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Distinct behaviors of infectious F-specific RNA coliphage genogroups at a wastewater treatment plant

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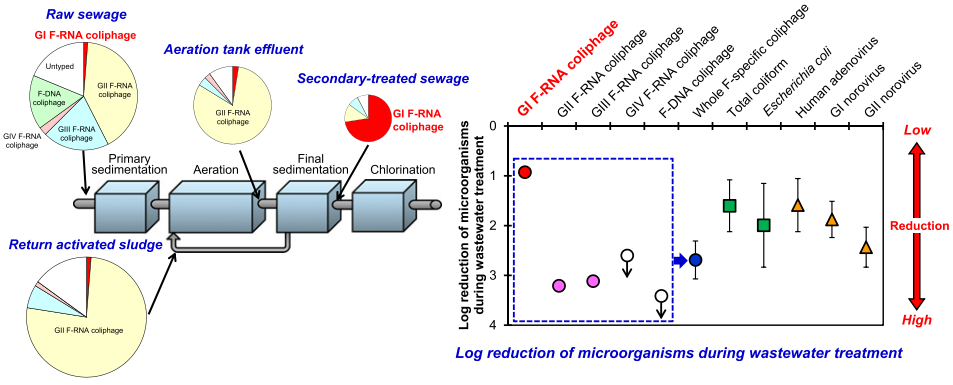
HIGHLIGHTS

- Behavior of F-specific coliphage types during wastewater treatment was examined.
- Phage plaques were classified into 4 F-RNA coliphage genogroups or F-DNA coliphages.
- GI F-RNA coliphages became the most abundant after wastewater treatment.
- GI F-RNA coliphages had much lower reduction ratios than other coliphage types.
- GI F-RNA coliphages were considered an appropriate indicator of virus reduction.

GRAPHICAL ABSTRACT



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ABSTRACT

The present study aimed to determine the differences in the behaviors of four F-specific RNA (F-RNA) coliphage genogroups (GI–GIV) during wastewater treatment. Raw sewage, aeration tank effluent, secondary-treated sewage, and return activated sludge were collected from a wastewater treatment plant in Japan at monthly intervals between March and December 2011 (n = 10 each). F-specific coliphages were detected by plaque assay in all tested samples, with a concentration ranging from −0.10 to 3.66 $\log_{10}$ plaque-forming units/ml. Subsequently, eight plaques were isolated from each sample, followed by genogroup-specific reverse-transcription quantitative PCR (qPCR) for F-RNA coliphages and qPCR for F-specific DNA (F-DNA) coliphages. GI F-RNA coliphages were the most abundant in the secondary-treated sewage samples (73% of the plaque isolates), while GII F-RNA coliphages were the most abundant in the other three sample types (41–81%, depending on sample type). Based on the results of the quantification and genotyping, the annual mean concentrations of each F-specific coliphage type were calculated, and their reduction ratios during wastewater treatment were compared with those of indicator bacteria (total coliforms and *Escherichia coli*) and enteric viruses (human adenoviruses and GI and GII noroviruses). The mean reduction ratio of GI F-RNA coliphages was the lowest (0.93 $\log_{10}$), followed by those of the indicator bacteria and enteric viruses (1.59–2.43 $\log_{10}$), GII–GIV F-RNA coliphages (>2.60–3.21 $\log_{10}$), and F-DNA coliphages (>3.41 $\log_{10}$). These results suggest that GI F-RNA coliphages may be used as an appropriate indicator of virus reduction during wastewater treatment.

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1. Introduction

F-specific coliphages are divided into F-specific DNA (F-DNA) and RNA (F-RNA) coliphages on the basis of morphological and genomic characteristics. The latter are subdivided into four distinct genogroups in two genera (GI and GII in the genus *Levivirus* and GII and GIV in the genus *Allolevivirus*) belonging to the family *Leviviridae* (Friedman et al., 2009). F-RNA coliphages have been widely used as indicators of human enteric viruses in aquatic environments and during water treatment processes, because of their morphological similarity with human enteric viruses and the relative simplicity of their analysis by culture-based and molecular biology-based techniques (Bae and Schwab, 2008; Charles et al., 2009; Flannery et al., 2013; Lucena et al., 2003; Zhang and Farahbakhsh, 2007).

Previous studies have reported that each of the four F-RNA coliphage genogroups shows a different persistence under various environmental conditions (Muniesa et al., 2009; Nappier et al., 2006; Schaper et al., 2002). GI F-RNA coliphages can survive for a longer duration in various types of water, such as river water, seawater, and treated sewage, than other genogroups (GII–GIV), with a duration particularly longer than that for GIII and GIV F-RNA coliphages (Muniesa et al., 2009; Schaper et al., 2002). Further, GIII F-RNA coliphages are inactivated by heat treatment more rapidly than GI F-RNA coliphages (Nappier et al., 2006). Meanwhile, no significant difference has been observed in persistence in water among multiple strains of GI F-RNA coliphages (Schaper et al., 2002). These results suggest that GI F-RNA coliphages have the highest environmental persistence among the four genogroups.

However, few studies have evaluated the differences in the reduction of indigenous F-RNA coliphage genogroups during water treatment processes quantitatively (Flannery et al., 2013; Hata et al., 2013), probably because reliable reverse-transcription quantitative PCR (RT-qPCR) assays for F-RNA coliphage genogroups have been unavailable until recently (Friedman et al., 2011; Ogorzaly and Gantzer, 2006; Wolf et al., 2008).

