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UV inactivation and resistance of rotavirus evaluated by integrated cell culture and real-time RT-PCR assay

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ABSTRACT

Rotaviruses are double-stranded RNA viruses which are among the most resistant waterborne enteric viruses to UV disinfection. An integrated cell culture and real-time RT-PCR (ICC real-time RT-PCR) assay was developed to detect the infectivity of rotaviruses in water, which uses real-time RT-PCR to detect RNA produced by infectious rotaviruses during replication in host cells. Detection of rotaviral RNA in host cells provides direct evidence of the presence of infectious rotavirus rather than just the presence of rotavirus RNA. Using this newly developed method, the inactivation and resistance of rotavirus to UV treatments at various doses was evaluated. With an initial concentration of 2×10^4 PFU/ml simian rotavirus (SA11), a first-order linear relationship was obtained at UV dose range of 0–120 mJ cm^{-2}, and the inactivation rate constant was estimated to be 0.0343 cm^2 mJ^{-1} ($R^2 = 0.966$). The dose-inactivation curve tailed off and reached plateau as the UV dose increased from 120 to 360 mJ cm^{-2}, indicating resistance phenomena of sub-populations of SA11 at very high UV doses. A maximal reduction of 4.8 $\log_{10}$ was observed. Through parallel comparison with traditional culture assay, the ICC real-time RT-PCR method demonstrated more effective, sensitive and faster infectivity detection of rotavirus and, the results reveal that rotaviruses are more resistant to UV irradiation than previously reported with traditional cell culture assays.

1. Introduction

Rotavirus is the most common cause of acute diarrhea in infants and young children, which causes approximately 111 million episodes of gastroenteritis and 352,000–592,000 deaths in children of less than 5-years-old worldwide each year (Parashar et al., 2003). China is estimated to have the second largest number of rotavirus deaths of about 35,000 deaths per year (Orenstein et al., 2006). Rotaviruses have been detected in wastewater (Gajardo et al., 1995), river water and even in drinking water (Gratacap-Cavallier et al., 2000). The outbreaks of rotavirus diarrhea are usually caused directly or indirectly by contaminated water. Therefore, rotavirus transmission via aquatic environment has been recognized as a major health problem worldwide. In order to effectively remove and inactivate rotaviruses, water treatment plants are required to provide multiple barrier controls of rotavirus including physical removal and disinfection. UV disinfection has gained more interest in the water industry since it has been demonstrated to be very effective against (oo)cysts of Cryptosporidium and Giardia, both of which are highly resistant to chlorination (Hijnen et al., 2006). In addition, unlike chemical

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disinfectants, UV disinfections produce much less toxic disinfection by-products (DBPs) (Haider et al., 2002). UV irradiation damages the nucleic acids (DNA/RNA) of the cell or virus, primarily through the formation of pyrimidine dimers, but also by other photoproducts of nucleic acids and nucleic acid lesions (Hijnen et al., 2006). If the damage is not repaired, the replication and transcription is blocked, leading to the inactivation of microorganisms.

A number of studies have demonstrated that UV disinfection can effectively inactivate most microorganisms including waterborne pathogens, such as enteric bacteria, viruses, bacterial spores and parasite cysts transmitted through water (Duan et al., 2003; Linden et al., 2002; Zimmer and Slawson, 2002). However, there have been only a few studies on UV inactivation of rotaviruses, and most of them were performed using conventional cytopathogenic effects (CPE) method for quantifying rotavirus infectivity and UV inactivation rates (Chang et al., 1985; Hijnen et al., 2006). Due to the fastidiousness of rotaviruses, it usually requires more than one week to obtain clear CPE and the inoculated host cells often deteriorate before the appearance of distinctive CPE, making it difficult to obtain reliable and reproducible data. Recently, real-time RT-PCR has been applied successfully for rapid detection and quantification of pathogenic microorganisms in environmental samples (Mina et al., 2006; Ranheim et al., 2006; Schwarz et al., 2002). One limitation associated with real-time RT-PCR method is that it only indicates the presence or absence of specific nucleic acid and does not reveal the infectivity of microorganisms. It is important to assess infectivity of microorganisms when evaluating the efficacy of disinfection and the assays used to quantify inactivation should also measure the ability of the microorganism to reproduce after disinfection (Gerba et al., 2002). To overcome this limitation, we have employed an integrated cell culture and real-time RT-PCR (ICC real-time RT-PCR) assay to detect and quantify the infective rotaviruses, which employs quantitative real-time RT-PCR to detect RNA from infected host cells produced by infectious viruses during cell culturing. This method does not depend on visual scoring for CPE but rather on a quantitative molecular examination of specific virus RNA in infected host cells, leading to more rapid, sensitive and reliable detection of infectious viruses.

