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ORIGINAL ARTICLE

Monitoring and evaluation of infectious rotaviruses in various wastewater effluents and receiving waters revealed correlation and seasonal pattern of occurrences

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Abstract

Aims: Sewage systems are important nodes to monitor enteric pathogens transmitted via water. The aim of this study was to assess the presence of rotaviruses in effluents from wastewater treatment plants (WWTPs) and receiving streams in Beijing, China, to evaluate the reductions of rotaviruses in WWTPs and to provide viral fate and transport data for further epidemiological studies.
Methods and Results: Two PCR-based methods, including an RT-qPCR and another quantitative RT-PCR (ICC-RT-qPCR), which was integrated with cell culturing, were applied to conduct a 1-year monitoring of infectious rotaviruses and viral genes in effluents from three WWTPs and the receiving waters in Beijing, China. The ICC-RT-qPCR was able to detect more positive samples than RT-qPCR, showing positive results for 67% of primary effluents, 47% of secondary effluents and 14% of tertiary effluents, in comparison with 44, 22 and 6% by RT-qPCR, respectively. Seasonal variations of rotaviruses were observed in all effluents with higher occurrences in winter than in summer, which correlated well with the seasonal pattern of rotaviruses in the river receiving wastewater effluents. The reduction efficiencies by different treatment processes were assessed. Secondary treatments can remove most of infectious rotaviruses in primary sewage, with annual average reduction values of $2{\cdot}08 \pm 0{\cdot}63$, $2{\cdot}83 \pm 0{\cdot}49$ and $2{\cdot}00 \pm 1{\cdot}10$ $\log_{10}$ for the three WWTPs, respectively. Tertiary treatments were able to further remove infectious rotaviruses.
Conclusions: The results showed a year-round distribution of rotaviruses in three WWTPs in Beijing and provided important information regarding the transport and susceptibility of rotaviruses to different levels of wastewater treatment processes.
Significance and Impact of the Study: This study, for the first time, revealed the whole year prevalence and reductions of rotaviruses in WWTPs and the corresponding receiving waters in China, and demonstrated the impact of wastewater discharge on the potential spreading of infectious rotaviruses and public health.

Introduction

The occurrences of enteric viruses in water bodies are well documented (Bosch *et al.* 1988; Divizia *et al.* 2004; Gerba *et al.* 1996), and they have been associated with waterborne gastroenteritis of nonbacterial origin (Divizia *et al.* 2004; Orenstein *et al.* 2006; Villena *et al.* 2003). Group A rotaviruses have been identified to be the dominant causative agent for severe gastroenteritis in infants and young children worldwide, responsible for nearly 50% of the total episodes and about 600 000 deaths of children per year (Grimwood and Bines 2007; Parashar *et al.* 2003,

2006). In China, rotaviruses lead to about 35 000 deaths in infants and children per year (Orenstein *et al.* 2007). The only proven route of rotavirus transmission is via the faecal–oral route, either by environmental contact with contaminated surfaces or by ingestion of contaminated water and food.

It has been recognized that bacterial indicators of faecal contamination used to assess environment water quality often do not reflect the presence of viruses in water samples (Contreras-Coll *et al.* 2002; Formiga-Cruz *et al.* 2005). This was evidenced by the outbreaks of viral diseases occurred as a result of the consumption of water that met the bacterial coliform standards (Le Guyader *et al.* 2008). Infectious rotaviruses originated from human faeces can be spread into receiving waters including rivers, lakes and seawater, from wastewater discharges because of their resistance to typical wastewater treatment processes (Gerba *et al.* 1996; Hot *et al.* 2003). Therefore, monitoring the occurrences and reductions of rotaviruses in various influents and effluents from WWTPs can provide insights into the susceptibility of rotaviruses to different treatment processes and the potential of their discharge into the receiving water bodies. The results will provide valuable information for epidemiological surveys.

Currently, there are very limited data regarding distributions and concentrations of rotaviruses in various wastewater effluents and their potential impact on the receiving water bodies (Gerba *et al.* 1996; Brassard *et al.* 2005; Villena *et al.* 2003). One of the main reasons for this knowledge void is because of the lack of feasible and reliable rotaviruses detection methods. Detection of rotaviruses in environmental water samples by conventional cell culture assay was fastidious, because of the difficulty of growing human rotaviruses in cell culture and the interference from more rapidly growing viruses (i.e. enteric viruses) (Gerba *et al.* 1996). Quantitative PCR (qPCR) is a rapid, sensitive, specific and quantitative method for detecting viral genes in environmental water; however, it suffers from several limitations (Ko *et al.* 2003, 2005; Min *et al.* 2006). One important shortcoming associated with direct PCR-based methods is the fact that it cannot distinguish infectious viral particles from those noninfectious (e.g. dead) ones, as they detect genes originated from both infectious and inactivated viruses (Griffin *et al.* 2003; Gregory *et al.* 2006). This might lead to misinterpreted implications in assessing risks to public health (Ko *et al.* 2005; Li *et al.* 2009). Another potential issue with qPCR-based virus detection is related to the selection of target gene fragments. Furthermore, various kinds of PCR inhibitory substances in environmental waters are concentrated together with viruses during sample concentration, and they often hinder the efficiency of PCR amplification (Haramoto *et al.* 2010).

