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Evaluation of the infectivity, gene and antigenicity persistence of rotaviruses by free chlorine disinfection

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Abstract

The effects of free chlorine disinfection of tap water and wastewater effluents on the infectivity, gene integrity and surface antigens of rotaviruses were evaluated by a bench-scale chlorine disinfection experiments. Plaque assays, integrated cell culture-quantitative RT-PCR (ICC-RT-qPCR), RT-qPCR, and enzyme-linked immunosorbent assays (ELISA), respectively, were used to assess the influence of the disinfectant on virus infectivity as well as genetic and antigenic integrity of simian rotavirus SA11 as a surrogate for human rotaviruses. The ICC-RT-qPCR was able to detect rotaviruses survival from chlorine disinfection at chlorine dose up to 20 mg/L (60 min contact), which suggested a required chlorine dose of 5 folds (from 1 to 5 mg/L) higher than that indicated by the plaque assay to achieve 1.8 $\log_{10}$ reductions in tap water with 60 min exposing. The VP7 gene was more resistant than the infectivity and existed at chlorine dose up to 20 mg/L (60 min contact), while the antigencity was undetectable with chlorine dose more than 5 mg/L (60 min contact). The water quality also impacted the inactivation efficiencies, and rotaviruses have a relatively higher resistant in secondary effluents than in the tap water under the same chlorine disinfection treatments. This study indicated that rotaviruses have a higher infectivity, gene and antigencity resistance to chlorine than that previously indicated by plaque assay only, which seemed to underestimate the resistance of rotaviruses to chlorine and the risk of rotaviruses in environments. Present results also suggested that re-evaluation of resistance of other waterborne viruses after disinfections by more sensitive infectivity detection method (such as ICC-RT-qPCR) may be necessary, to determine the adequate disinfectant doses required for the inactivation of waterborne viruses.

Key words: rotaviruses; free chlorine disinfection; infectivity; genes; antigenicity; ICC-RT-qPCR

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Introduction

Rotaviruses are major agents of epidemic gastroenteritis for young children and animals, and there are about 111 million episodes of gastroenteritis and 352,000–592,000 deaths in children of less than five years old worldwide each year (Parashar et al., 2003). China has the second largest number of rotaviruses incidents with about 35,000 deaths per year (Parashar et al., 2003; Orenstein et al., 2007). Many researchers have proven that water is one of the most important routes of rotaviruses transmission (Gerba et al., 1996; Parashar et al., 2003; Espinosa et al., 2008). Chlorination is the most commonly used method for water disinfection worldwide and currently, however, there are only a few studies on the inactivation and persistence of waterborne rotaviruses to chlorination and the results are not always consistent (American Water Works Association, 2000; White, 1998; Espinosa et al., 2008).

The observed inconsistency of those results is partially due to the differences in experimental conditions, such as the preparation procedure for viruses seeded in samples, the pH and temperature of water samples, as well as the organic matter in water samples (Barbeau et al., 2005; Casteel et al., 2008; Shin and Sobsey, 2003; Page et al., 2009). Another important factor that contributes to the inconsistency of the previous results is related to the limitations of the virus detection method applied in those studies (Blackmer et al., 2000; Ko et al., 2005; Li et al., 2009).

When evaluating the efficiencies of disinfection, it is important to assess the infectivity of viruses, namely the ability of viruses to reproduce after disinfection (Gerba et al., 2002; Ko et al., 2005; Li et al., 2009). Most previous studies relied on the observation of cytopathogenic effects (CPE) produced by infected cells to quantify virus infectivity and chlorine disinfection efficiency. The plaque assay usually requires several days even more than one week to obtain clear CPE and the inoculated host cells

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Table 1 Water quality parameters of water samples used for rotaviruses inactivation study

	pH	Chemical oxygen demand (mg/L)	Total nitrogen (TN) (mg/L)	Total phosphorus (TP) (mg/L)	Turbidity (NTU)
Tap water	7.3	3.3	0	0	0
Secondary effluents	7.1	44.3	36.2	4.3	2.3

often deteriorate before the appearance of distinctive CPE due to the toxic disinfection by products (DBPs) produced, which makes it difficult to obtain reliable and reproducible data on response to chlorine disinfection (Min et al., 2006; Smith et al., 1979).

In addition, detection methods applied should be rapid to afford timely information. Quantitative RT-PCR has emerged as a rapid method for the detection and quantification of rotavirus in environmental samples (Kittigul et al., 2005; Min et al., 2006). However, it detects both viable and nonviable particles including the naked genome released in water samples and non-infectious single shelled particles, thus it only indicates the presence of nucleic acid and does not necessarily reveal the infectivity of viruses (Ko et al., 2005). To overcome these limitations, we have developed and applied an integrated cell culture and quantitative RT-PCR (ICC-RT-qPCR) assay to detect and quantify infective rotaviruses, which employs quantitative RT-PCR to detect RNA from infected host cells produced by infectious viruses during cell culturing (Li et al., 2009). This method does not depend on visual scoring of CPE but rather on a quantitative molecular detection of specific virus RNA in infected host cells, facilitating more rapid, sensitive and reliable detection of infectious viruses (Ko et al., 2003; Li et al., 2009).

