0) and 100 μL of 100x Tris-EDTA buffer (pH 8.0) for neutralization.

The concentrated sample was applied to centrifugation using a Centriprep YM-50 (Millipore, Tokyo, Japan) at 2500 rpm for 10 minutes. The remaining portion (approximately 2 mL) was applied to further ultrafiltration at 2500 rpm for 5 minutes to obtain a final volume of 700 μL.

RNA extraction and reverse transcription. Viral RNA was extracted from 140 μL of the final concentrated sample using a QIAamp viral RNA mini kit (Qiagen, Tokyo, Japan) to obtain a final volume of 60 μL.

Of the extracted RNA, 20 μL was added to a reaction mixture containing 2 μL of 200 U/μL SuperScript II reverse transcriptase (Invitrogen, Tokyo, Japan), 2 μL of 100 mM dithiothreitol (Invitrogen, Tokyo, Japan), 8 μL of 5x first-strand buffer (Invitrogen, Tokyo, Japan), 1 μL of 20 U/μL RNase inhibitor (Applied Biosystems, Tokyo, Japan), 2 μL of the four 2.5 mM deoxynucleoside triphosphate stocks (Applied Biosystems, Tokyo, Japan), 2 μL of 50 μM random hexamers (Applied Biosystems, Tokyo, Japan), and 3 μL of MilliQ water (Millipore, Tokyo, Japan). The reaction mixture was incubated at 42 °C for 60 minutes, and at 99 °C for 5 minutes with the GeneAmp PCR system 9600 (Applied Biosystems, Tokyo, Japan).

Real-time PCR. The concentration of cDNA of human noroviruses was quantitatively determined by real-time PCR using the ABI PRISM 7000 sequence detection system (SDS; Applied Biosystems, Tokyo, Japan) as follows.

About 5 μL of each resulting cDNA sample was mixed with 45 μL of a reaction buffer containing 25 μL of 2x TaqMan universal PCR master mix (Applied Biosystems, Tokyo, Japan), 400 nM of each primer, and 300 nM TaqMan probe (Kageyama *et al.*, 2003). The mixtures were added to a 96 well micro plate (Applied Biosystems, Tokyo, Japan), and incubated as follows: 50 °C for 2 minutes, followed by at 95 °C for 10 minutes, 50 cycles at 95 °C for 15 s and at 56 °C for 1 minutes.

In order to draw a standard curve, standard samples of human noroviruses of genotypes 1 and 2 (10^7 copies/μL each) were diluted by serial 10-fold dilution. The wastewater samples and the standard samples were applied to the real-time PCR at the same time, followed by analysis using the SDS software (version 1.1; Applied Biosystems, Tokyo, Japan) to obtain the quantitative data on the concentration of human noroviruses in a well. Two and three wells were used for the wastewater and standard samples, respectively, and the average was used for the subsequent calculation. The concentration of human noroviruses in the original wastewater sample was calculated assuming that no viruses were lost during the detection processes, such as the concentration of water samples, the extraction of RNA, and the synthesis of cDNA. A concentration of 0.0001 copies/mL was given to a negative result for the quantitative analysis.

Quantification of indicator bacteria

Total coliforms and Escherichia coli. Total coliforms and *E. coli* in 1 mL of the sample were quantified by a single-agar-layer method using chromocult-coliform agar (Merck, Tokyo, Japan). After incubation at 37 °C for 24 hours, blue colonies were counted as *E. coli*, while both blue and red colonies were counted as total coliforms.

F-specific phages. Except for the samples in July 2003, the concentration of F-specific phages in 1 mL of the sample was determined by a double-agar-layer method using the host strain *Salmonella enterica* serovar Typhimurium WG49 as described in ISO 10705 (Mooijman *et al.*, 2002). A concentration of 0.1 PFU/mL was given to a negative result for the quantitative analysis.

