

Occurrence and molecular characterization of Torque teno virus (TTV) in a wastewater treatment plant in Tehran

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ABSTRACT

Torque teno virus (TTV) is a single-stranded DNA virus which is predominantly transmitted by the fecal–oral route and may be excreted in the absence of the clinical symptoms. TTV was previously considered a probable cause of hepatitis, but further studies could not strongly connect TTV to any serious health problem. TTV is highly resistant to water and wastewater treatment processes and can be a useful indicator for determining the fecal contamination of water. The purpose of the present study was to assess the prevalence and molecular characterization of TTV in treated wastewater in Tehran. Thirteen effluent samples were collected monthly from the biggest wastewater treatment plant in Tehran, Iran (from September 2017 to August 2018). The presence of the TTV was monitored in the samples by the nested polymerase chain reaction (PCR) method. The TTV genome was found in 76.9% of the samples, and TTV of groups 1 and 3 were determined using phylogenetic analysis. Therefore, treated wastewater can play a key role in the transmission of TTV and the usage of treated wastewater as a source of potable water needs to be carefully controlled.

Key words | detection, Iran, phylogenetic analysis, Torque teno virus, virus concentration, wastewater treatment plant

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INTRODUCTION

Recently, indicator microorganisms have been widely used to assess the potential risks of infection from fecal contamination of waters such as drinking water, groundwater, wastewater, and treated wastewater. The Council of European Union, the World Health Organization (WHO), and also the United States Environmental Protection Agency (US EPA) have used bacterial indicators including fecal coliforms in their guidelines and standard methods for water supply systems. There are many outbreaks around the world which have happened in water systems in the absence of bacterial indicators and the water systems were in accordance with the applied standard methods (Craun *et al.* 2006). However, establishing a direct correlation between bacteria and viruses present in water and wastewater is not always possible (Charest *et al.* 2015).

Since viral pathogens are more resistant to the harsh aquatic environment than bacterial indicators, using the fecal viral indicators would overcome these limitations (Charest *et al.* 2015). The enteric viruses, such as enterovirus, adenovirus, rotavirus, astrovirus, and norovirus, are predominantly transmitted by the fecal–oral mode and are one of the common causes of acute diarrhea in humans, especially in newborn and children less than 5 (Fong & Lipp 2005; Dalla Vecchia *et al.* 2013). All the enteric viruses possess similar structural properties such as the absence of an envelope that causes it to be more resistant to the common water treatment systems (Fong & Lipp 2005). There are many reports around the world on molecular identification of enteric viruses in wastewater and treated wastewater samples (Petrinca *et al.* 2009; La Rosa *et al.*

2010; Myrmele *et al.* 2015). Recently, Torque teno virus (TTV), which is also a non-enveloped virus particle and highly stable in the aquatic environments, has been suggested as a novel viral indicator of fecal contamination in water and wastewater systems and in aquatic environments (Haramoto *et al.* 2010). Epidemiological studies on hemophilic patients revealed that after treatment with dry heat at 65 °C during 96 h, the infectivity of TTV was not completely inactivated (Verani *et al.* 2006).

TTV was first detected in post-transfusion Japanese hepatitis patients; nonetheless, further studies have implied that the virus is not related to any serious health problem (Takahashi *et al.* 1998; Haramoto *et al.* 2005). Many people, with asymptomatic infection, may carry TTV, and it might lead to the hypothesis that TTV can be normal flora of the human body (Nishizawa *et al.* 1997; Mushahwar *et al.* 1999).

TTV virions are non-enveloped and icosahedral, with a diameter of 30–32 nm, and possess a single-stranded circular DNA genome with negative polarity (Mushahwar *et al.* 1999; Peng *et al.* 2002). TTV is classified under the genus Anellovirus and recently has divided into seven different phylogenetic groups with more than 30 genotypes (Peng *et al.* 2002; Diniz-Mendes *et al.* 2008; Hsiao *et al.* 2016). It is present in Europe, North America, and Asia with a frequency of 40–90% in the general population, blood donors or specific groups including viral hepatitis patients (Bendinelli *et al.* 2001). TTV DNA may be detected in human body fluids such as saliva, plasma, maternal milk, and feces of infected individuals. Because of the high levels of viral shedding in the stool, the fecal–oral route can be one of the most important transmission routes (Okamoto *et al.* 1998a, 1998b; Diniz-Mendes *et al.* 2008).

Many studies have been conducted to detect and quantify the TTV genome in different sources of water. These surveys revealed widely variable detection rates which can be possibly associated with geographic location, sources of water, and detection methods (Diniz-Mendes *et al.* 2008; Hamza *et al.* 2011).

In this study, 13 effluent samples before the chlorination step were collected from a wastewater treatment plant (WWTP) in Tehran, from September 2017 to August 2018, and were analyzed for detecting TTV genome using the nested polymerase chain reaction (PCR) technique.