The objective of this study was to apply the newly developed ICC real-time RT-PCR method to examine the inactivation and resistance of rotavirus SA11 to UV treatments and, to compare the results with those obtained by the conventional cell culture method and real-time RT-PCR assay conducted in parallel in this study, as well as with those from previous studies.

2. Materials and methods

2.1. Host cells and virus

The MA-104 cells (ATCC number: CRL-2378.1™) and simian rotavirus strains (SA11) (ATCC number: VR-1565™) were provided by Professor Hong Meng of the Medicine Academe in Shandong Province, China. The MA-104 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM)-high glucose, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1.7 g L^{-1} 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 were frozen and thawed three times and then clarified using low-speed centrifugation (900×*g*) in order to remove residual cell debris. The suspensions were divided into aliquots and were stored at −80 °C.

2.2. Cell culture assay based on CPE

Rotavirus SA11 was enumerated using a modified plaque-forming unit (PFU) method based on MA-104 cytopathogenic effects (CPE) (Smith et al., 1979; Hansen et al., 2007). Mammalian MA-104 cells were grown to confluency with 5% CO_2 in 24-well cell culture plates with DMEM supplemented with 10% FBS. MA-104 cells were thoroughly washed prior to infection with serum-free DMEM. Then serial dilutions of SA11 were made in DMEM and then were treated with 20 U trypsin mL^{-1} for 60 min at 37 °C. MA-104 culture plates that were infected with the serial dilution of SA11 were incubated under 5% CO_2 for 120 min with periodical rocking at 15 min interval for efficient adsorption. Following adsorption, 2 mL of maintenance medium consisting of DMEM with 2% FBS was added and incubated for 4 days. Then an agar overlay maintenance medium consisting of 2 × DMEM supplemented with 1 μg mL^{-1} trypsin and 2% agar was added. Following addition of overlay, the agar was permitted to solidify and the plates were returned to the 5% CO_2 incubator. Plates were incubated for 3 days and then 2 mL of 10% formaldehyde in normal saline solution was added to each well. Then the plates were returned to the CO_2 incubator for 12 h 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. Plaques were quantified and results from duplicate flasks averaged to calculate a titer.

2.3. Real-time RT-PCR assay

2.3.1. VP7-specific primers for real-time RT-PCR assay

The specific primers in our study were designed in the conservative sequence of the VP7 gene of rotavirus SA11, which play an important role in the infection of cells. And many researchers designed primers in this gene (Kittigul et al., 2005). However, most of primers in previous studies were for RT-nested PCR and the amplified products are too long for real-time RT-PCR. The primers in this study were designed using Primer Premier 5.0, and the amplified product was 318 bp in length. The primer VP7-F (sense, 5′-CCTCACTTA TACACTTTGCC-3′) was used as forward primer and the primer VP7-R (antisense, 5′-TTCGCTTCGTCAGTTTGCT-3′) was used as reversed primer. The designed primers were checked against NCBI blast to ensure that they are specific for target organisms.

2.3.2. Preparation of rotavirus VP7 cDNA standard

To prepare the rotavirus cDNA standard for real-time RT-PCR assay, total RNA was extracted from SA11 using the QIAamp® UltraSens™ Virus Kit (Cat.: 53706, QIAGEN, Germany), and then was reverse-transcribed in 10 μL volumes using the ExScript™ RT reagent Kit (TaKaRa, Cat.: DRR041, Dalian). The cDNA was amplified by PCR with the consensus primers VP7-F and VP7-R. The amplified product was separated on a 2% agarose gel, purified with the Gel Extraction Kit (TaKaRa, Cat.: DV810A, Dalian) and cloned into the pCR® 2.1-TOPO vector (Invitrogen,, USA). The VP7 gene sequence in the plasmid was analyzed by digestion with *Eco*RI and subsequent DNA sequencing. Quantification of the cDNA standard was carried out by UV spectrophotometry at 260 nm. The quantity (copies mL^{-1}) of cDNA standard was calculated according to the following formula (18): copies/mL = {$6.022 \times 10^{23} \times [C] \times$ OD260}/{molecular weight}. Final values for absolute levels of viral genome are given as 9×10^{11} copies/μL of the cDNA standard.