It is well established that the free chlorine species can react with various amino acids in the virus capsid proteins as well as with the nucleic acid protected by the capsid (Shannon et al., 2008). However, the actual limiting step (that is, the molecular target and its level of damage) responsible for inactivation is not yet known. Direct PCR can detect the viral gene reduction and the enzyme-linked immunosorbent assay (ELISA) can evaluate the viral capsid antigencity during chlorine disinfection.

The objectives of this study are to apply the newly developed ICC-RT-qPCR method to investigate the resistance of rotaviruses to chlorine disinfection in both tap water and typical secondary effluents, and to compare the results with those by employing other established conventional and molecular methods for virus detection including plaque assay, direct RT-qPCR, and ELISA. In addition to virus inactivation, destruction of virus gene and antigencity were also evaluated during free chlorine disinfection process.

1 Materials and methods

1.1 Host cells and virus strains

The MA-104 cells (ATCC number: CRL-2378.1™) and simian rotavirus strains (SA11) (ATCC number: VR-1565™) were generously donated by Prof. Hong Meng of the Medicine Academy in Shandong Province, China. The MA-104 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM)-high glucose (Hylcone, Logan, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hylcone, Logan, USA), 1.7 g/L sodium bicarbonate. The SA11 strains were propagated in MA-104 cells for 7–8 days, with the maintenance medium supplemented with 2% fetal bovine serum (Jean et al., 2002). Then the rotavirus SA11 were collected from infected cell culture suspensions, which was frozen and thawed three times followed by centrifugation (900 ×g) to remove residual cell debris. The suspensions were divided into aliquots and stored at –80°C.

Table 2 Initial chlorine dose and residual free chlorine concentrations in inactivation treatment water samples after 60 min contact time*

Initial free chlorine dose concentration (mg/L)	Residual free chlorine (mg/L)	
	Tap water	Secondary effluents
1	0.61	0. 13
5	3.32	1.12
10	7.93	4.79
20	14.94	8.21

* pH of water samples was about 6.5–6.9.

1.2 Chlorine disinfection

The inactivation efficiency and potential resistance of rotaviruses to chlorine disinfection were evaluated in both tap water and secondary effluent matrix. The secondary effluent before the disinfection process was obtained from Qinghe Wastewater Treatment Plant (WWTP), Beijing, China and the water quality parameters are shown in Table 1.

Free chlorine (5% sodium hypochlorite; Sigma, Japan) stock solution (100 mg/L) was prepared in chlorine-demand free (CDF) water. The free chlorine concentration was measured by the N,N-diethyl-*p*-phenylenediamine (DPD) colorimetric method (American Public Health Association, 1985). The rotavirus SA11 were seeded into each virus control glass flask containing 50 mL of phosphate buffer solution (PBS, pH 7.2), and a series of disinfection reaction glass flasks containing 50 mL of water sample with a final concentration of about 4×10^3 plaque forming units per mL (PFU/mL), and the water samples were added with different free chlorine concentrations at 1, 5, 10 and 20 mg/L, respectively. A contact time of 60 min was chosen because the median contact time for chlorine disinfection in most water treatment plants is 60 min (American Water Works Association, 2000; White, 1998). Samples (1 mL) were then taken after 60 min contact time followed by immediate dechlorination with 0.1% (W/V) sterile sodium thiosulfate addition and subject to different virus detection assays as described in Section 2.3. The residual free chlorine at the end of the test (60 min) in each test was also measured and the results were summarized in Table 2.

1.3 Rotavirus detection assays

1.3.1 Plaque assay

A modified plaque assay based on MA-104 cytopathogenic effects (CPE) (Smith et al., 1979; Hansen et al., 2007) has been developed in our lab and was reported previously (Li et al., 2009). Briefly, serial dilutions of SA11 were treated with trypsin and inoculated onto confluent cells in 24-well plates and incubated for 120 min, with gentle rocking every 20 min for viral adsorption. And 2 mL of maintenance medium consisting of DMEM with 2% fetal bovine serum (FBS) was added and incubated for 4 days. Then the infected cells were overlaid with 2% agar, containing nutrients, trypsin and antibiotics. Plates were incubated for 3 days and then 2 mL of 10% formaldehyde in normal saline solution was added to each well. After 12 hours incubation, solid overlay was removed from wells by rinsing under warm tap water and 2 mL of a 0.1% Crystal Violet solution was added to each well to permit visualization of plaques. Cytopathogenic plaques were quantified and results from duplicate flasks were averaged to calculate a titer (PFU/mL).

