Quantification and Genotyping of Torque Teno Virus at a Wastewater Treatment Plant in Japan[▽]

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Torque teno virus (TTV) DNA was quantitatively detected in influent and effluent samples collected from a wastewater treatment plant in Japan, with the highest concentration being 4.8×10^4 copies/liter. Genogroup-specific nested PCR demonstrated that TTV of genogroup 3 was the most abundant in wastewater among the five genogroups tested.

Torque teno virus (TTV) is an emerging virus which was discovered as an agent of unknown hepatitis in Japan in 1997 (17). TTV has a single-stranded and circular DNA of approximately 3,800 bp packaged within a 30- to 32-nm nonenveloped virion (8, 15, 18). TTV is currently classified into the family *Circoviridae*, genus *Anellovirus*, and divided into five genogroups (1 to 5) consisting of more than 30 genotypes (6, 14, 19–21). Although it is indicated that TTV may not cause serious health problems, several genotypes of TTV are thought to be responsible for human diseases (22, 24). TTV is isolated not only in hepatitis patients but also in healthy persons (1, 7, 10, 18, 22); thus, it is strongly suspected to be transmitted by the fecal/oral route.

To date, there have been several studies on the detection of TTV DNA in aquatic environments using PCR, suggesting that TTV is ubiquitous in these environments worldwide (2–5, 16, 25). Only one of these studies provided quantitative data on TTV DNA; TTV DNA was detected in 48 (92%) of 52 river water samples in Brazil, with the highest concentration being 7.46×10^5 genome equivalents per 100 ml (3).

In this study, the occurrence of TTV in wastewater in Japan was determined quantitatively based on monthly monitoring for 1 year. Influent (100 ml each) and effluent (1,000 ml each) samples were collected from a wastewater treatment plant with a conventional activated sludge process from March 2005 to February 2006. The water samples were concentrated by an adsorption-elution method using an electronegative membrane (Millipore, Tokyo, Japan) (9), followed by further concentration by using a Centriprep YM-50 device (Millipore) to obtain a final volume of 700 μ l.

Viral DNA was extracted from 200 μ l of the concentrated sample by using a QIAamp DNA mini kit (Qiagen, Tokyo, Japan), and then 5 μ l of the resulting DNA (200 μ l) was subjected to real-time PCR using a LightCycler DX400 (Roche Diagnostics, Tokyo, Japan). The primers and the TaqMan probe used here were designed to amplify TTV of all known

genogroups (23). Serial dilutions of the chemically synthesized oligo-DNA with a length of 79 bp (range, 1.0×10^0 to 1.0×10^6 copies per reaction) were used to make a standard curve, resulting in a high correlation ($r = -1.00$) between log standard concentration and crossing point. As few as three copies of the oligo-DNA were successfully quantified using this real-time PCR assay, while one copy of the DNA was always found negative. Therefore, the sample with a TTV DNA concentration of three copies per reaction or more was regarded as positive.

For TTV DNA-positive samples, 5 μ l of the extracted DNA was subjected to nested PCR using genogroup-specific primers (13) to determine the genetic diversity of TTV in wastewater. All PCR amplifications were performed with a GeneAmp PCR system 9700 (Applied Biosystems, Tokyo, Japan) in 50- μ l reaction volumes, using an AmpliTaq Gold PCR master mix (Applied Biosystems). The PCR products were fractionated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining (0.1 μ g/ml).

Table 1 summarizes the results of detection of TTV DNA in the wastewater samples. TTV DNA was detected in all 12 influent samples tested, with a geometric mean concentration of 1.7×10^4 copies/liter (range, 4.8×10^3 to 4.8×10^4 copies/liter). The concentration of TTV DNA in the influent samples showed no clear seasonal pattern, suggesting that TTV infections occur constantly throughout the year.