To overcome some of the above limitations associated with direct qPCR method, an integrated cell culture and quantitative RT-PCR (ICC-RT-qPCR) method for detecting and quantifying infectious rotaviruses has been developed in our laboratory, which was shown to have higher sensitivity and shorter incubation time compared to conventional plaque assay (Li *et al.* 2010). The concentrations of rotaviruses in water samples were estimated and numbered using the standard curves in previous studies, which indicated a linear relationship (R^2 of 0·9575) between the logarithm of copy numbers detected by ICC-RT-qPCR and PFU concentration (from 0·2 to 200 PFU ml^{-1}) of inoculated rotaviruses SA11 and Wa (Li *et al.* 2010). In this study, we applied this developed method, in parallel with the conventional direct PCR method, to conduct a 1-year monthly monitoring of infectious rotaviruses and viral genes in various effluents from three WWTPs (Q, G and X) and in their receiving surface waters, including a river and a lake in Beijing, China. The results revealed the prevalence, fate and transport of the rotaviruses in various wastewater effluents treated with different treatment processes, and the continuous field monitoring facilitated by the detection method allowed for observation of seasonal changes in the occurrences of rotaviruses in the receiving surface waters.

Materials and methods

Environmental water samples

The wastewater samples were collected monthly from three municipal WWTPs for a 1-year period from May 2007 to April 2008. The description of unit treatment processes and basic water characteristics were summarized in Tables 1 and 2. Particularly, WWTP Q discharges about 80% of the effluent into a recreational river (R), and a small portion of the secondary effluents is further treated by 0·02-μm hollow fibre membrane ultrafiltration before its discharge into a recreational lake (L). Samples from both river (R) and lake (L) were also collected 30 cm below the water surface for analysis. Twenty litres of water samples were collected and stored in plastic bottles on ice for delivery. All samples were concentrated within 24 h after collection. Suspended solids (SS), chemical oxygen demand and total nitrogen were analysed for each sample upon arrival using the standard methods (Chinese Standard Press, 1996).

Water samples concentration and pretreatment

The water sample was concentrated to obtain virus concentrate according to methods reported elsewhere with some modifications (Katayama *et al.* 2002; Kocwa-Haluch

Table 1 Description of treatment processes in three wastewater treatment plants (WWTPs), surface waters and sampling sites for rotavirus occurrence study in Bejing, China

WWTP	Size (m^3 day^{-1})	Inhabitants	Wastewater treatment process	Sampling sits (ID)
G	1 000 000	2 400 000	Aerated grit chamber and Primary setting	Primary effluents (G1)
			Activated sludge process (retention time: 5 h), and secondary setting	Secondary effluents (G2)
			Coagulative precipitation and sand filtration	Tertiary effluents (G3)
Q	400 000	800 000	Aerated grit chamber and primary setting	Primary effluents (Q1)
			Anaerobic–anoxic–oxic (A^2/O) activated sludge (Retention time: 6 h), and secondary setting	Secondary effluents (Q2)
			0·02- μm hollow fibre membrane ultrafiltration	Tertiary effluents (Q3)
X	900 000	1925 000	Aerated grit chamber and Primary setting	Primary effluents (X1)
			A^2/O activated sludge (Retention time: 6 h), and secondary setting	Secondary effluents (X2)
			Reverse osmosis membrane ultrafiltration	Tertiary effluents (X3)

Table 2 Basic water quality characteristics of various water samples analysed in this study

Water samples	SS (mg l^{-1})	Chemical oxygen demand (mg l^{-1})	Total nitrogen (mg l^{-1})
G			
Primary effluents (G1)	150–220	250–350	40–60
Secondary effluents (G2)	10–20	30–60	30–50
Tertiary effluents (G3)	0–3	20–50	20–40
Q			
Primary effluents (Q1)	120–200	200–300	40–60
Secondary effluents (Q2)	10–20	30–60	15–25
Tertiary effluents (Q3)	5–10	20–30	10–20
X			
Primary effluents (X1)	150–250	200–400	50–70
Secondary effluents (X2)	10–20	10–30	10–20
Tertiary effluents (X3)	0	0	0
River (R)	10–20	20–40	10–25
Lake (L)	5–10	10–30	10–25