MATERIALS AND METHODS

Collection of the treated wastewater samples

The secondary effluent samples were collected from a WWTP located in the south of Tehran, Iran with a treatment capacity of 4.5×10^8 m³ of wastewater per year. The final effluent treated wastewater is used for agricultural irrigation. The samples (5 L for each time) were collected monthly for one year from September 2017 to August 2018.

The time of collecting and quality parameters of the samples such as pH, turbidity, biochemical oxygen demand (BOD), and chemical oxygen demand (COD) were recorded and the samples were transferred to the laboratory by cool boxes within 2 h.

Sample preparation

Presumptive viruses in the secondary effluent samples were concentrated using an adsorption–elution method with positively charged membranes (0.45 µm pore size and 47 mm in diameter, Zeta Plus 1MDS, 3M, USA) (Fout *et al.* 2010).

Putative viruses were eluted by passing 7.5 mL of 3% beef extract (wt/vol) with 0.05 M glycine (pH 9.5) (Fout *et al.* 1996; Eaton *et al.* 2005). Then, the organic flocculation process was conducted as described by Fout *et al.* (1996) and Borchardt *et al.* (2004). Briefly, the eluted sample was acidified, and the pH was adjusted to 3.5 ± 0.1 . Then, the acidified elute was centrifuged for 15 min at 4 °C at $2,500 \times g$, and the precipitate was resuspended in 30 mL of sterile 0.15 M sodium phosphate solution (pH 9.0–9.5). The suspended pellet was centrifuged at 4 °C for 10 min at $6,000 \times g$, and the pH of the supernatant was adjusted to 7.0–7.5. A 10 mL of the elute was reconcentrated using a Centrifugal Concentrator 30,000 MWCO (Amicon ultra-15, Millipore, USA) to a final volume of 500 µL (Fout *et al.* 2010).

Viral DNA extraction

Viral nucleic acids were extracted from 140 µL of the final elute obtained from the Centrifugal Concentration step using a QIAamp RNA Mini Kit (Qiagen, Germany)

according to the manufacturer's protocol. The extracted nucleic acids were stored at -70°C .

Nested PCR

The extracted nucleic acids were examined by the nested PCR to diagnose the presence of TTV as described previously (Hu *et al.* 2005).

A 5 μL of extracted DNA was amplified using 10 pmol of the first-round primers (NG054 and NG147). Subsequently, 2 μL of the first-round PCR product was used as the template for the second-round amplification using NG132 and NG133 primers (Al-Moslih *et al.* 2004; Hu *et al.* 2005).

Amplified products were electrophoresed on 1.5% agarose gel to visualize DNA bands. The first-round and the second-round product sizes were 230 and 132 bp, respectively.

Assessment of TTV sequences and phylogenetic analysis

Re-amplified products of the positive samples ($n=4$) (230 bp in length) obtained from the first round of PCR were used for sequencing. Amplicons were cleaned up using a QIA Quick Gel Extraction kit (Qiagen, Germany). The BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) was employed for sequencing. The ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems, USA) was used for conducting the electrophoresis process and the data collection phase. All the selected amplified products have bi-directionally sequenced. Previous reference sequences were retrieved from the GenBank and compared with sequenced samples (166 bp) from the present survey, and a maximum-likelihood phylogenetic tree was created based on the UTR sequence region of the TTV genomes using the Kimura 2-parameter model by the MEGA 6 software (with 1,000 bootstrap replicates).

Reference sequences were downloaded from the GenBank (Accession Nos AY456101, AJ309728, AJ402218, AF060547, AB008394, AB017772, AB017774, AB017775, AB017776, AB017777, AB037926, AF261761, AB017779, AB054647, AB017778, AB008394, AB030489, AB030488, AF298585, AM712034, AM712032, AF079173, AF129887, AF116842, AB017610, AF122915, AB041007, AF122913, DQ187003,

DQ187000, DQ186999, DQ187001, DQ187002, AB030486, KT163886, AX025667, AB017613, AX025718, AX025677, AB038620, AB038619, AB025946, AB028669, AB059353, AX025822, AB038621, AB038624, AB038623, AB038622, FJ392105). The studied sequences were submitted to the GenBank under accession numbers MN245270–MN245273.