1.3.2 RT-qPCR assay for quantification of virus genes

Two step RT-qPCR was carried out to quantify the genome segments of rotavirus after free chlorine disinfection. For RT-qPCR, the rotavirus genes was extracted from 100 μL of the chlorine treated samples using QIAamp UltraSens Virus Kit (Catalog number: 53706, Qiagen, Germany). RT-qPCR protocols and primers were essentially the same as the previously reported (Hu et al., 2008; Li et al., 2009). The primer VP7-F (5'-CCTCACTTATACACTTTGCC-3') was used as the forward primer and the primer VP7-R (5'-TTCGCTTCGTCAGTTTGCT-3') was used as the reversed primer, which targets the VP7 segments. Reverse transcription (RT) reaction was performed in 10 μL reaction mixtures using ExScript™ RT reagent Kit (TaKaRa, Cat.: DRR041, Dalian, China). qPCR was carried out in 25 μL reaction mixtures consisting of 12.5 μL of 2× SYBR Premix Ex Taq™ (TaKaRa Cat.: DRR037A, Dalian, China), 0.25 μL of each primer (20 μmol/L final concentration), 2 μL of cDNA template, and 10 μL of dH_2O. The thermocycling profile for rotavirus includes 95°C for 10 sec, then followed by 40 cycles of 95°C for 5 sec, 59°C for 20 sec, and 72°C for 30 sec, and at last, the melting curve analysis at 95°C for 15 sec, annealing at 60°C for 1 min. The serially diluted VP7-plasmid was used to establish the standard curve (Hu et al., 2008), and the ddH_2O was used as the negative control.

1.3.3 ICC-RT-qPCR assay for quantification of infectious rotavirus

Details of the ICC-RT-qPCR protocols and primers were described in our previous studies (Li et al., 2010). Briefly, serial dilutions of SA11 subjected to chlorination were treated with trypsin and inoculated onto confluent cells in cell culture plates (10 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 culturing at 37°C. After 2-day incubation, the viral RNA was extracted from the cell monolayer with Trizol (Invitrogen, Carlsbad, USA), and subjected to reverse transcription immediately. The reverse transcription reaction was performed in 10 μL volumes using ExScript™ RT reagent Kit (TaKaRa, Cat.: DRR041, Dalian, China). qPCR was performed in 25 μL volumes containing 2 μL of the cDNA as the template. An incubation time of 2 days was selected because it allowed for detection of rotavirus at concentration as low as 2×10^{-1} PFU per cell culture, which is sensitive enough for this study (Li et al., 2010).

1.3.4 ELISA assay for detection of rotaviral antigencity

To quantify antigencity of rotavirus, an ELISA kit was used according to the manufacturer's instruction (Rotavirus ELISA Kit, Lanzhou Institute of Biological, China). The ELISA kits contained anti-rotavirus antibodies containing a human polyclonal anti-rotavirus serum collected after natural infection and a rabbit monoclonal anti-rotavirus antibody. Briefly, a 96-well micro titer plate coated with rabbit polyclonal anti-rotavirus antibody was used for the assay. The 50 μL of each sample (containing rotavirus) was added into each well, supplemented with 50 μL of horseradish peroxidase-labeled anti-rotavirus antibody, and incubated for 30 min at 37°C with constant horizontal rocking (60 r/min). After removing the solution and washing the plate, the reagent A (tetramethylbenzidine, TMB) and B (peroxide) provided in the kit were added into each well, and mixed thoroughly. The plate was placed in the dark at 37°C for 10 min before added with 50 μL/well of stopping buffer to terminate the reaction. The absorbance at 450 nm was read immediately using a SpectraMax M_5 microplate reader (Molecular Devices Co., California, USA) and corrected with blank well 96-well plate as the background control. The data were analyzed according to the manufacturer's instructions. P is the OD_{450} value of the samples, and N is the OD_{450} values of negative control samples. The sample is considered positive if the P/N ratio is above 2.1 and otherwise as negative.

1.3.5 Data analysis

The virus inactivation (I) efficiency by chlorination evaluated by plaque assay, RT-qPCR and ICC-RT-qPCR methods, respectively, was determined using the following Eq. (1):

$$\log I = \log \frac{N_0}{N_t} \tag{1}$$

where, N_0 (copies/mL) is the concentration of rotaviruses in control glass flask with no chlorine exposure; N_t (copies/mL) is the concentration of rotaviruses in reaction glass flasks after exposure to free chlorine disinfectant at time t (min).

The data presented was average of results from at least two independent experiments and the inactivation curve was obtained by plotting the $\log_{10}(N_0/N_t)$. The antigencity reductions (R) evaluated by ELISA were calculated by

Eq. (2):

$$R = \frac{P_t - P_0}{N_t} \quad (2)$$

where, P_0 is the OD_{450} value of the samples in control glass flask, P_t is the OD_{450} value of the samples in reaction glass flasks after exposure to free chlorine disinfectant at time t (min), N is the OD_{450} values of negative control samples.