and Zalewska 2002; Li *et al.* 2010). Briefly, water samples were first amended with 5 mmol l^{-1} $AlCl_3$ and followed by pH adjustment (to pH 3·5) using 1 mol l^{-1} HCl. Then, 8 g SiO_2 was added into water samples, which was stirred for 15 min to allow complete adsorption of viral particles to the SiO_2 particles, and followed by filtration through a membrane to collect the virus-SiO_2 complex. Forty millilitres of H_2SO_4 (pH 3·0) was added and passed through to rinse out the cation, and the virus–SiO_2 complex was collected into a 50- ml tube with 18 ml NaOH (pH 10·5), and vortexed for 5 min to release the virus particles to the eluant. The SiO_2 particles, as well as the bigger particles, were removed by centrifugation at 4000 ***g***, 4°C for 10 min, while most colloidal and fine particles were left in the eluate to reduce loss of viral particles adhering to them. Two millilitres of 10× TE buffer (100 mmol l^{-1} Tris–HCl, 10 mmol l^{-1} EDTA, pH 8·0) was added into the elute that contained virus for neutralization. The virus concentrate (with a total volume of 20 ml) was re-concentrated into 4 ml with Millipore's Amicon Ultra-4 centrifugal filter devices (100 000 NMWL; Millipore, Tullagreen, Ireland) and stored at –80°C until analysis. Two 1-ml concentrated water samples were analysed for rotaviruses using both ICC-RT-qPCR and direct RT-qPCR in parallel for comparison.

Direct RT-qPCR method for detection of rotavirus VP7 genes

For quantification of viral genes in concentrated water samples, total RNA was extracted from concentrated water samples using the QIAamp® UltraSens™ Virus Kit (Cat.: 53706; Qiagen, Hilden, Germany) and then was reverse-transcribed in 10 μl volumes using the ExScript™ RT reagent Kit (TaKaRa, Cat.: DRR041, Dalian, China) (Li *et al.* 2010). Quantification of viral genes was conducted by qPCR. The primers designed for the detection of group A rotaviruses (Li *et al.* 2010) were applied (vp7 sense: 5-CTGACGAAGCGAATAAATGG-3; vp7 antisense: 5-GGTCACATCATACAATTCT-3).

The quantified plasmid DNA carrying the same gene region was constructed by our laboratory as standards for RT-qPCR. Quantitative PCR was performed in 20 μl volumes with a SYBR® Premix Ex TaqTM kit (TaKaRa, DaLian, China) in an iQ icycler (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. Briefly, the reaction contained 4 μl of cDNA or quantified plasmid DNA, 10 μl of 2 × SYBR buffer, 0·2 μl of each primer

(0·2 μmol l^{-1} finial concentrations) and 5·6 μl of PCR-grade water. The qPCR amplification was performed with one cycle of preheating at 95°C for 10 s, 40 cycles of melting at 95°C for 5 s, annealing at 56°C for 20 s and extension at 72°C for 30 s. The amplification was followed by a melting curve analysis with 70 cycles of dissociation; from 95 to 60°C with a temperature ramp of 0·5°C every 30 s. After the melting curve analysis, the products were held at 4°C. Melting curve analysis showed the specific melting peek at 81·0 ± 0·5°C. Negative controls (autoclaved distilled water) and positive controls (rotaviruses suspensions) were included with each set of test samples and taken through nucleic acid extraction and enzymatic amplification assays. The concentration of rotaviral VP7 genes in water samples were calculated by the standard curve using plasmid DNA showed that the reliable detection ranged from $1{\cdot}2 \times 10^2$ to $1{\cdot}2 \times 10^8$ copies per qPCR (Hu *et al.* 2008; Li *et al.* 2010).

ICC-RT-qPCR assay for detection of infectious rotaviruses

The ICC-RT-qPCR protocols were described in detail in our previous reports (Li *et al.* 2010). Briefly, the concentrated water samples were further clarified by filtering through 0·2-μm-pore-size filters [PES (4 mm, low protein binding); Whatman, Piscataway, NJ, USA] to remove bacteria contamination, then treated with trypsin and inoculated onto confluent cells in cell culture flasks (25 cm^2) and incubated for 120 min, with rocking every 20 min for viral adsorption. Then with the inoculums removed, 4 ml of DMEM containing 2% FBS was added to the inoculated cell monolayer for culture at 37°C. After 2-day incubation, the viral RNA was extracted from the cell monolayer with Trizol (Invitrogen, Carlsbad, CA, USA) and subjected to reverse transcription immediately or stored at −80°C. The reverse transcription reaction was performed in 10 μl volumes using ExScriptTM RT reagent Kit (TaKaRa, Cat.: DRR041, Dalian) and qPCR was performed in 25 μl volumes containing 4 μl of the cDNA as the template as described before. An incubation time of 2 days was selected because it allowed for detection of rotaviruses at concentration as low as 2×10^{-1} PFU per cell culture, which is sensitive enough for this study. Negative controls (autoclaved distilled water) and positive controls (rotaviruses suspensions) were included with each set of test samples and taken through incubation to host cells, nucleic acid extraction and enzymatic amplification assays. The original concentrations of infectious rotaviruses in concentrated water samples were estimated by the relationship between the logarithm of copy numbers detected by ICC-RT-qPCR and PFU concentration of inoculated rotaviruses (Li *et al.* 2010), and the concentrations in initial water samples were calculated based on the concentration times.

Calculation of log reductions of infectious rotaviruses by different wastewater treatment processes

The reductions by different levels of wastewater treatment processes were calculated by the following equation:

$$\text{Log reductions} = \text{Log}_{10}\frac{N_0}{N_t}$$

where: N_0 = concentration (PFU/L) of infectious rotaviruses before the water treatment process; N_t = concentration (PFU/L) of infectious rotaviruses after the water treatment process.

