

One-year monthly survey of rotavirus, astrovirus and norovirus in three sewage treatment plants (STPs) in Beijing, China and associated health risk assessment

X. Q. He, L. Cheng, D. Y. Zhang, X. M. Xie, D. H. Wang and Z. Wang

ABSTRACT

To evaluate the presence and distribution of the three main viruses (rotavirus, astrovirus, and norovirus) responsible for human acute gastroenteritis in sewerage systems, a one-year study was carried out in Beijing, China. A total of 96 samples of influent and effluents from three sewage treatment plants (STPs) were collected from November 2006 to October 2007. Silica was used to concentrate viral particles from water samples and a reverse transcriptase-nested polymerase chain reaction (RT-nested PCR) method was used for detection of viruses. Virus(es) could be detected in 35.4% (34/96) of the water samples analysed, where human rotavirus was the most frequently detected one (32.3%, 31/96), followed by human astrovirus (6.3%, 6/96) and human norovirus (3.1%, 3/96). According to the quantitation results of rotaviruses, which were gained by the real-time quantitative RT-PCR method with SYBR Green I, it was known that the distributions of rotaviruses in influents and effluents of three STPs were quite similar, i.e., abundant in cold weather (from October to March) and less prevalent in warm weather (from April to September). According to the estimated exposure dose and exposure frequency, as well as the acceptable annual risk level, it was shown that the rotaviruses in the reused wastewater after conventional treatment process presented potential risk to human health through both occupational and accidental exposure.

Key words | astrovirus, health risk, norovirus, rotavirus, STP

INTRODUCTION

Enteric viruses play an important role in pediatric diarrhoea during the winter season in China. The reported frequency of infection by rotavirus, astrovirus, and norovirus was 59%, 8% and 6%, respectively, during the winter season (from December 2000 to March 2001) in Beijing Children's Hospital (Liu *et al.* 2006). The infection was most commonly seen among children <5 years old hospitalized with acute diarrhoea (Zhang *et al.* 2007) and 96.8% of children were infected under 3 years of age (Tong *et al.* 2003). The number of deaths caused by rotavirus diarrhoea accounted for 40% of all diarrhoea deaths and 11.1% of total deaths. Astrovirus seemed an important aetiological agent in young children with acute diarrhoea in China, and over 95% of astrovirus infections occurred in children before 2 years of age in seven regions of China during 1998–2005 (Fang *et al.* 2006). Norovirus was one of the important pathogens causing

acute gastroenteritis outbreaks among infants and young children from January 2002 to December 2006 in Beijing Children's Hospital (Deng *et al.* 2007), which also caused outbreaks of acute gastroenteritis in six hospitals of Beijing from November to the end of 2006 (Jia *et al.* 2007).

Enteric viruses are able to persist under environmental conditions, and the release of infectious viruses into the water environment may cause public health problems, particularly with regard to the use of contaminated water for drinking, recreation, park irrigation, and road washing etc. (Meleg *et al.* 2006; Rutjes *et al.* 2009). Outbreaks of viral acute gastroenteritis by consumption of contaminated water have been frequently reported in the world, and viruses are known as the aetiological agents for the infectious gastroenteritis (Divizia *et al.* 2004). While rotaviruses, astrovirus, and norovirus have been involved in these outbreaks