Results and discussion

Profile of human noroviruses in wastewater

The seasonal profiles of human noroviruses in wastewater samples are shown in Figure 1. Human noroviruses were detected in all raw sewage samples. The concentration of

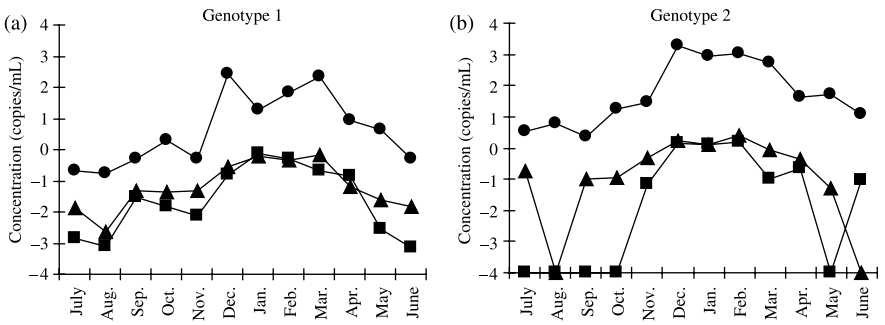

Figure 1 Seasonal profile of human noroviruses at WWTP; ●, raw sewage; ▲, secondary-treated sewage; ■, final effluent

human noroviruses of each genotype increased in winter (from December 2003 to March 2004), compared to other seasons (*t*-test, $P < 0.05$). The sample in December 2003 showed the highest concentration of human noroviruses of each genotype: 260 copies/mL for genotype 1 and 1900 copies/mL for genotype 2. On the other hand, the lowest concentration was obtained from the sample in August 2003 for genotype 1 (0.17 copies/mL) and the sample in September 2003 for genotype 2 (2.4 copies/mL). In other words, the concentration of genotype 1 or 2 varied within a range of 3.19 $\log_{10}$ or 2.91 $\log_{10}$, respectively, during the survey for one year. A simple interpretation of these data can expect approximately 1000 times (3 $\log_{10}$) more infections in winter than in summer, although the asymptomatic infection or the number of viruses excreted per infection might be different among seasons or symptoms. Further studies are needed to discuss the relationships between the epidemiology and the prevalence of viruses in raw sewage.

Human noroviruses of genotype 2 were always more abundant in raw sewage than those of genotype 1. The ratio of the concentration of genotype 2 to that of genotype 1 ranged from 2.4 to 60.9 with a geometric average of 13.3. According to the epidemiological reports (the National Institute of Infectious Disease, Tokyo, Japan), the number of isolation cases of genotype 2 from hospitalized patients' feces (1,592 cases) was 12.2 times as many as that of genotype 1 (130 cases) during the survey period. The institute also reported the increasing number of isolation cases of human noroviruses in winter, which agreed with the results of this study.

Human noroviruses of genotype 1 were detected in all secondary-treated sewage and final effluent samples, while those of genotype 2 were detected in 10 (83%) of 12 secondary-treated sewage samples and in 7 (58%) of 12 of final effluent samples. Similarly to the raw sewage, secondary-treated sewage and final effluent samples contained human noroviruses at the higher concentration in winter (*t*-test, $P < 0.05$). The highest value of the concentration of human noroviruses was 0.64 copies/mL for genotype 1 and 2.6 copies/mL for genotype 2.

According to other quantitative or semi-quantitative studies, the concentration of human noroviruses in raw sewage or secondary-treated sewage ranged from 5 to 1000 copies/mL or from 0.1 to 10 copies/mL, respectively (Lodder and de Roda Husman, 2005; Pusch *et al.*, 2005; van den Berg *et al.*, 2005), which are similar to the results obtained in this study.

Profile of indicator bacteria in wastewater

Figure 2 shows the seasonal profiles of total coliforms, *E. coli*, and F-specific phages in wastewater samples. Unlike human noroviruses, the concentration of these bacteria in raw sewage was almost constant throughout the year. The concentration of total coliforms, *E. coli*, or F-specific phages varied within a range of 0.57 $\log_{10}$, 0.30 $\log_{10}$, or 0.94 $\log_{10}$, respectively.