For the samples with negative results, the log reductions were not determined; however, the minimum log reductions were estimated by using the detection limits. Microsoft Excel 2007 was used for the data analysis.

Results

Occurrences and seasonal distributions of rotaviruses in various WWTPs effluents

The occurrences of rotaviruses in various effluents from three WWTPs were examined using both ICC-RT-qPCR and direct RT-qPCR methods, and the results are

Table 3 Summary of rotavirus detection results by ICC-RT-qPCR and RT-qPCR assay for various effluents from three different WWTPs

	Percentages of positive samples analysed by ICC-RT-qPCR			Percentages of positive samples analysed by RT-qPCR		
ID of WWTPs	Primary effluent (%)	Secondary effluents (%)	Tertiary effluents (%)	Primary effluent (%)	Secondary effluents (%)	Tertiary effluents
G	75	58	33	42	33	17%
Q	67	42	8	42	17	0
X	67	42	0	50	17	0
Total	69	47	14	44	22	6%

The samples were collected monthly from three municipal wastewater treatment plants (WWTPs) for one period from May 2007 to April 2008. There were 12 samples for each site.

summarized in Table 3. As expected, the occurrences of rotaviruses in the effluents decreased with the higher level of treatment processes. For all the samples analysed, higher percentages of positive results were yielded by ICC-RT-qPCR methods than direct RT-qPCR method for the same effluent sample. As shown in Table 3, 67% primary effluents, 47% of secondary effluents and 14% of tertiary effluents were detected rotavirus-positive by ICC-RT-qPCR assay, compared with those 44% primary effluents, 22% secondary effluents and 6% of tertiary effluents that were VP7-positive by direct RT-qPCR assay.

The seasonal profiles of infectious rotaviruses detected by ICC-RT-qPCR and viral genes detected by RT-PCR method, in primary, secondary and tertiary effluents were shown in Figs 1 and 2, respectively. Similar seasonal variation patterns of infectious rotaviruses and rotaviruses VP7 gene concentrations were observed in various effluents for all three WWTPs studied, which showed higher viral concentrations detected in the fall and winter months (from November 2007 to March 2008) and lower concentrations observed in the summer months (from June to September 2008). The seasonal profile, however, was more clearly shown with ICC-RT-PCR method because of its lower detection limit and higher sensitivity as previously discussed. The annual average concentrations of infectious rotaviruses in the similar level effluents were found to be comparable among the three WWTPs studies. For example, the annual average concentrations of infectious rotaviruses in primary sewage were similar for the three WWTPs, with average values of 16·6, 7·6 and 6·5 PFU l^{-1} in G, Q and X WWTPs, respectively. However, the highest incidence of infectious rotaviruses had occurred in winter months (from November 2007 to March 2008), with average values of 288, 446 and 689 PFU l^{-1} in influents in G, Q and X WWTPs, respectively.

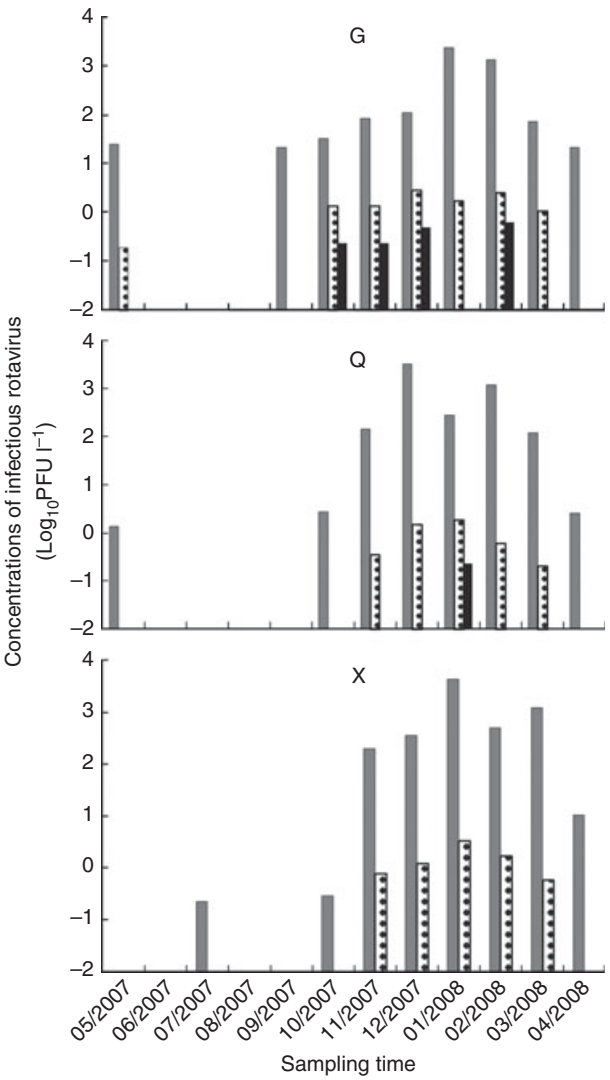

Figure 1 Seasonal variations of infectious rotaviruses in primary, secondary and tertiary effluents from three (G, Q and X) WWTPs. The *Y* axis indicates the infectious rotavirus concentrations detected by ICC-RT-qPCR. The samples with negative results were not shown in the figures. (■) primary effluents; (◪) secondary effluents; (■) tertiary effluents.