X. Q. He (corresponding author)

L. Cheng

D. Y. Zhang

X. M. Xie

School of Biological Sciences and Technology,

Beijing Forestry University,

P.O. Box 162,

Beijing 100083,

China

E-mail: lenahe@bjfu.edu.cn

D. H. Wang

Z. Wang

State Key Laboratory of Environmental Aquatic

Chemistry,

Research Center for Eco-Environmental Sciences,

Chinese Academy of Sciences,

P.O. Box 2871,

Beijing 100085,

China

(Hung *et al.* 1984; Boccia *et al.* 2002; Divizia *et al.* 2004; Hoebe *et al.* 2004; Gally *et al.* 2006; Hewitt *et al.* 2007; Martinelli *et al.* 2007; Maunula *et al.* 2009), routine procedures for monitoring these viruses in water samples had not been drawn up for the water-microbiology screening panel. Sewage water contains much higher numbers of viruses (Meleg *et al.* 2006) and sewage workers frequently exposed to raw sewage were found to be at higher risk of intestinal disease (Rylander 1999; Venczel *et al.* 2003). The effluent with enteric pathogens from the sewerage systems, which directly entered into the water environment, was found to be one of the most important causes of waterborne outbreak (Hafliger *et al.* 2000); therefore, sewerage systems should be regarded as important nodes to monitor and control human enteric pathogen transmission (Katayama *et al.* 2008). Consequently, viral contamination resulting from the use of these treated waters will produce a risk that needs to be addressed (Arraj *et al.* 2008). Information on the monthly concentration distribution of enteric viruses in sewage treatment plants (STPs) will be useful, not only for understanding their fate but also for assessing the risk of infection through different exposure scenarios (Katayama *et al.* 2008).

In Beijing, the treated water from STPs, regarded as a second source water, had been widely used as toilet flushing water, industrial process water, environmental water, garden water and road washing water (He *et al.* 2008). Viruses discharged from STPs represent a risk of infection via various exposure processes such as the reused water for the park irrigation or road sprinkling. An important step in addressing the safety of reused wastewater was to determine likely risks of exposure to treated water. A sound understanding of such risks will not only be of significant value in managing low-technology reuse schemes but form the basis of risk assessments for advanced reuse proposals (Hamilton *et al.* 2006).

Different types of filters and filtration methods, such as cartridge filters, glass wool filters, cation-coated filter, silica etc., had been used to collect and concentrate rotavirus, astrovirus, and norovirus from sewage water samples (Baggi *et al.* 2001; van Zyl *et al.* 2004; Katayama *et al.* 2008). Traditional methods such as cell culture for enteric virus detection remained cumbersome, costly and time-consuming (Jean *et al.* 2002). Recently, more molecular approaches had been used to detect rotavirus, astrovirus, and norovirus from sewage water samples (Baggi *et al.* 2001; Jean *et al.* 2002; Meleg *et al.* 2006; Min *et al.* 2006; He *et al.* 2008; Katayama *et al.* 2008).

In China, most studies focused on the epidemiologic characteristics of viral diarrhoea in hospitals. However,

knowledge of the occurrence of rotavirus, astrovirus, and norovirus in water environment was very limited. The goal of this study was to assess viral contamination of the three viruses responsible for acute gastroenteritis (RV, AV, and NV) from the urban STPs in Beijing. And obtained quantitative data of RV were used to provide a rough estimate of the risk of infection from occupational and accidental exposure to treated waters in Beijing.

MATERIALS AND METHODS

Collection of water samples

From November 2006 to October 2007, influent and effluent samples were collected monthly for 1 year and 96 water samples in total were drawn from three urban STPs. The three municipal STPs received complex mixtures of industrial and domestic sewage with a treatment capacity of 1 (A), 0.04 (B) and 0.1 (C) million tons daily, respectively. All the three plants used activated sludge and included a sedimentation step at the end of the process, and in plants A and C part of the secondary effluent was further treated by coagulant and micro-filtration, respectively, resulting in reclaimed effluent (He *et al.* 2008).

We have taken 36 samples (1 mL) from influents, 36 samples (1L) from effluents of secondary treatment, as well as 24 samples (1L) from reclaimed treatment. All samples were processed within 12 h after collection. The method used for the concentration of water samples was described previously (He *et al.* 2008).

Extraction of nucleic acids from water specimens

RNA was extracted using the Qiagen viral RNA extraction kit (QIAGEN, Germany) following the manufacturer's instruction. The 50 μ L RNA eluates were stored at -80°C until amplification of nucleic acid was performed.