RESULTS

In this study, the TTV genome was identified in 76.9% (10 out of 13 samples, including September, two samples in October, November, December, February, March, April, June, and August) of the secondary effluent and 23.1% (3 out of 13 samples, including January, May, and July) of samples were negative for TTV (Table 1). The high prevalence of TTV in the treated wastewater samples suggests the fecal–oral way of TTV transmission. As results revealed, TTV did not show a seasonal distribution pattern, and there

Table 1 | Quality parameters and the results of nested PCR of the secondary-treated wastewater samples

Sample period	pH	BOD ^a (mg/L)	COD ^b (mg/L)	Turbidity (NTU)	Results of nested PCR
2017					
September	7.87	71	129	8	Positive
October	7.63	38	101	10	Positive
October (second time)	7.87	71	129	8	Positive
November	7.50	180	448	8	Positive
December	7.62	157	322	7	Positive
2018					
January	7.32	122	260	6	Negative
February	7.22	87	130	N.M ^c	Positive
March	7.30	64	95	N.M	Positive
April	7.62	21	36	N.M	Positive
May	7.69	5.6	23	N.M	Negative
June	7.65	9	44	N.M	Positive
July	7.61	29	48	N.M	Negative
August	7.73	19	35	N.M	Positive

^aBiological oxygen demand.

^bChemical oxygen demand.

^cNot measured.

was no meaningful relation between the presence of TTV in samples and pH, BOD, turbidity, and COD of the samples.

The quality parameters of the treated wastewater samples, which were recorded after sampling, are shown in Table 1.

The phylogenetic analysis identified two groups of TTV in this study (groups 1 and 3) (Figure 1).

Sequences from four secondary-treated wastewater samples (October, February, April, and June) were aligned and compared with 35 retrieved sequences from the GenBank and allocated into the five known major groups. Two

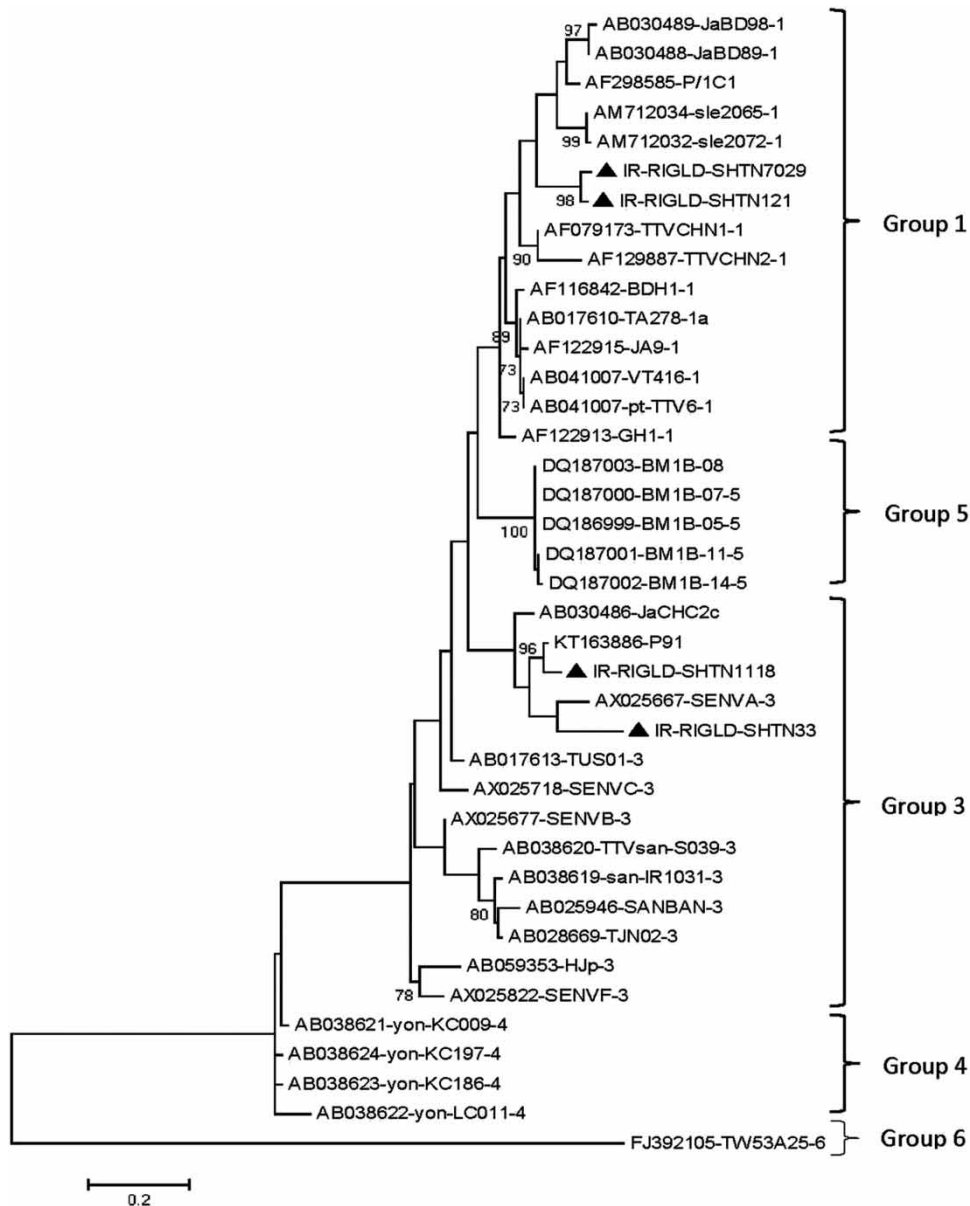


Figure 1 | Phylogenetic analysis of a 166-bp fragment from UTR regions of different TTV isolates using the Kimura two-parameter nucleotide substitution model and the maximum-likelihood method. Four TTV sequences from secondary-treated wastewater samples (labeled with black triangles) were analyzed with 35 TTV sequences of groups 1–6 available from the GenBank as reference genes.