Reductions of infectious rotaviruses by different levels of treatment processes in WWTPs

Reduction/inactivation efficiencies of infectious rotaviruses by secondary and tertiary treatments processes in three WWTPs were listed in Table 4. The secondary treatments reduced most of infectious rotaviruses, with annual average log reduction value of 2·08 ± 0·63, 2·83 ± 0·49 and 2·00 ± 1·10 $\log_{10}$ for the three WWTPs (G, Q and X), respectively. Virus removal in the secondary treatments would be mostly attributed to the adsorption of microorganisms on the activated sludge as well as inactivation (Gerba *et al.* 1996; Carducci *et al.* 2008). Therefore, the treatment processes configurations and operation condition would affect the virus inactivation efficiency.

The tertiary treatments in two WWTPs (G and Q) achieved reductions of 0·72 ± 0·08 and 0·91 $\log_{10}$ in average, respectively. As the infectious rotaviruses were not detected in any tertiary effluents in X WWTPs, the reduction/inactivation efficiency of reverse osmosis treatment process was not evaluated. However, the minimum reductions were calculated as 0·143 $\log_{10}$ for WWTP X based on the average concentration of infectious rotaviruses in secondary effluents and the detection limit of ICC-RT-qPCR assay.

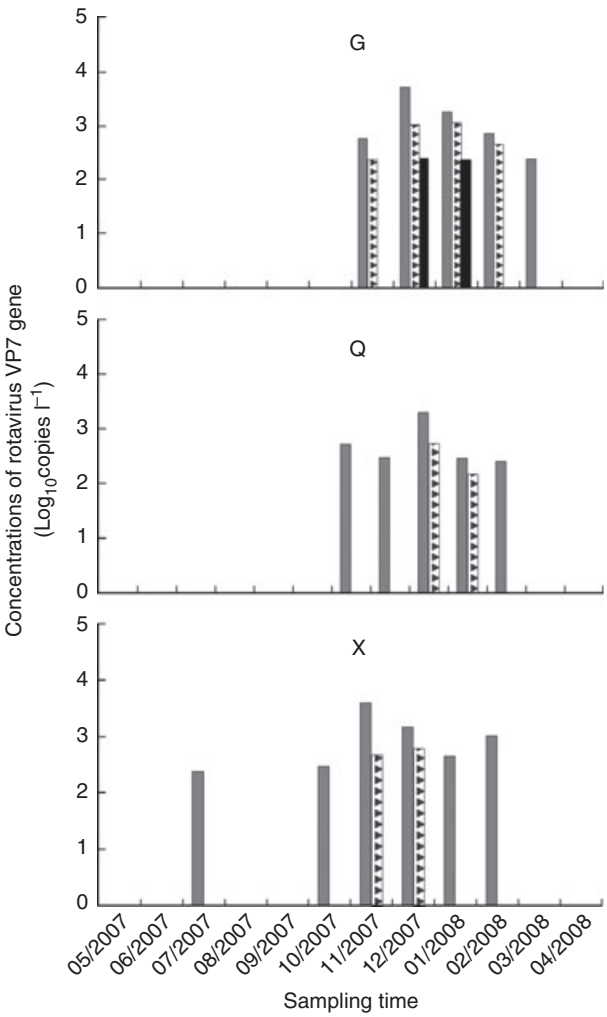

Figure 2 Seasonal variations of rotaviral VP7 gene in primary, secondary and tertiary effluents from three (G, Q and X) WWTPs. The *Y* axis indicates the concentrations of VP7 gene detected by qPCR. The samples with negative results were not shown in the figures. (▩) primary effluents; (▶) secondary effluents; (■) tertiary effluents.

The occurrences of rotaviruses in receiving waters that receives the wastewater discharges

Figure 3 shows the occurrences of infectious rotaviruses in the secondary and tertiary effluent from WWTP Q, as well as that in the receiving River and Lake Water. The river receives secondary effluents from Q WWTP and the lake receives tertiary effluents from WWTP Q. Three of the 12 samples showed positive results for rotaviruses for the river water sample, and they are all in winter months when the rotaviruses concentration was the higher in the discharge effluents, indicating a very likely correlation (average correlation coefficient = 83%) of the effluents discharge and the occurrences of the rotaviruses in the river water. None of the lake samples, however, were positive for rotaviruses, suggesting that the virus concentration in the tertiary effluents is relatively low or absent, leading to undetectable rotaviruses in the lake water.