2 Results

2.1 Inactivation of rotaviruses by chlorine disinfection evaluated by plaque assay and ICC-RT-qPCR

Inactivation efficiency and resistance to chlorine disinfection was evaluated by exposing SA11 (with initial concentration of 4×10^3 PFU/mL) to free chlorine for 60 min and at varying concentrations of 1, 5, 10 and 20 mg/L, respectively. The inactivation was assessed simultaneously by both plaque assay and ICC-RT-qPCR method, and in both tap water and secondary effluents, respectively. The maximum reduction detected by plaque assay was about 3.6 $\log_{10}$ due to the detect limits of plaque assay (about 2 PFU/mL examined in our previous studies). As shown in Fig. 1, based on the plaque assay, no infectious rotaviruses residual could be detected when chlorine dose was higher than 5 mg/L in tap water or higher than 10 mg/L in secondary effluents with exposure time of 60 min. In comparison, however, for both tap water and secondary effluent samples, ICC-RT-qPCR method detected more residual SA11 than those determined by plaque assay after the same chlorine treatments, resulting in more chlorine dose required than those evaluated by plaque assay. For example, the ICC-RT-qPCR results suggested that to achieve 1.8 $\log_{10}$ reductions in tap water, a chlorine dose of 5 folds higher than that indicated by the plaque assay (5 mg/L versus 1 mg/L, with 60 min exposure time) is required. Furthermore, the higher sensitivity of ICC-RT-qPCR method allowed for chlorine inactivation assessment of SA11 with much wider chlorine dose range (0–20 mg/L) than those by the traditional plaque assay (chlorine dose of 0–10 mg/L).

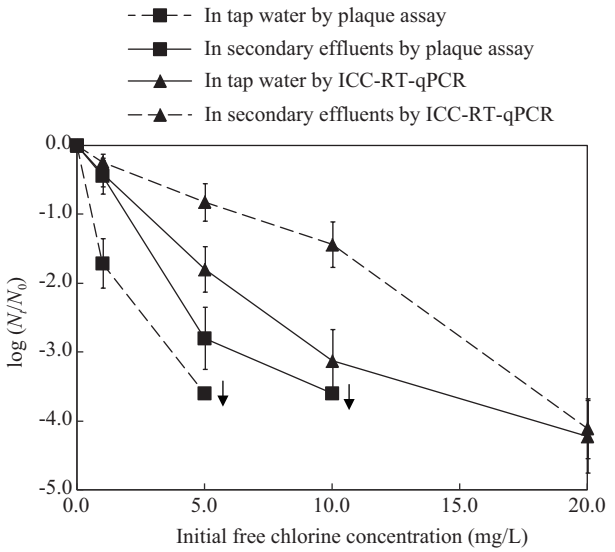

Fig. 1 Inactivation curves of rotaviruses by chlorine disinfection evaluated by plaque assay and ICC-RT-qPCR assay, either in tap water and secondary effluent matrix, with 60 min exposure time. The arrows indicate detection limits of plaque assay, which had maximum reduction of 3.6 $\log_{10}$ with initial concentration of 4×10^3 PFU/mL rotavirus.

Table 3 Comparison of rotaviruses inactivation results between ICC-RT-qPCR method and two-passage cell culture plaque assays (60 min contact time)

Chlorine dose (mg/L)	ICC-RT-qPCR assay	Plaque assay	
		First passage (7 days)	Second passage (14 days)
1	+	+	+
5	+	+	+
10	+	–	+/–
20	+/–	–	+/–

The samples were inculcated into host cells for 7 days with the maintenance medium supplemented with 2% fetal bovine serum. Then the cell culture suspensions were collected and frozen and thawed three times, and then inculcated into fresh host cell for 7 days again.
+: infectious rotavirus was found in all three cell culture plates; –: infectious rotavirus was not found in all three cell culture plates; +/–: infectious rotavirus was found at least in one cell culture plate.

To verify the results of ICC-RT-qPCR (with 2-day incubation), CPE assay with two passages of continuous cell culturing was also applied to evaluate the inactivation of rotaviruses by chlorine disinfections. As shown in Table 3, second passage cell culturing for additional 7 days confirmed that the treatment with chlorine dose of 20 mg/L, which was detected partially positive with ICC-RT-PCR method, indeed had rotavirus present and was cytopathogenic. Those results demonstrated that ICC-RT-qPCR-method with only 2 days of incubation in cell culturing can detect virus level that would otherwise require up to 14 or more days conventional cell culturing by observing CPE. Therefore, ICC-RT-PCR method is faster and has lower detection limit than CPE method.

2.2 Gene persistence of rotaviruses after chlorine disinfection

The persistence of virus genes was evaluated through the detection of a fragment of rotavirus VP7 gene by direct RT-qPCR. The results showed that VP7 gene was very resistant to chlorine disinfection, and was detectable even at the chlorine dose of 20 mg/L in both tap water and secondary effluents. The reductions of rotavirus genes as well as the infectivity inactivation in either tap water or secondary effluents were also compared. As shown in Fig. 2, the reductions of rotaviral VP7 gene segments quantified by RT-qPCR were less than the virus quantity and infectivity detected by ICC-RT-qPCR. For example, the reductions of VP7 segments at chlorine dose of 10 mg/L determined by RT-qPCR were 1.7, and 0.7 $\log_{10}$ in tap water and secondary effluents respectively, while the infectivity reductions evaluated by ICC-RT-qPCR were 3.1 and 1.4 $\log_{10}$, respectively (Fig. 2).