Detection of rotavirus, astrovirus and norovirus in water samples

Primer characteristics and references for the amplification conditions of different PCR or nested PCR protocols used for nucleic acid detection of rotavirus, astrovirus, and norovirus were all described previously (Liu *et al.* 2006;

He *et al.* 2008). Briefly, primers END9, R1, R3, and Rp were used for the amplification of sequences from the VP7 gene of group A rotaviruses. Norovirus was analysed using RT-PCR with primers JV12a and JV13b. Astrovirus was detected by RT-PCR with primers Mon 269 and Mon 270. To avoid false-positive results, quality control measures were followed as recommended and for each set of amplifications, negative and positive control samples were included. All methodologies were standardized with reference strains of each virus. In the present study, previously characterized virus strains obtained from faecal samples were used as positive controls. The PCR products were resolved on 1.8% agarose gel, followed by ethidium bromide staining (0.5 mg/mL), and images were obtained with the image capture system (BioRad) with the Quantity One software program. PCR products were sequenced at the Chinese National Human Genome Center, Beijing using the same primers. The sequences were aligned and searched for nearly identical sequences using the Basic Local Alignment Search Tool (blast) program available on the NCBI network server.

Quantitative detection of rotavirus genomes in water samples

Real-time PCR was performed in a MX3005P real-time quantitative polymerase chain reaction system (Stratagene, USA) in a total volume of 25 μ L, consisting of the Brilliant II SYBR Green QPCR master mix (Stratagene, USA), 300 nM forward primer R3 and 300 nM reverse primer Rp (Baggi & Peduzzi 2000). The thermal cycling conditions were as follows: an initial denaturation at 95 °C for 10 min followed by 45 cycles of 30 s at 95 °C, 40 s at 50 °C and 30 s at 72 °C. Each run ended with a melting curve analysis. Fluorescence data were collected at the end of each cycle, and determination of the cycle threshold line was carried out automatically by the instrument. PCR amplicons that were positive with SYBR green real-time PCR were further analysed by gel electrophoresis through 1.8% agarose gels to confirm the amplicon size.

In order to make a standard curve, standard samples of rotavirus-DNA plasmid (10^{15} copies/ μ L) were diluted by serial 10-fold dilution. The water and standard samples were subjected to real-time PCR simultaneously, followed by analysis using Mx3000P software (Stratagene) to obtain quantitative data on the concentration of rotavirus cDNA in a well. Three wells were used for the water and standard samples, respectively, and the average was used for subsequent calculations.

Risk assessment

For both professional and accidental contact with secondary effluent and/or reclaimed water during road sprinkling and park irrigation in Beijing, the risk of infection with rotavirus per exposure event was estimated. The probability of an infection (P_i) resulting from ingestion of some number of rotavirus was calculated based on the beta-Poisson model (Gerba *et al.* 1996). Yearly infection risks were calculated using the following equation: $P_a = 1 - (1 - P_i)^n$, where n is the exposure frequency in 1 year. The range of estimated ingested volumes per contact event used and the exposure frequency in 1 year in the calculations were based on the results obtained in a survey of water ingestion (He *et al.* 2006a). In this study, we assumed that rotavirus genomes detected were 1,000 times more abundantly present than infectious rotavirus particles (Rutjes *et al.* 2009).

RESULTS

Profile of rotavirus, astrovirus and norovirus in sewage samples

Virus(es) could be detected in 35.4% (34/96) of the water samples analysed. Rotavirus was the most frequently detectable (32.3%, 31/96), followed by human astrovirus (6.3%, 6/96) and human norovirus (3.1%, 3/96). All positive samples gave the correct sequence after using blast on NCBI. In plant A, all three viruses were detected and rotavirus was the highest in frequency (36.1%, 13/36). No astrovirus was detected in plant C, and no norovirus was detected in plant B. Rotaviruses were detected in 44.4% (16/36) of the influent samples and 25% (17/60) of the effluent samples from three STPs. The seasonal distribution of rotavirus, astrovirus and norovirus in influent and effluent samples from the three plants is shown in Figure 1. It was observed that the positive signals for rotavirus occurred in all samples taken between winter and spring (from October to April), and was less detectable during summer (from June to August). The highest percentage of positive detection for rotaviruses occurred in October 2007 (100%), followed by November 2006 (87.5%) and December 2006 (87.5%). All positive detections for astroviruses were found in winter and spring (December, January, April and October). Three positive detections for noroviruses were all in February 2007.