Discussions

As sewerage systems are important nodes for human enteric pathogens transmitted via water, study of rotaviruses in raw and treated wastewater is important with regard to public health interests, especially in developing countries where there is a lack of information concerning microbiological surveillance, risk assessment and epidemiologic studies. In this study, a whole-year monitoring programme was conducted in various effluents by different level treatments from three WWTPs, as well as in receiving surface waters that received effluents from these WWTPs, in Beijing, China. A newly developed ICC-RT-qPCR was employed, to determine the occurrence of infectious rotaviruses, and the results were compared with rotaviral VP7 gene using direct RT-qPCR.

The results of this study indicated that higher percentages of positive results were determined by ICC-RT-qPCR assay than direct RT-qPCR method alone in raw and treated wastewaters. There are many reasons accounting for those results. In the direct RT-qPCR assay, the concentrated samples were directly extracted RNA and then subjected to RT and qPCR. The compounds matrices in sewage effluents were also concentrated accompanying with the virus concentration, which can largely inhibit the RNA extraction, RT and PCR, resulting less positive samples (Ijzerman *et al.* 1997; Ko *et al.* 2003). However, in the ICC-RT-qPCR method, rotaviruses present in the concentrated water samples were used to infect the host cells for 2 days, and then the specific infectious viral RNA in host cells was quantified. The replication of rotavirus RNA in host cells definitely indicates there are infectious rotaviruses present in water samples. The preinfection step increased sensitivity and allowed for differentiating the RNA associated with infectious virus from those originated from lysed virus. In addition, the host-culturing step was shown to be able to reduce the PCR inhibitors in the concentrated samples because the culture medium was removed before the RNA extraction from the host cells (Grimm *et al.* 2004; Ko *et al.* 2003; Li *et al.* 2010). This helps to overcome the RT-qPCR inhibition issue often encountered because of the concentrated substances in the wastewater matrix that inhibit PCRs (Ijzerman *et al.* 1997; Gallagher and Margolin 2007). The results demonstrated that ICC-PCR allows for more rapid, sensitive and reliable method than the traditional cell culture method or direct PCR approach for detection of infectious viruses in water environments (Gallagher and

Table 4 Log removals of infectious rotavirus by secondary and tertiary treatments from G, W and Q wastewater treatment plants

	Log removal of infectious rotavirus by secondary treatments (log_{10})			Log removal of infectious rotavirus by tertiary treatments (log_{10})		
Sampling time	G	Q	X	G	Q	X
05/2007	2·11	–	–	–	–	–
06/2007	–	–	–	–	–	–
07/2007	–	–	0·66	–	–	–
08/2007	–	–	–	–	–	–
09/2007	–	–	–	–	–	–
10/2007	1·39	–	0·54	0·76	–	–
11/2007	1·79	2·60	2·41	0·74	–	–
12/2007	1·59	3·32	2·47	0·77	–	–
01/2008	3·13	2·17	3·11	–	0·91	–
02/2008	2·73	3·30	2·47	0·60	–	–
03/2008	1·84	2·76	3·31	–	–	–
04/2008	–	–	1·00	–	–	–
AVG ± SD	2·08 ± 0·63	2·83 ± 0·49	2·00 ± 1·10	0·72 ± 0·08	0·91	–

–, could not be determined (influent and/or effluent concentrations were lower than detection limit).

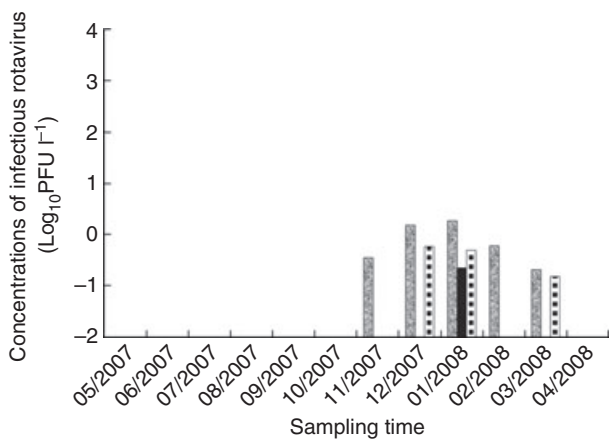

Figure 3 The occurrences of infectious rotaviruses in effluents of Q WWTP and recreational water receiving the effluents. There were three positive samples from the river that received the secondary effluents of Q WWTP, but none of the samples was positive from the lake that received the tertiary effluents from Q WWTP. The *Y* axis indicates the infectious rotavirus concentrations detected by ICC-RT-qPCR. The samples with negative results were not shown in the figures. (▨) secondary effluents; (■) tertiary effluents; (▣) receiving river.

Margolin 2007; Ko *et al.* 2003; Lee *et al.* 2005; Li *et al.* 2010).