The geometric average concentration of total coliforms or *E. coli* in secondary-treated sewage was 390 CFU/mL or 66 CFU/mL, respectively, while that in final effluent was 230 CFU/mL and 36 CFU/mL, respectively. F-specific phages were detected in all 11 secondary-treated sewage samples, ranging from 1.0 PFU/mL to 17 PFU/mL, while 9 (82%) of 11 final effluent samples were positive for F-specific phages.

Removal efficiency of microorganisms at WWTP

Table 1 shows the removal ratio of the tested microorganisms at the WWTP. The removal ratio of the microorganisms by physical and biological treatment processes was calculated using the data on raw sewage and secondary-treated sewage, while the data on

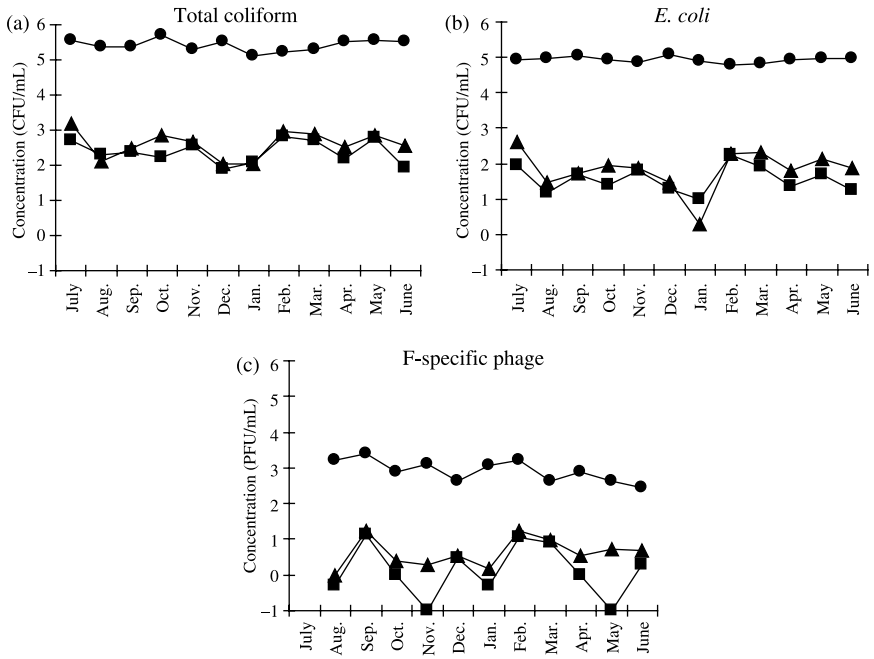

Figure 2 Seasonal profile of indicator bacteria at WWTP; ●, raw sewage; ▲, secondary-treated sewage; ■, final effluent

secondary-treated sewage and final effluent was used to determine the removal ratio by chemical treatment, i.e, chlorination.

By physical and biological treatment processes, microorganisms in raw sewage were generally removed from 2 to 3 $\log_{10}$, which was probably attributed to the adsorption of microorganisms to activated sludge (Gerba, 1984; Sano *et al.*, 2004). On the other hand, the removal ratio of bacteria by chlorination was mostly lower than 1 $\log_{10}$. Interestingly, the concentration of human noroviruses also decreased by chlorination as well as bacteria, although they are considered to have high resistant to chlorine (Keswick *et al.*, 1985). In this study, human noroviruses were determined by PCR because no host cell is available for the cultivation of human noroviruses (Wolfaardt *et al.*, 1997). Therefore, viable viruses might have been inactivated more efficiently than the results by PCR.

Through all treatment processes at the WWTP, human noroviruses of genotype 2 were removed most effectively (3.69 $\log_{10}$ on average), followed by *E. coli* (3.37 $\log_{10}$), total coliforms (3.05 $\log_{10}$), F-specific phages (2.81 $\log_{10}$), and human noroviruses of genotype 1 (2.27 $\log_{10}$). The reasons why human noroviruses of genotype 2 were removed more efficiently than those of genotype 1 (t-test, $P < 0.01$) should be studied in the future.