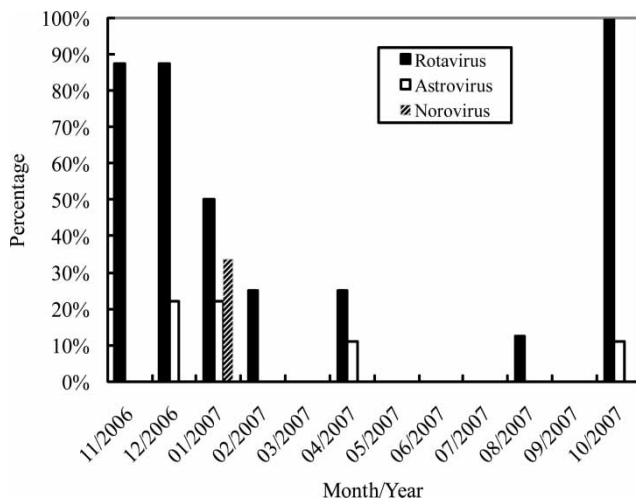


Figure 1 | Distribution of rotavirus, astrovirus and norovirus in sewage samples.

Quantitative survey of rotavirus genomes in sewage samples

The genomes of rotavirus in samples of positive detection were further quantified by real time PCR. Melting curve analysis was undertaken for the amplified DNA. The rotavirus real time PCR gave distinct melting curve diagrams, indicating that the PCR product was that of the human rotavirus sequence and thus the primers used in the study were specific for human rotavirus (Figure 2(a)). The efficiency of replication was 99%, falling within the requested range of 90%–110%. A good linear relationship was consistently observed with rotavirus cDNA ranging from 3.62×10^2 to 3.62×10^6 copies per reaction with a mean correlation coefficient of 0.999 in three independent runs. All the Ct values from the sewage samples fall within the range of linearity. Gel electrophoresis also confirmed that DNA of the expected size was amplified using the human rotavirus cDNA standard and the human rotavirus positive sample (Figure 2(b)). In contrast, other non-specific products were not observed.

The quantitative detections of rotavirus in three STPs were carried out for each month of the 1-year period, from November 2006 to October 2007. The seasonal profile of rotaviruses in influent, secondary effluent and reclaimed water is shown in Figure 3. As it is shown, the concentrations of rotavirus in influents of three STPs were quite similar, i.e., abundant in cold seasons (3.68, 2.82 and 2.86 log copies L^{-1} on average, respectively, from October to March), and less in warm seasons (0.81, 1.42 and 0 log

copies L^{-1} on average, respectively, from April to September). In secondary effluents, abundance of rotavirus was lower than that in influent, e.g., 1.0, 0.6 and 0.88 log copies L^{-1} in cold weather on average, respectively, and no detection in warm seasons. The distribution profiles in reclaimed water also showed seasonal variation and the concentrations were the highest (0.67 and 0.36 log copies L^{-1} on average in plant A and plant C, respectively) during cold weather. No virus was detected during warm seasons in reclaimed water.

Potential health risk exposure to rotavirus in treated waters

Estimated average volumes of water ingested by an occupational worker of road sprinkling (77 mL) and park irrigation (58 mL) in 1 day were used to calculate the risk of infection with rotavirus for an exposed occupational individual (He *et al.* 2006b). The estimated average volume of water ingested in 1 day for a passer-by during road sprinkling was 10 mL and for young and old during park irrigation was 40 and 50 mL, respectively (He *et al.* 2006b).

The P_a value at ingestion of 10 to 77 mL water ranged from 5.0×10^{-4} to 1.0×10^{-1} based on the average detected rotavirus concentrations in three STPs (Table 1). And maximum rotavirus concentrations resulted in a P_a value of 9.1×10^{-3} to 4.5×10^{-1} (Table 1). For the occupational workers, the maximum virus concentration resulted in an estimated P_a value of 4.5×10^{-1} during road sprinkling, whereas during park irrigation the maximum virus concentration led to an estimated P_a value of 2.8×10^{-2} if secondary effluents were used directly (plant B). To non-occupational people, the maximum virus concentration resulted in an estimated P_a of 1.0×10^{-1} during road sprinkling, whereas the maximum virus concentration led to an infection risk of 2.4×10^{-2} during park irrigation. The infection risks for people who were accidentally exposed to the water were generally lower, because they swallowed less water than occupational workers.