Hata et al. (2013) demonstrated by RT-qPCR that the efficiency in the reduction of F-RNA coliphage genogroups at a wastewater treatment plant (WWTP) installing an activated sludge process and sand filtration was the lowest for F-RNA coliphages of GI (0.49 ± 0.29 $\log_{10}$), followed by that for GII (2.04 ± 0.50 $\log_{10}$) and GIII (3.39 ± 0.55 $\log_{10}$). Considering that the reduction ratios of various viruses, such as noroviruses and sapoviruses, were ranked between the reduction ratios for GI and GIII, these genogroups have been suggested as indicators that predict the magnitude of virus reduction at a WWTP (Hata et al., 2013).

In that paper of Hata et al. (2013), a process control was inoculated into water concentrates to check the efficiency of RNA extraction and RT-qPCR, but this was not used for original water samples. Because recovery of viruses by the virus concentration method used in the study was reported to vary greatly depending on sample and virus types (Haramoto et al., 2009a; Katayama et al., 2008), the reduction ratios of F-RNA coliphages examined from the water concentrates by RT-qPCR may have over- or underestimated compared with the actual values. Meanwhile, the obtained results did not provide any information about the reduction of the infectivity of F-RNA coliphage genogroups (Hata et al., 2013).

Flannery et al. (2013) determined the reduction of GII F-RNA coliphages at a WWTP using both plaque assay and RT-qPCR: the reduction ratio determined by plaque assay (mean, 2.30 $\log_{10}$) was significantly higher than that determined by RT-qPCR (mean, 0.54 $\log_{10}$). These results indicate that the use of RT-qPCR alone underestimates the reduction of infectious F-RNA coliphages during wastewater treatment. However, other F-RNA coliphage genogroups were unfortunately not examined.

This study aimed to determine the differences in the behavior of infectious F-RNA coliphage genogroups during wastewater treatment. F-specific coliphages were examined by plaque assay in four types of samples from a WWTP in Japan, followed by plaque isolation and (RT-)qPCR for four F-RNA coliphage genogroups and F-DNA coliphages. Subsequently, the annual mean concentrations of each F-specific coliphage types were calculated. Their reduction ratios at the WWTP were then compared with those of indicator bacteria and enteric viruses to evaluate the applicability of using F-RNA coliphage genogroups as an indicator of virus reduction during wastewater treatment plant.

2. Materials and methods

2.1. Collection of WWTP samples

During the 10-month period between March and December 2011, monthly sampling was conducted at a WWTP in Yamanashi Prefecture, Japan, where an activated sludge process was installed. On each sampling day, raw sewage (RS), aeration tank effluent (ATE), and secondary-treated sewage (STS) were collected at the site between screening and the primary sedimentation tank, the outlet end of the aeration tank, and the outlet end of the final sedimentation tank, respectively. STS samples were further treated with chlorine and then discharged into a nearby river. However, we were unfortunately not permitted to collect the effluent. In addition, return activated sludge (RAS) was collected prior to being mixed back into the aeration tank. All the samples were collected as a grab sample in autoclaved polypropylene bottles. Once collected, the samples were placed in a cool box with refrigerants and transported to the laboratory within 2 h.

2.2. Detection and isolation of F-specific coliphage plaques

Undiluted and/or 10-fold serially diluted samples were processed to detect the presence of F-specific coliphages by plaque assay using the host strain *Salmonella enterica* serovar Typhimurium WG49, according to ISO standard 10705-1 (Anonymous, 1995) with some modifications. A duplicate was used for each sample, and a negative control, which contains no water sample, was always included in the analysis. F-specific coliphage plaques were counted after incubation at 37 °C for approximately 24 h, and coliphage concentration was expressed as plaque-forming units (PFU)/ml.

For each sample eight plaques were randomly isolated from agar plates using a sterile loop and inoculated in a 2-ml microtube containing 500 μl of autoclaved ultrapure water (Merck Millipore, Billerica, USA). The microtubes were vigorously vortexed for approximately 1 min and stored as a plaque suspension at −20 °C until further analysis.

2.3. Genogroup-specific RT-qPCR for F-RNA coliphages

After thawing at room temperature, RNA was extracted from 10 μl of the plaque suspension by heating at 95 °C for 5 min using a Veriti 96-Well Thermal Cycler (Life Technologies, Carlsbad, USA). RT was performed for the RNA using a High Capacity cDNA Reverse Transcription Kit (Life Technologies) following the manufacturer's protocol.

First, each synthesized cDNA sample was subjected to TaqMan-based qPCR specific for GI F-RNA coliphages. In brief, a 2-μl aliquot of the cDNA was mixed with 23 μl of a qPCR mixture containing 12.5 μl of Premix Ex Taq (Perfect Real Time) (Takara Bio, Otsu, Japan), 10 pmol each of forward and reverse primers, and 5 pmol of TaqMan probe (Wolf et al., 2008). qPCR runs were performed with a Thermal Cycler Dice Real Time System TP800 (Takara Bio) under the following thermal conditions: 95 °C for 30 s, 40 cycles of 95 °C for 10 s and 58 °C for 30 s. Molecular biology-grade water was used as the template for negative controls.

Based on the criteria proposed by previous studies (Haramoto et al., 2009b, 2012b), GI was considered to be an unrepresentative genogroup of the plaque when the threshold cycle (Ct) value was higher than 30, corresponding to $<10^3$ genome copies/reaction. In contrast, when the tested sample yielded a Ct value of ≤20, GI was judged as the