Our monthly monitoring results showed that occurrences of rotaviruses were higher in both WWTP effluents and in receiving waters in the autumn and winter, when rotaviral gastroenteritis are more prevalent. Similar seasonal finding was reported by others as well (Gerba *et al.* 1996; Brassard *et al.* 2005). The increase in rotaviruses in winter was consistent with higher clinical cases of rotaviruses gastroenteritis of infants and children during the cold months in China (Orenstein *et al.* 2006, 2007), and the same pattern has also been described elsewhere (Parashar *et al.* 2003). This study provided seasonal profiles of infectious rotaviruses and rotaviral VP7 genes in water sources that seem to be consistent with the epidemic epidemiology of rotaviruses. Another important reason for the abundance of rotaviruses in the winter months is the lower temperature because rotaviruses were reported to be more stable at lower temperature (Gerba *et al.* 1996; Mahony *et al.* 2000). Previously, it was showed that rotaviruses can persist 10 days at 20°C and 32 days at 4°C in river water (Gerba *et al.* 1996).

The presence of rotaviruses in wastewater effluent in summer revealed in this study suggests the possible circulation of rotaviruses in the human environment throughout the year. The survival strategy of rotaviruses in summer is important from the epidemiological viewpoint and might be a key for reducing the outbreaks in the winter season as rotaviruses are removed or inactivated more efficiently in summer than in winter, because of the higher temperature and stronger UV sunlight in summer.

This study, for the first time, evaluated the reduction efficiencies of infectious rotaviruses by different levels of wastewater treatment processes in Beijing, China. The results showed that the secondary treatment processes such as active sludge and A^2/O can inactive/remove most of infectious rotaviruses, and the tertiary treatments were able to further remove rotaviruses. However, the correlation of infectious rotaviruses in receiving water (River) with those in the effluents suggested that wastewater discharges were likely an important source of rotaviral contamination for the corresponding receiving waters. As

the low concentrations of infectious rotaviruses, the tertiary effluents from Q WWTP, dilution by the water in Lake, and even the inactivation in environments, there were no positive samples from Lake, which received tertiary effluents from Q WWTP.

This study provided new information on the fate, transport and susceptibility of rotaviruses to different levels of wastewater treatment processes and demonstrated the impact of wastewater discharge on the receiving surface water quality in terms of potential spreading of infectious rotaviruses. Further studies will be conducted on larger scale and longer-term monitoring of rotaviruses in different WWTPs effluents and receiving streams to obtain more geographical insights into the fate and transport of rotaviruses through wastewater treatment processes and impact on public health. We will also research on the development of ICC-RT-qPCR assay for other viruses to evaluate the risk of other virus infection transmitted in water.

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References

Bosch, A., Pinto, R.M., Blanch, A.R. and Jofre, J.T. (1988) Detection of human rotavirus in sewage through two concentration procedures. *Water Res* **22**, 343–348.

Brassard, J., Seyer, K., Houde, A., Simard, C. and Trottier, Y.L. (2005) Concentration and detection of hepatitis A virus and rotavirus in spring water samples by reverse transcription-PCR. *J Virol Method* **123**, 163–169.

Carducci, A., Morici, P., Pizzi, F., Battistini, R., Rovini, E. and Verani, M. (2008) Study of the viral removal efficiency in a urban wastewater treatment plant. *Water Sci Technol* **58**, 893–897.

Chinese Standard Press (1996) The standard of Chinese Environmental protection: water quality analysis methods. 5066-2432-X

Contreras-Coll, N., Lucena, F., Mooijman, K., Havelaar, A., Pierzo, V., Boque, M., Gawler, A., Holler, C. *et al.* (2002) Occurrence and levels of indicator bacteriophages in bathing waters throughout Europe. *Water Res* **36**, 4963–4974.

Divizia, M., Gabrieli, R., Donia, D., Macaluso, A., Bosch, A., Guix, S., Sa′nchez, G., Villena, C. *et al.* (2004) Waterborne gastroenteritis outbreak in Albania. *Water Sci Technol* **50**, 57–61.

Formiga-Cruz, M., Hundesa, A., Clemente-Casares, P., Albinana-Gimenez, N., Allard, A. and Girones, R. (2005) Nested multiplex PCR assay for detection of human enteric viruses in shellfish and sewage. *J Virol Methods* **125**, 111–118.

Gallagher, E.M. and Margolin, A.B. (2007) Development of an integrated cell culture – Real-time RT-PCR assay for detection of reovirus in biosolids. *J Virol Methods* **139**, 195–202.

Gerba, C.P., Rose, J.B., Haas, C.N. and Crabtree, K.D. (1996) Waterborne rotavirus: a risk assessment. *Water Res* **30**, 2929–2940.

Gregory, J.B., Litaker, R.W. and Noble, R.T. (2006) Rapid one-step quantitative reverse transcriptase PCR assay with competitive internal positive control for detection of enteroviruses in environmental samples. *Appl Environ Microbiol* **72**, 3960–3967.

Griffin, D.W., Donaldson, K.A., Paul, J.H. and Rose, J.B. (2003) Pathogenic human viruses in coastal waters. *Clin Microbiol Rev* **16**, 129–134.

Grimm, A.C., Cashdollar, J.L., Williams, F.P. and Fout, G.S. (2004) Development of an astrovirus RT-PCR detection assay for use with conventional, real-time, and integrated cell culture/RT-PCR. *Can J Microbiol* **50**, 269–278.

Grimwood, K. and Bines, J.E. (2007) Rotavirus vaccines must perform in low-income countries too. *Lancet* **370**, 1739–1740.