2.3 Antigenicity persistence of rotaviruses after chlorine disinfection

In present study, a commercial ELISA kit (Bluegene, Shanghai, China) was used to detect the antigencity of

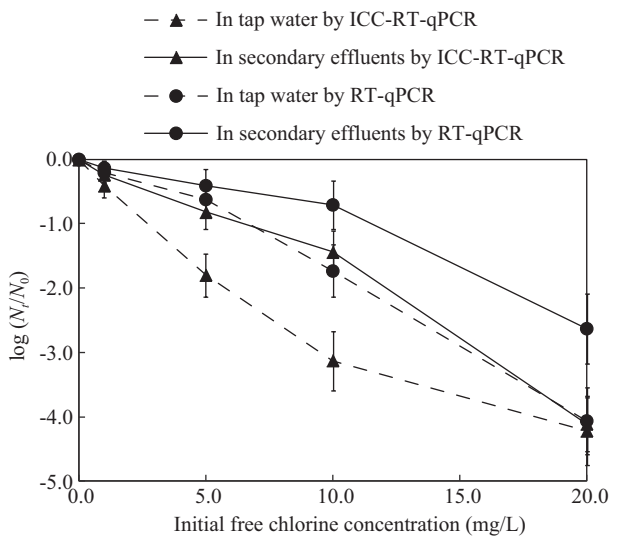

Fig. 2 Reductions of rotavirus gene (VP7 gene segment, indicative of infectivity) by chlorine disinfection with 60 min exposure, determined by both direct RT-qPCR and ICC-RT-qPCR methods, in either tap water or secondary effluents.

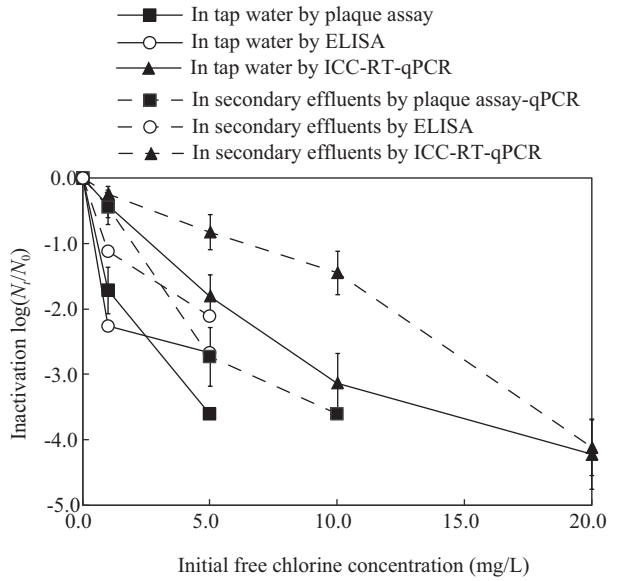

Fig. 3 Reductions of rotaviruses antigencity (ELISA) and infectivity by chlorine disinfection with 60 min exposure in either tap water or secondary effluents.

rotavirus. The average value of P_0/N is 4.9 with initial rotavirus concentration of 4×10^3 PFU/mL, 2.2 with 4×10^2 PFU/mL rotavirus. As sample with P/N value below 2.1 is considered negative according to the instruction of manufacturer, and therefore the detect limit of the ELISA methods applied is about 4×10^2 PFU/mL rotaviruses and maximum antigencity reductions was about 2.7 in the present study. As shown in Fig. 3, the antigencity of rotavirus was undetectable with chlorine dose more than 5 mg/L, while there still existed infectious SA11 detected by both plaque assay and ICC-RT-qPCR. The reductions of antigencity in tap water were similar to that in secondary effluents, which reduced fast at first, and had a tailing with chlorine dose increasing from 1 to 5 mg/L mostly likely due to the higher detection limit of ELISA.

2.4 Water quality largely affected the inactivation efficiencies

Comparing the disinfection efficiencies of infectivity, gene and antigencity of rotaviruses, less reduction was achieved with spiked secondary effluent samples than that in tap water with the same chlorine treatments (Figs. 1, 2 and 3). For example, when rotaviruses were treated with 10 mg/L free chlorine with 60 min contact, there were 3.2 $\log_{10}$ reductions in tap water, however, there was only 1.4 $\log_{10}$ reductions in secondary effluents (evaluated by ICC-RT-qPCR assay); the VP7 gene reductions were 1.6 $\log_{10}$ in tap water, while only 0.7 $\log_{10}$ in secondary effluents.

3 Discussion

Disinfection with chlorine has been adopted worldwide to ensure the safety of drinking water and wastewater effluents. This study represents an attempt to evaluate the inactivation/reductions of rotaviruses by various methods in tap water and secondary wastewater effluents disinfected by free chlorine.