2.3.3. Real-time PCR condition

Real-time PCR conditions using a Bio-RAD iCycler instrument to detect rotavirus cDNA were optimized. Each 25 μl reaction contained 2 μl of RV cDNA template, 12.5 μl of 2 × SYBR Premix Ex TaqTM (TaKaRa Cat.: DRR037A, Dalian), 0.25 μl of each primer (20 μM final concentration), and 10 μl of dH_2O. The real-time PCR consists of three consecutive programs that include pre-incubation and denaturation of the cDNA template for 10 s at 95 °C, followed by 40 cycles of PCR-amplification in the quantitative analysis mode: denaturation at 95 °C for 5 s, annealing at 59 °C for 20 s in the single acquisition mode, and amplification at 72 °C for 30 s, and at last, the third program was set to confirm the PCR product identity by one cycle of melting curve analysis at 95 °C for 15 s, annealing at 60 °C for 1 min. In every real-time PCR run, negative (no template) and positive rotavirus controls were processed as a routine quality control of the assay.

2.4. Integrated cell culture and real-time RT-PCR (ICC real-time RT-PCR) assay

MA-104 cells in T25 flasks (25 cm^2) were inoculated with SA11 stocks, which were treated with 20 U trypsin ml^{-1} for 60 min at 37 °C, and then incubated for 2 h at 37 °C, rocking every 20 min for efficient adsorption. Then with the inoculums removed, 4 ml of DMEM containing 2% fetal bovine serum was added to the inoculated cell monolayer for culture at 37 °C. After 2-days incubation for virus replication, the supernatant was discarded and the viral RNA was extracted from the cell monolayer with Trizol (Invitrogen, Carlsbad, CA, USA). The nucleic acid was suspended in 20 μl of RNase-free distilled H_2O and subjected to reverse transcription immediately or stored at −80 °C. The reverse transcription reaction was performed in 10 μL volumes using ExScript™ RT reagent Kit (TaKaRa, Cat.: DRR041, Dalian). Real-time PCR was performed in 25 μL volumes containing 2 μL of the cDNA as the template. The diluted SA11 (2×10^4, 2×10^3, 2×10^2, 2×10^1, 2×10^0, 2×10^{-1} PFU) were incubated per cell culture for 2-day and subjected to real-time RT-PCR, in order to evaluate the relationship of the results of ICC real-time RT-PCR assay to the PFU detected by cell culture assay based on CPE. Two-day incubation time was selected because it allowed for detection of rotavirus at concentration as low as 2×10^{-1} PFU per cell culture, which is desired for this study. In addition, long time incubation would lead to host cells cracking off especially at high level of SA11 incubation, and contamination of bacteria or fungi.

2.5. UV disinfection

A collimated beam UV apparatus containing one 30-W, low-pressure mercury vapor germicidal lamp emitting nearly monochromatic UV irradiation at 254 nm was used. The UV irradiance was measured with a radiometer that had been factory calibrated prior to the study. Approximately 2×10^4 PFU/mL SA-11 were suspended in 10 ml phosphate buffered saline (PBS) with a concentration of approximately 2×10^4 PFU/ml and added to a glass petri dish (r = 3 cm) for different durations of UV irradiation exposure, which were placed on a magnetic stir plate, and continuously stirred. The average irradiance in the mixed suspension was determined mathematically by the Beer–Lambert law over the sample depth, accounting for UV absorbance of the test suspension and incident average irradiance (U.S. Environmental Protection Agency Office of Water, 2006). In our study, the depth of viral suspension was 0.35 cm, and 254 nm UV absorbance of the viral suspension was approximately 0.050/cm. The fluence rate was about 0.40 mW cm^{-2} and the UV fluence was varied by the exposure time. Experiments were performed at room temperature. The UV-disinfected SA11 were evaluated using three different methods in parallel, including conventional cell culture CPE method, ICC real-time RT-PCR and direct real-time RT-PCR method.

2.6. UV dose–response

Microbial UV response is a measurement of the sensitivity of the microorganism to UV light and is unique to each microorganism. The microbial response is calculated using the following equation (U.S. Environmental Protection Agency Office of Water, 2006):

$$\text{Log Inactivation} = \log_{10}\frac{N_0}{N_t}$$

where N_0 is the concentration of infectious microorganisms before exposure to UV light and N_t is the concentration of infectious microorganisms after exposure to UV light.

The correlation of log inactivation and doses usually follows the first-order kinetic model, at least for certain UV dose range (Hijnen et al., 2006) and, the shape of the dose–response curve can reflect the inactivation kinetics and potential resistance phenomena.

2.7. Quality control

Special cautions were made to avoid false-positive results due to cross contamination. The plasmid positive controls, as well

as negative controls, were included in all PCR assays to ensure the validity of the method that there was no carryover contamination.