Haramoto, E., Kitajima, M., Katayama, H. and Ohgaki, S. (2010) Real-time PCR detection of adenoviruses, polyomaviruses, and torque teno viruses in river water in Japan. *Water Res* **44**, 1747–1752.

Hot, D., Legeay, O., Jacques, J., Gantzer, C., Caudrelier, Y., Guyard, K., Lange, M. and Andreoletti, L. (2003) Detection of somatic phages, infectious enteroviruses and enterovirus genomes as indicators of human enteric viral pollution in surface water. *Water Res* **37**, 4703–4710.

Hu, X.H., He, M., Liu, L., Li, D. and Shi, H.C. (2008) Construction of external standard for detection of rotavirus in water using the quantitative real-time polymerase chain reaction. *Environ Sci* **29**, 380–385.

Ijzerman, M.M., Dahling, D.R. and Fout, G.S. (1997) A method to remove environmental inhibitors prior to the detection of waterborne enteric viruses by reverse transcription-polymerase chain reaction. *J Virol Methods* **63**, 145–153.

Katayama, H., Shimasaki, A. and Ohgaki, S. (2002) Development of a virus concentration method and its application to detection of enterovirus and Norwalk virus from coastal seawater. *Appl Environ Microbiol* **68**, 1033–1039.

Ko, G., Cromeans, T.L. and Sobsey, M.D. (2003) Detection of infectious adenovirus in cell culture by mRNA reverse transcription-PCR. *Appl Environ Microbiol* **69**, 7377–7384.

Ko, G.P., Cromeans, T.L. and Sobsey, M.D. (2005) UV inactivation of adenovirus type 41 measured by cell culture mRNA RT-PCR. *Water Res* **39**, 3643–3649.

Kocwa-Haluch, R. and Zalewska, B. (2002) Presence of Rotavirus hominis in sewage and water. *Polish J Environ Studies* **11**, 751–755.

Le Guyader, F.S., Le Saux, J.C., Ambert-Balay, K., Krol, J., Serais, O., Parnaudeau, S., Giraudon, H., Delmas, G. *et al.*

(2008) Aichi virus, norovirus, astrovirus, enterovirus, and rotavirus involved in clinical cases from a French oyster-related gastroenteritis outbreak. *J Clin Microbiol* **46**, 4011–4017.

Lee, S.H., Lee, C., Lee, K.W., Cho, H.B. and Kim, S.J. (2005) The simultaneous detection of both enteroviruses and adenoviruses in environmental water samples including tap water with an integrated cell culture–multiplex-nested PCR procedure. *J Appl Microbiol* **98**, 1020–1029.

Li, D., Gu, A.Z., He, M., Shi, H.C. and Yang, W. (2009) UV inactivation and resistance ofrotavirus evaluated by integrated cell culture and real-time RT-PCR assay. *Water Res* **43**, 3261–3269.

Li, D., Gu, A., Wan, Y., He, M., Hu, X. and Shi, H.C. (2010) An integrated cell culture and reverse transcription quantitative PCR assay for detection of infectious rotaviruses in environmental waters. *J Microbiol Methods* **82**, 59–63.

Mahony, J.O., Donoghue, M.O., Morgan, J.G. and Hill, C. (2000) Rotavirus survival and stability in foods as determined by an optimised plaque assay procedure. *Int J Food Microbiol* **61**, 177–185.

Min, B.S., Noh, Y.J., Shin, J.H., Baek, S.Y., Min, K.I., Ryu, S.R., Kim, B.G., Park, M.K. *et al.* (2006) Assessment of the quantitative real-time polymerase chain reaction using a cDNA standard for human group A rotavirus. *J Virol Methods* **137**, 280–286.

Orenstein, E.W., Fang, Z.Y., Xu, J., Liu, C., Shen, K., Qian, Y., Jiang, B., Kilgore, P.E. *et al.* (2006) The epidemiology and burden of rotavirus in China: a review of the literature from 1983 to 2005. *Vaccine* **25**, 406–413.

Orenstein, E.W., Fang, Z.Y., Xu, J., Liu, C., Shen, K., Qian, Y., Jiang, B., Kilgore, P.E. *et al.* (2007) The epidemiology and burden of rotavirus in China: a review of the literature from 1983 to 2005. *Vaccine* **25**, 406–413.

Parashar, U.D., Hummelman, E.G., Bresee, J.S., Miller, M.A. and Glass, R.I. (2003) Global illness and deaths caused by rotavirus disease in children. *Emerg Infect Dis* **9**, 565–572.

Parashar, U.D., Gibson, C.J., Bresse, J.S. and Glass, R.I. (2006) Rotavirus and severe childhood diarrhea. *Emerg Infect Dis* **12**, 304–306.

Villena, C., El-Senousy, W.M., Abad, F.X., Pinto′, R.M. and Bosch, A. (2003) Group A rotavirus in sewage samples from Barcelona and Cairo: emergence of unusual genotypes. *Appl Environ Microbiol* **69**, 3919–3923.