The more sensitive infectivity detection method (ICC-RT-qPCR) was employed to evaluate the inactivation of rotaviruses, and the results were compared and confirmed with the results determined by conventional plaque assay based on CPE. ICC-RT-qPCR method detects the infectious rotaviral-specific RNA replicated in host cells after 2-day incubation, while the plaque assay counts the plaque produced in host cells for more than 1 week incubation. Usually, the inhibitors in water samples such as the disinfection by-products (DBPs) after free chlorine disinfection may exhibit toxicity to host cells, making cells cytoclasis before forming observed CPE (Venkobachar et al., 1977; Nishikiori et al., 2008). As a result, the plaque assay based on visible CPE would yield false-negative results and lead to higher log reductions than what would be detected with ICC-RT-qPCR assay.

The higher sensitive ICC-RT-qPCR showed that rotaviruses were very resistant to chlorine disinfection and can survival at the chlorine dose of 10 and 20 mg/L with 60 min contact, when the traditional plaque assay detected on infectious rotaviruses. The two passages cell culturing results confirmed the results of ICC-RT-qPCR assay. Similar results were also reported by Blackmer et al. (2000), in which chlorine disinfection efficiency for poliovirus evaluated by ICC-RT-PCR indicated a required contact time that was 5-fold longer than that determined by detecting visible CPE. These results indicated that previous studies using plaque assay only may have underestimated the resistance of enteric viruses to chlorine disinfection, and highlight the necessity to re-evaluation the chlorine dose requirement using more sensitive methods.

Research on the mechanisms of chlorine inactivation of rotaviruses has been inadequate (Shannon et al., 2008). With the development of molecular biology methods, PCR-based methods have been used to evaluate the effects of virus disinfection and to research disinfection mechanisms. However, direct RT-qPCR method detects

both virus genes associated with infectious virus, as well as those naked genomes released from virus. The results in the present work showed that VP7 gene was more resistant than infectivity detected by ICC-RT-qPCR to chlorine disinfection (Fig. 2). Shin and Sobsey (2003) also reported that although the infectivity of poliovirus cannot be tested by cell culture method, its nucleic acid can still be revealed by RT-PCR when the virus is treated with free chlorine. These results also indicate that the molecular virus detection methods that capture molecular marker (e.g., genome) only, such as direct RT-PCR, may underestimate the disinfection efficiency due to its inability to differentiate the gnome of infectious virus from those residual released from viruses. The main reason to those results is that the length of the virus nucleic acid segment detected is limited and fails to reflect the overall status of viral nucleic acid. More powerful methods such as gene chip are recommended to evaluate the whole gene change during disinfection.

The protein of the viruses serves some crucial functions, including RNase protection, attachment to host cell receptors (as part of the entry process), and also interaction with the host cellular immune system (Flint, 2000; Nuanualsuwan and Cliver, 2003; Shannon et al., 2008). The infectivity of viruses requires the functional integrity of both the viral RNA and protein. Even though the antigenicity of a virus is not an infectivity determinant, changes in antigenic function may provide insight into the protein conformational changes that accompany inactivation (Nuanualsuwan and Cliver, 2003; Li et al., 2002). ELISA has been adopted to evaluate the antigencity of virus after inactivation due to its fast, specific, and simple assay procedure (Wang et al., 1995; Li et al., 2002). The higher detection limit of ELISA than ICC-RT-qPCR and RT-qPCR methods reveal different inactivation efficiencies with varying chlorine ranges, highlighting the importance of detection methods for evaluating disinfection phenomena. Although the extent of protein (the antigencity) damage did not seem to be affected noticeably by the matrix of effluents comparing to the clean tap water, the infectivity reduction of virus in effluents was much less than that in tap water at low chlorine dose (< 1 mg/L) with exposing 60 min due to the "shielding" effects brought upon by the background matrix.

The water quality impacts the disinfection efficiencies during chlorine disinfection. The reduced disinfection inactivation in secondary effluents may be due to several reasons such as free chlorine consumption due to the instant chlorine demand created by oxidisable compounds (Winward et al., 2008; Page et al., 2009), and the shielding of virus by particles in secondary effluents (Winward et al., 2008). The residual free chlorine in secondary effluents was less than those in tap water, indicating presence of chlorine-consuming reduced compounds in the secondary effluents (Table 2). Previous study showed that organic concentration in water samples did not affect resistance of coliform bacteria to chlorine disinfection at a fixed free chlorine residual, however, the particles in grey water harbored particle associated coliforms, which were highly resistant to chlorine disinfection (Winward et al., 2008). Therefore, the turbidity in secondary effluent might contributed to the persistence of virus.