In our previous study, TTV DNA was qualitatively detected in 97% (93/96) of influent samples collected from eight wastewater treatment plants in Japan (4). Compared with studies conducted in other countries (2, 25), a very high prevalence of TTV DNA was obtained from influent samples collected from various wastewater treatment plants in Japan. This might be explained by the fact that TTV DNA was isolated in nearly 90% of healthy persons tested in Japan (10, 22).

TTV DNA was also detected in 2 (17%) of 12 effluent samples tested, at 5.5×10^3 and 2.4×10^3 copies/liter in June and November, respectively. Considering the sample volumes tested for real-time PCR, the detection limit of TTV DNA in the effluent sample was calculated to be 4.2×10^2 copies/liter. Therefore, it is expected that at least 1.06 to 2.06 log reductions were achieved at the wastewater treat-

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TABLE 1. Detection of TTV in influent and effluent samples at a wastewater treatment plant in Japan

Sample type	Date of sample collection	Concn of TTV (no. of copies/liter)	Genogroup(s) detected ^a
Influent	March 2005	2.2×10^4	3
	April 2005	4.7×10^4	1, 3
	May 2005	2.4×10^4	1, 3
	June 2005	1.3×10^4	3, 5
	July 2005	2.3×10^4	3
	August 2005	1.3×10^4	3
	September 2005	2.7×10^4	3, 4
	October 2005	4.8×10^3	1, 3
	November 2005	2.1×10^4	3
	December 2005	1.1×10^4	3
	January 2006	5.9×10^3	3
	February 2006	4.8×10^4	3
Effluent	March 2005	$<4.2 \times 10^2$	N.T.
	April 2005	$<4.2 \times 10^2$	N.T.
	May 2005	$<4.2 \times 10^2$	N.T.
	June 2005	5.5×10^3	None
	July 2005	$<4.2 \times 10^2$	N.T.
	August 2005	$<4.2 \times 10^2$	N.T.
	September 2005	$<4.2 \times 10^2$	N.T.
	October 2005	$<4.2 \times 10^2$	N.T.
	November 2005	2.4×10^3	None
	December 2005	$<4.2 \times 10^2$	N.T.
	January 2006	$<4.2 \times 10^2$	N.T.
	February 2006	$<4.2 \times 10^2$	N.T.

^a N.T., not tested.

ment plant for TTV DNA-negative samples. On the other hand, the removal ratio of TTV DNA was calculated to be only 0.37 and 0.94 log in June and November, respectively. Because the water samples were collected only once a month, these values may not be representative of actual removal efficiency at the wastewater treatment plant. The fate of TTV at wastewater treatment processes should be addressed further in future studies.

Genogroup-specific nested PCR for TTV successfully produced PCR products of the expected length (260 to 306 bp, depending on the genogroup) on agarose gels. TTV of genogroup 3 was detected in all 12 influent samples tested (Table 1). TTV of genogroup 1, 4, or 5 was detected in 3 (25%), 1 (8%), or 1 (8%) out of 12 influent samples tested, respectively, while no sample was positive for TTV of genogroup 2. Based on the epidemiological data from human specimens, TTV of genogroup 3 is the most abundant out of the five genogroups (11–13). Therefore, it is strongly suggested that the monitoring of influent samples of a wastewater treatment plant may be a suitable approach for understanding the actual incidence of TTV in the service area of the plant. On the other hand, two effluent samples, which were positive for TTV DNA by real-time PCR, were found to be negative for any genogroup. This is probably because real-time PCR provides a lower detection limit than does conventional nested PCR.

To our knowledge, this is the first study that demonstrated the quantitative detection of TTV DNA in water samples at a wastewater treatment plant. From the results that showed that the concentration of TTV DNA in influent samples was relatively constant throughout the year, TTV may be an appropriate indicator of fecal contamination. Further studies should be

focused on the quantitative monitoring of TTV in various kinds of environmental samples, such as river water, seawater, or shellfish, because these samples receive effluents from wastewater treatment plants.

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