DISCUSSION

For the first time, the monthly distributions of rotavirus, astrovirus and norovirus in wastewater and in the reclaimed water were surveyed quantitatively in China. The detection frequencies for rotavirus, astrovirus and norovirus were 30.6, 5.6 and 2.8%, respectively, which were in accordance with the reported infection frequencies of 59, 8 and 6% by rotavirus, astrovirus and norovirus, respectively (Liu *et al.*

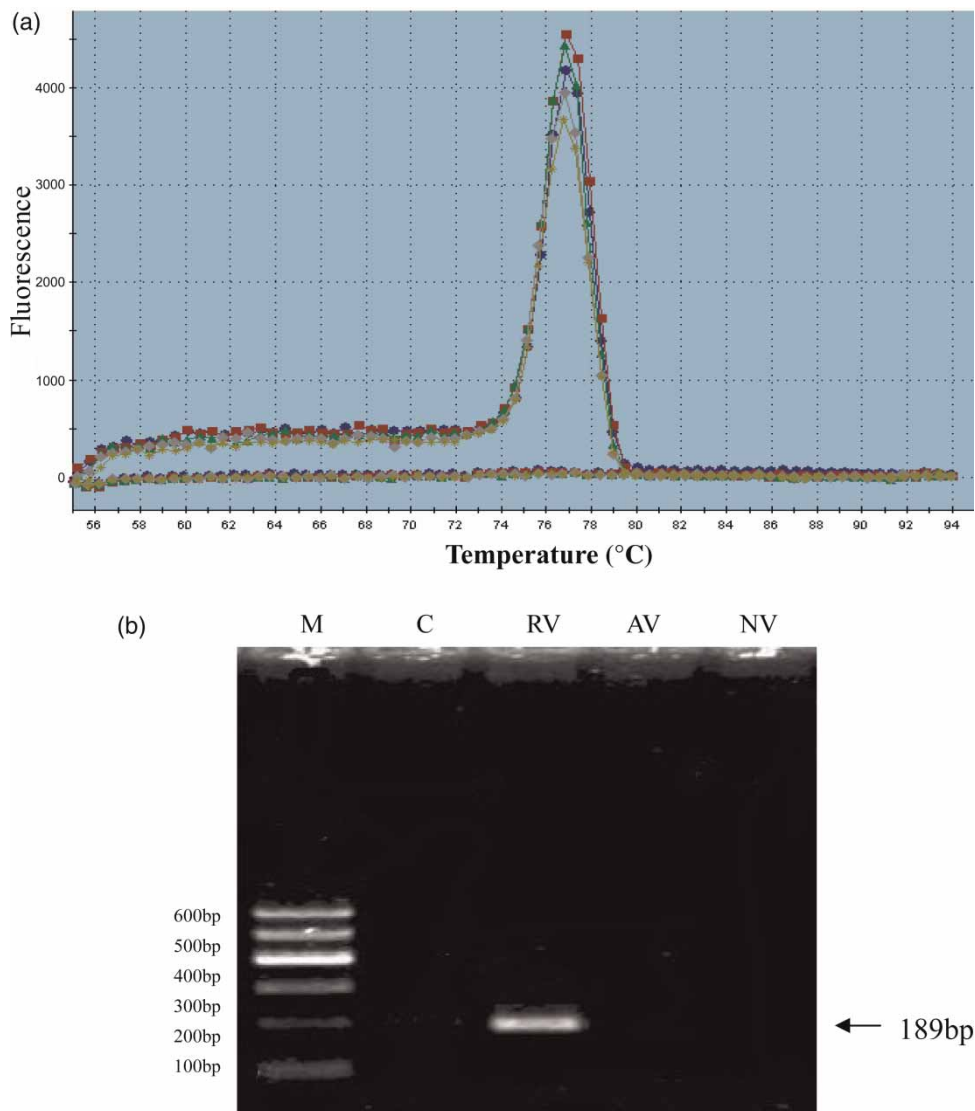


Figure 2 | Specificity of the real-time PCR: (a) melting curve analyses for RV (b) agarose gel electrophoresis of amplified products. Lanes: M, 100 bp DNA ladder; C, negative control; RV, rotavirus; AV, astrovirus; NV, norovirus. The positive band denotes 189 bp products.