The pH, temperature of water samples also has impacts on the efficacy of free chlorine disinfection (Barbeau et al., 2005; Page et al., 2009; Vaughn et al., 1986; Winward et al., 2008). In this study, we did not particularly evaluate the impact of pH and temperature on the disinfection efficacy of SA11 because it is beyond the scope of this investigation. In our experiments, no pH adjustment was made to either tap water or secondary water after adding chlorine and the pH was about 6.5–6.7, which is similar to typically pH condition in drinking and secondary effluents chlorine disinfection process in practice. The experimental temperature was 4°C, which was similar to many previous bench-scale studies conducted by other researches (Vaughn et al., 1986; Shin and Sobsey, 2003).

In the United States, the reported average free chlorine residual used in drinking water treatment is approximately 1.0 mg/L, and the median contact time is 60 min (American Water Works Association, 2000; White, 1998). Four logs (99.99%) reduction of enteric virus must be removed or inactivated from surface water or groundwater under the direct influence of surface water by filtration and disinfection, or a combination of these technologies according to the National Primary Drinking Water Standards (U.S. Environmental Protection Agency, 2001). As shown in Fig. 2, there was only 0.2 and 0.4 $\log_{10}$ reduction under this chlorine condition in tap water and secondary effluents, which were far below the requirements of water standards. The results of this study suggest that current chlorine dose was inadequate to effectively inactive rotaviruses. This may explain why infectious viruses have been detected in drinking water and treated wastewater even after "considered" adequate disinfection (Brassard et al., 2005; Gratacap-Cavallier et al., 2000).

4 Conclusions

In this study, inactivation of rotaviruses by chlorine disinfection in typical tap water and secondary effluents condition was characterized by plaque assay based on CPE, ICC-RT-qPCR, direct RT-qPCR and ELISA assays, respectively. The results showed that ICC-RT-qPCR assay can reveal more detailed assessment of virus inactivation and resistance to free chlorine than that of plaque assay. The results indicated that rotaviruses have a higher infectivity, and gene resistance to free chlorine disinfection that previously indicated by plaque assay, the later seemed to underestimate the resistance of rotaviruses to chlorine disinfection, and the risk of rotavirus in environments. The water quality largely impacts the efficiency of disinfection, and rotaviruses have a relatively higher resistant in secondary effluents than in the tap water under the same chlorine dose and exposure. Current chlorine disinfection condition cannot inactive rotaviruses adequately according to US EPA water standards. Our results suggest that re-evaluation of resistance of other waterborne viruses after free chlorine and other disinfections may be necessary.

using more sensitive infectivity detection method (such as ICC-RT-qPCR), to determine the appropriate disinfectant doses required for the inactivation of waterborne viruses.

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References

American Public Health Association, 1985. Standard Methods for the Examination of Water and Wastewater (17th ed.). American Public Health Association, Washington, DC.

American Water Works Association, 2000. Committee report: disinfection at large and medium-sized systems. *Journal of the American Water Works Association*, 92(5): 32–43.

Barbeau B, Desjardins R, Mysore C, Prévost M, 2005. Impacts of water quality on chlorine and chlorine dioxide efficacy in natural waters. *Water Research*, 39(10): 2024–2033.

Blackmer F, Reynolds K A, Gerba C P, Pepper I L, 2000. Use of integrated cell culture-PCR to evaluate the effectiveness of poliovirus inactivation by chlorine. *Applied and Environmental Microbiology*, 66(5): 2267–2268.

Brassard J, Seyer K, Houde A, Simard C, Trottier Y L, 2005. Concentration and detection of hepatitis A virus and rotavirus in spring water samples by reverse transcription-PCR. *Journal of Virological Methods*, 123(2): 163–169.

Casteel M J, Schmidt C E, Sobsey M D, 2008. Chlorine disinfection of produce to inactivate hepatitis A virus and coliphage MS2. *International Journal of Food Microbiology*, 125(3): 267–273.

Espinosa A C, Mazari-Hiriart M, Espinosa R, Maruri-Avidal L, Méndez E, Arias C F, 2008. Infectivity and genome persistence of rotavirus and astrovirus in groundwater and surface water. *Water Research*, 42(10-11): 2618–2628.

Flint S J, 2000. Principles of Virology: Molecular Biology, Pathogenesis, and Control. ASM Press, Washington, DC.

Gerba C P, Gramos D M, Nwachuku N, 2002. Comparative inactivation of enteroviruses and adenovirus 2 by UV light. *Applied and Environmental Microbiology*, 68(10): 5167–5169.

Gerba C P, Rose J B, Haas C N, Crabtree K D, 1996. Waterborne rotavirus: a risk assessment. *Water Research*, 30(12): 2929–2940.

Gratacap-Cavallier B, Genoulaz O, Brengel-Pesce K, Soule H, Innocenti-Francillard P, Bost M et al., 2000. Detection of human and animal rotavirus sequences in drinking water. *Applied and Environmental Microbiology*, 66(6): 2690–2692.

Hansen J J, Warden P S, Margolin A B, 2007. Inactivation of adenovirus type 5, rotavirus WA and male specific coliphage (MS2) in biosolids by lime stabilization. *International Journal of Environmental Research and Public Health*, 4(1): 61–67.