Table 1 Removal ratio of microorganisms at WWTP

Microorganism	Log removal of microorganism (mean ± standard deviation)		
	Physical and biological treatments	Chemical treatment (chlorination)	Whole process
Human norovirus			
Genotype 1	1.82 ± 0.61	0.45 ± 0.49	2.27 ± 0.67
Genotype 2	2.74 ± 1.19	0.95 ± 1.80	3.69 ± 1.21
Bacteria			
Total coliform	2.83 ± 0.36	0.22 ± 0.25	3.05 ± 0.40
E. coli	3.11 ± 0.61	0.26 ± 0.37	3.37 ± 0.43
F-specific phage	2.30 ± 0.51	0.51 ± 0.53	2.81 ± 0.77

Table 2 Correlation coefficient among microorganisms in final effluent

Microorganism	Genotype 1	Genotype 2	Total coliform	E. coli	F-specific phage
Human norovirus					
Genotype 1	1.00	0.69	−0.04	0.08	0.44
Genotype 2	0.69	1.00	−0.29	−0.06	0.23
Bacteria					
Total coliform	−0.04	−0.29	1.00	0.85	−0.03
E. coli	0.08	−0.06	0.85	1.00	0.32
F-specific phage	0.44	0.23	−0.03	0.32	1.00

The removal ratio of human noroviruses was almost constant independently of the initial concentration of the viruses in raw sewage, which led to the increasing concentration of human noroviruses in the final effluent discharged from the WWTP in winter. The seasonal profile of human noroviruses in the final effluent was similar to that in the Tamagawa River, Tokyo, Japan, where effluent from several WWTPs covers nearly half of the river water (Haramoto *et al.*, 2005). In case that river water contaminated with effluents from WWTPs is used for the production of tap water, the risk of infection of human noroviruses via tap water could be an issue especially in winter.

Correlation among microorganisms in final effluent

A correlation coefficient (r) among the tested microorganisms in the final effluent is summarized in Table 2. The highest r-value was obtained between total coliforms and *E. coli* ($r = 0.85$) and the second was obtained between the two serotypes of human noroviruses ($r = 0.69$).

According to Water Pollution Control Law, total coliforms are the only indicator microorganism in the regulation for the discharge from a WWTP in Japan: the concentration of total coliforms should not exceed 3000 CFU/mL. In this study, not only all final effluent samples but also all secondary-treated sewage samples before chlorination met this regulation. However, no positive correlation was found between total coliforms and human noroviruses of genotype 1 ($r = -0.04$) or 2 ($r = -0.29$). F-specific phages are considered as one of the reliable indicators of human enteric viruses (IAWPRC, 1991), while those in the final effluent did not show a high positive correlation with human noro viruses of each genotype: the r-value was 0.44 for genotype 1 and 0.23 for genotype 2.

Lack of applicability of the conventional bacteria items as an indicator of human noroviruses may pose a need for direct monitoring of the viruses in effluent from a WWTP. The reduction of human enteric viruses at WWTPs should be more emphasized, because WWTPs are one of the most effective nodes to control the concentration of viruses circulating around human society. The introduction of advanced wastewater treatment systems, such as UV-irradiation or ozonation, would directly contribute to reducing the viruses in the water environment and result in the reduction of risk infection.

Conclusions

The concentration of human noroviruses in raw sewage ranged from 0.17–260 copies/mL for genotype 1 and from 2.4–1900 copies/mL for genotype 2, showing much higher values in the epidemic season, i.e, winter.

Human noroviruses of genotype 2 were removed most effectively (3.69 $\log_{10}$ on average) at the wastewater treatment plant, followed by *E. coli* (3.37 $\log_{10}$), total coliforms (3.05 $\log_{10}$), F-specific phages (2.81 $\log_{10}$), and human noroviruses of genotype 1 (2.27 $\log_{10}$). The removal ratio of human noroviruses was almost constant, independent of the initial concentration of the viruses in raw sewage.

None of the tested bacteria was judged to be a reliable indicator of human noroviruses in final effluent.

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