2006). Three viruses were detected mostly in winter, which supported epidemiological reports that these viruses were more frequently detected in hospitalized patients' faeces in the winter. It was very difficult to eliminate the three viruses of the influents by the sewerage system and the three viruses also occurred in the secondary and tertiary effluents of the sewerage system, which demonstrated the permanence property of the three viruses and the dissemination possibility by the reused effluent water of sewerage system.

The present study showed a higher prevalence of rotavirus, astrovirus and norovirus during the cold season, but the difference was statistically significant only for rotavirus. Increased viral stability in the environment due to lowered

temperature, as found in studies on some enteric viruses, could promote waterborne gastroenteritis during winter, and thereby a higher viral load in sewage (Myrmel *et al.* 2006).

The prevalence of positive PCRs for rotaviruses was similar compared with some previous studies. For example, a study from Oslo in Norway reported that rotaviruses were detected in 38, 72 and 83% of the inlet samples and 36, 56% and 68% of the outlet samples from three STPs by RT-nested PCR (Myrmel *et al.* 2006). In Bangkok, 25% of the sewage samples were positive for rotavirus using RT-nested PCR (Kittigul *et al.* 2005). An RT-PCR hybridization was used in a study from Barcelona and Cairo, where 66.9

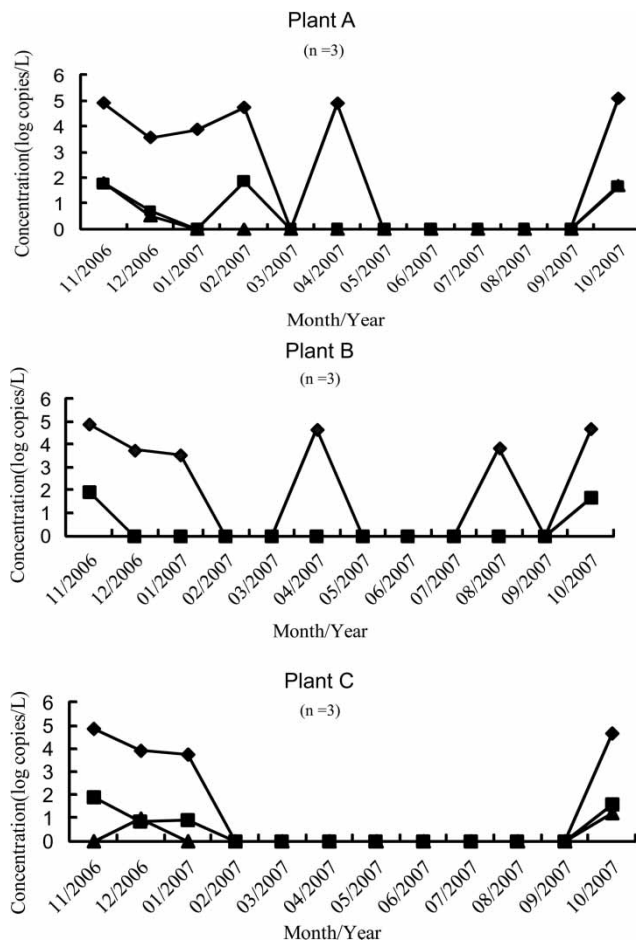


Figure 3 | Seasonal profile of rotaviruses at three STPs. (◆) Influent, (■) secondary effluent and (▲) reclaimed water.

and 85.7% of sewage samples were positive for rotavirus, respectively (Villena *et al.* 2003). A study in France reported a prevalence of rotavirus in raw sewage of 42% (Dubois *et al.* 1997).

The positive samples for detectable astrovirus and norovirus were rare in present study. A similar study in Norway, however, reported a higher prevalence of astrovirus in raw sewage of 46–94% (Myrmel *et al.* 2006). A study on astrovirus in Hungary also showed a relatively high prevalence (43%) (Meleg *et al.* 2006). Tajima *et al.* (2007) reported that norovirus was detected in 20 of 22 specimens (91%) in the secondary effluent and 20 of 28 specimens (71%) in the reclaimed water after tertiary treatments. In a study from the Netherlands (Lodder & de Roda Husman 2005), the concentration of norovirus in sewage was higher than that of rotavirus. The difference might be attributed to the epidemiological situation, the method used to concentrate the viruses, or even to the primer sets used for detection.