Hu X H, He M, Liu L, Li D, Shi H C, 2008. Construction of external standard for detection of rotavirus in water using the quantitative real-time polymerase chain reaction. *Environmental Science*, 29(2): 380–385.

Jean J, Blais B, Darveau A, Fliss I, 2002. Rapid detection of human rotavirus using colorimetric nucleic acid sequence-based amplification (NASBA)-enzyme-linked immunosorbent assay in sewage treatment effluent. *FEMS Microbiology Letters*, 210(1): 143–147.

Kittigul L, Ekchaloemkiet S, Utrarachkij F, Siripanichgon K, Sujirarat D, Pungchitton S et al., 2005. An efficient virus concentration method and RT-nested PCR for detection of rotaviruses in environmental water samples. *Journal of Virological Methods*, 124(1-2): 117–122.

Ko G, Cromeans T L, Sobsey M D, 2003. Detection of infectious adenovirus in cell culture by mRNA reverse transcription-PCR. *Applied and Environmental Microbiology*, 69(12): 7377–7384.

Ko G, Cromeans T L, Sobsey M D, 2005. UV inactivation of adenovirus type 41 measured by cell culture mRNA RT-PCR. *Water Research*, 39(15): 3643–3649.

Li D, Gu A Z, He M, Shi H C, Yang W, 2009. UV inactivation and resistance of rotavirus evaluated by integrated cell culture and real-time RT-PCR assay. *Water Research*, 43(13): 3261–3269.

Li D, Gu A Z, He M, Yang W, Hu X H, Shi H C, 2010. An integrated cell culture and reverse transcription quantitative PCR assay for detection of infectious rotaviruses in environmental waters. *Journal of Microbiological Methods*, 82(1): 59–63.

Li J W, Xin Z T, Wang X W, Zheng J L, Chao F H, 2002. Mechanisms of inactivation of hepatitis A virus by chlorine. *Applied and Environmental Microbiology*, 68(10): 4951–4955.

Min B S, Noh Y J, Shin J H, Baek SY, Min K I, Ryu S R et al., 2006. Assessment of the quantitative real-time polymerase chain reaction using a cDNA standard for human group A rotavirus. *Journal of Virological Methods*, 137(2): 280–286.

Nishikiori R, Nomura Y, Sawajiri M, Masuki K, Hirata I, Okazaki M, 2008. Influence of chlorine dioxide on cell death and cell cycle of human gingival fibroblasts. *Journal of Dentistry*, 36(12): 993–998.

Nuanualsuwan S, Cliver D O, 2003. Capsid functions of inactivated human picornaviruses and feline calicivirus. *Applied and Environmental Microbiology*, 69(1): 350–357.

Orenstein E W, Fang Z Y, Xu J, Liu C Y, Shen K L, Qian Y et al., 2007. The epidemiology and burden of rotavirus in China: A review of the literature from 1983 to 2005. *Vaccine*, 25(3): 406–413.

Page M A, Shisler J L, Mariñas B J, 2009. Kinetics of adenovirus type 2 inactivation with free chlorine. *Water Research*, 43(11): 2916–2926.

Parashar U D, Hummelman E G, Bresee J S, Miller M A, Glass R I, 2003. Global illness and deaths caused by rotavirus disease in children. *Emerging Infectious Diseases*, 9(5): 565–572.

Shannon M A, Bohn P W, Elimelech M, Georgiadis J G, Mariñas B J, Mayes A M, 2008. Science and technology for water purification in the coming decades. *Nature*, 452(7185): 301–310.

Shin G A, Sobsey M D, 2003. Reduction of Norwalk virus, poliovirus 1, and bacteriophage MS2 by ozone disinfection of water. *Applied and Environmental Microbiology*, 69(7): 3975–3978.

Smith E M, Estes M K, Graham D Y, Gerba C P, 1979. A plaque-assay for the simian rotavirus SAII. *The Journal of General Virology*, 43(3): 513–519.

U.S. Environmental Protection Agency, 2001. National primary drinking water standards. EPA816.-F-01–007. Office of Water, U.S. Environmental Protection Agency, Washington, DC.

Vaughn J M, Chen Y S, Thomas M Z, 1986. Inactivation of human and simian rotaviruses by chlorine. *Applied and Environmental Microbiology*, 51(2): 391–394.

Venkobachar C, Iyengar L, Prabhakara Rao A V S, 1977. Mechanism of disinfection: effect of chlorine on cell-membrane functions. *Water Research*, 11(8): 727–729.

Wang F, Liang Z, Yang Z, 1995. Comparative study of chlorine on the HAV antigen and infectivity. *China Journal of Disinfection*, 12: 94–96.

White G C, 1998. Handbook of Chlorination and Alternative Disinfectants (4th ed.). John Wiley & Sons, Inc., New York.

Winward G P, Avery L M, Stephenson T, Jefferson B, 2008. Chlorine disinfection of grey water for reuse: effect of organics and particles. *Water Research*, 42(1-2): 483–491.