Iranian isolates were classified as group 1 and two categorized as group 3.

DISCUSSION

In this study, TTV was detected in 76.9% of secondary effluent samples (Table 1), and phylogenetic analysis revealed TTV genogroups 1 and 3.

Most enteric viruses responsible for acute diseases are excreted for a short time. However, TTV is a highly prevalent virus which is not related to any serious health problems and pathology with tenacious viremia (Diniz-Mendes *et al.* 2008). The presence of TTV in water and wastewater samples can indicate the fecal contamination and possibly the existence of other enteric viruses (Ahmed *et al.* 2009).

As we know, there is no report about TTV frequency in environmental water samples in Iran, and this is the first survey on wastewater. However, many studies have performed on clinical samples. Koohi *et al.* (2012) detected TTV in 92% of chronic hepatitis C patients, and Najafimemar *et al.* (2018) found it in 54%, 48%, and 49.3% of chronic hepatitis B-infected individuals, co-infected patients with HIV/HBV, and healthy individuals, respectively. The results of both studies indicate the circulation TTV of groups 1 and 3 among the studied cases (Koohi *et al.* 2012; Najafimemar *et al.* 2018). The findings of the present study were in accordance with the previous surveys in Iran on clinical samples.

In the present study, the phylogenetic analysis revealed that TTV of genogroup 1 was detected in October and February, and TTV of genogroup 3 was detected in April and June. As mentioned before, the previous epidemiological studies on human specimens in Iran indicate that TTV of genogroups 3 and 1 are circulating with the high frequency in Iran. The similarities of the detected TTV sequences in the present study might be due to the sample collection from only one single WWTP in Tehran.

The changes in the TTV circulating groups in the Tehran population could be led to the variations in the detected viral types of the secondary-treated wastewater samples. Monitoring of viruses in the influent and effluent of a WWTP provides useful information to assess their real occurrence in its service area.

The frequencies of TTV in environmental water and influent and effluent wastewater samples widely vary in different parts of the world. The TTV positivity rates in final wastewater samples were reported 50% in Germany (Hamza *et al.* 2011), 41.7% and 42% in the USA, and 39% in Italy (Plummer *et al.* 2014; Charest *et al.* 2015).

Verani *et al.* (2006) detected TTV genogroups 1, 3, and 6 in 25% of river water samples. Also, TTV of genogroups 3, 1, 4, and 5 were identified in influent wastewater samples in Japan (Haramoto *et al.* 2008). The identification of the other genogroups in various studies could be due to the differences in analysis methods and sample sizes.

Haramoto *et al.* (2005, 2008) detected TTV in 97% and 100% of influent samples. Nevertheless, lower frequencies of TTV were detected (24% and 17%) in the final effluent samples.

Dalla Vecchia *et al.* (2013) detected TTV in 11.7% of tap water samples in Brazil which illustrates the resistance of TTV to drinking water treatment processes and the risk of the transmission of the virus by tap water.

In a survey of drinking water, wastewater, and animal feces in the USA, Plummer *et al.* (2014) found no correlation between the incidence of TTV and fecal indicator bacteria.

CONCLUSION

Monitoring of TTV in the secondary effluent samples of the WWTP in Tehran was performed (September 2017–August 2018) for the first time and provided new insights on TTV frequency and circulating groups in the area. The results of the present study indicate the presence of TTV DNA of genogroups 1 and 3 in the samples. The high occurrence of TTV in the secondary effluent samples implied that TTV is epidemic widely in Tehran throughout the studding period and could be continuously discharged into the environmental water. Therefore, further studies are required to be conducted on final effluent (after chlorination) and influent samples to assess the efficiency of the wastewater treatment protocol and the infectivity of TTV in wastewater and treated wastewater.

The presence of TTV genomes in the Tehran secondary effluent and the associated risk of using the treated wastewater as a source of reclaimed water suggest that the

quality of wastewater treatment needs to be reconsidered and viruses should be employed as a fecal indicator. It seems in order to control gastroenteritis in Iran, the treatment of water and wastewater should be improved, and the discharge of wastewater needs to be minimized.

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CONFLICT OF INTEREST

The authors declare that there is not any conflict of interest.

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