In this study, the concentration of rotaviruses in influent of the three STPs in Beijing was quantitatively determined monthly for a year, which indicated that rotavirus infections should occur in the service area of the STPs. The data also suggested that a virus detection of the influent in a STP would be an appropriate measure to understand the exact occurrence of viruses in the area.

For the purposes of risk assessment, rotavirus was chosen as the model organism. Rotavirus is a subgroup of enteric viruses that are known to cause gastroenteritis. It is highly infectious (Parashar *et al.* 2003) and has been widely used as a representative organism for enteric viruses in risk assessment of wastewater reuse (Hamilton *et al.* 2006; Seidu *et al.* 2008). Detection of viral genomes may be considered insufficient to identify infectious risk for the human population (Gassilloud *et al.* 2003; Rutjes *et al.* 2009). In this study, it was assumed that 1/1,000 of rotavirus genomes detected were infectious rotavirus particles, though it may underestimate the health risks (Rutjes *et al.* 2009). A disease risk of 10^{-3} per person per year, which was used by WHO as the tolerable risk of waterborne disease from

Table 1 | Rotavirus infection risk per year by effluents from three municipal STPs

Sampling sites	Road sprinkling				Park irrigation						
	Occupational		Passerby		Occupational		Nonoccupational				
	Max	Mean	Max	Mean	Max	Mean	Young	Old			
							Max	Mean	Max	Mean	
A	Secondary effluent	0.3965	0.1036	0.0882	0.0197	0.0235	0.0051	0.0162	0.0035	0.0203	0.0044
	Reclaimed water	0.3682	0.0682	0.0805	0.0127	0.0213	0.0033	0.0148	0.0023	0.0184	0.0028
B	Secondary effluent	0.4509	0.0758	0.1039	0.0142	0.0278	0.0037	0.0193	0.0025	0.0240	0.0032
C	Secondary effluent	0.4341	0.0775	0.0989	0.0146	0.0264	0.0038	0.0183	0.0026	0.0228	0.0032
	Reclaimed water	0.2469	0.0143	0.0504	0.0026	0.0132	0.0007	0.0091	0.0005	0.0114	0.0006

drinking fully treated drinking water, was unduly cautious (Haas 1996). It means one occurrence of disease per person per 1,000 years (for a community this risk of 10^{-3} means that every year 0.1% of the community becomes diseased as a result of drinking fully treated drinking water) (Mara *et al.* 2007). A more acceptable level for the risk of infection (rather than of disease) is 10^{-2} (i.e. once in every 100 years, essentially once in a lifetime; or 1% of the community per year) (Mara *et al.* 2007). Even this level of risk is relatively cautious, given the much higher actual incidence of diarrhoeal disease occurring in both industrialized and developing countries. In our study, a risk of rotavirus infection per year of 10^{-2} was used as the reference level of acceptable risk (Seidu *et al.* 2008). The observed maximum P_a value was 4.5×10^{-1} , which was much higher than this acceptance level. All the P_a values during road sprinkling and 46.9% of P_a values during park irrigation exceeded the line of 10^{-2} . Based on this assumption, the results indicated that there was a certain health risk for both occupational workers and people who were accidentally exposed to rotaviruses in the effluents of STPs. A study in Uganda also showed a higher infection risk per year (8.87×10^{-1} and 9.85×10^{-1}) (Howard *et al.* 2006). The estimated risks from rotavirus infections recorded in this assessment should be interpreted with some caution. The health risks presented here should not be misconstrued as a representative for all the possible enteric virus infections because of the diverse symptoms of enteric virus infections (Seidu *et al.* 2008).

The results of this study illustrated the presence of waterborne viruses in sewage water systems of Beijing and showed that both occupational and accidental exposure to rotaviruses in treated water may pose a health risk. The present results demonstrated the benefits of employing a more advanced treatment of sewage (Myrmel *et al.* 2006). The reduction of viruses in STPs will directly contribute to reducing viruses in the environment and accordingly result in the reduction of infection risk.

In conclusion, this study provided the first direct evidence that human viruses were prevalent in sewage water systems in Beijing and showed that both occupational and accidental exposure to rotaviruses in treated water may pose a health risk.

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