3. Results and discussion

3.1. Correlation of rotavirus detected with CPE method and ICC real-time RT-PCR assay

A non-linear correlation was observed between the non-inactivated SA11 dose used for inoculation determined by PFU and VP7 copies detected by ICC real-time RT-PCR per cell culture after 2-day incubation (Fig. 1). The diluted SA11 concentrations ranged from 2×10^{-1} to 2×10^{4} PFU were evaluated. The detect limits of the cell culture assay based on CPE was about several PFU per cell culture (25 cm^2 MA-104 cells) which was in agreement with previous study (Chang et al., 1985). In contrast, the ICC real-time RT-PCR can detect as low as 0.2 PFU/per cell culture rotaviruses in water samples after 2-day incubation. No rotavirus specific RNA in host cells was detected when SA11 was inoculated in MA-104 cells at time zero without any incubation.

It is observed that the virus reproduction rate during the 2-day incubation varied in cells detected by ICC real-time RT-PCR. It showed that when the amount of rotavirus was below 2×10^{1} PFU per cell culture, the replication of rotaviral genome was very slow, and it became fast when the amount of rotavirus increased from 2×10^{1} to 2×10^{3} PFU per cell culture; then the replication was inhibited when the amount of rotavirus was more than 2×10^{3} PFU per cell culture. This phenomenon was in agreement with the results reported by others (Xu et al., 1996; Schalk et al., 2007). The results indicated that the ICC real-time RT-PCR method is sensitive to initial virus concentration, incubation length and therefore requires standardized cell culturing. Detailed evaluation on how these factors affect the ICC real-time RT-PCR results are beyond the scope of this study and they are discussed in another study (He et al., 2008).

The results also showed that detection of virus RNA using ICC real-time RT-PCR method is correlated with conventional PFU method and therefore is valid for quantifying rotavirus. The main advantage of ICC real-time RT-PCR is that the method is rapid and more sensitive, which can detect virus at concentration of 0.2 PFU within 2-day in contrast to conventional cell culture assays which take up more than 1 week to become detectable. Another advantage of ICC real-time RT-PCR assay is that it reduces chances of bacterial or fungal contamination because of relatively short incubation periods (Schalk et al., 2007).

3.2. Comparison of UV dose–response curve of SA11 with cell culture assay and with ICC real-time RT-PCR method

Inactivation of SA11 with initial concentrations (2×10^{4} PFU/ml) by UV (254 nm) dose raning from 0 to 360 mJ cm^{-2} were assessed using both cell culture method and ICC real-time RT-PCR simultaneously. Fig. 2 showed the inactivation curve of rotavirus SA11 detected by the conventional cell culture assay and an apparent inactivation rate constant was estimated to be 0.096 $cm^2\,mJ^{-1}$ ($R^2 = 0.9368$). The curve seems to show a saturation pattern at UV dose greater than 24 mJ cm^{-2}. Due to the detection limits of cell culture assay, at UV doses higher than 48 mJ cm^{-2}, the residual virus could not be detected any more by cell culture method. At initial concentration of 2×10^{4} PFU/ml, the reduction at 48 mJ cm^{-2} is about more than 4.3 $\log_{10}$ reductions and this is the detection limit as shown in Fig. 2. The UV inactivation first-order rate constant of SA11 detected by the cell culture assay in our study was comparable to those reported in previous studies (Chang et al., 1985; Hijnen et al., 2006) and the results validate our experimental methods.

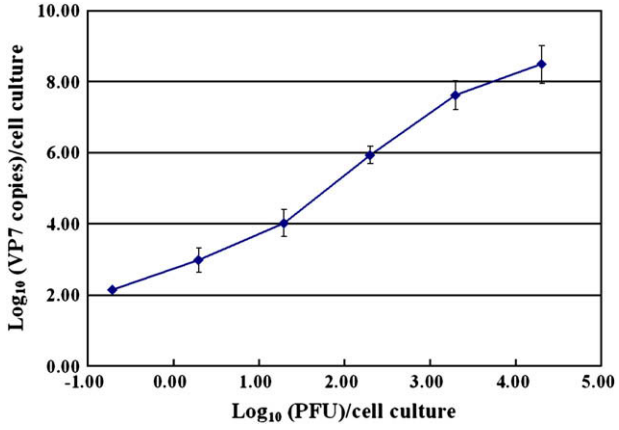

Fig. 1 – Correlation of concentrations of SA11 detected by cell culture based on CPE and those determined by ICC real-time RT-PCR assay. X-axis: the $\log_{10}$ PFU of rotavirus SA11 detected by CPE per cell culture (25 cm^2 of MA-104 cells). Y-axis: the $\log_{10}$ VP7 copy number per cell culture detected by ICC real-time RT-PCR with two-day cell culture incubation.

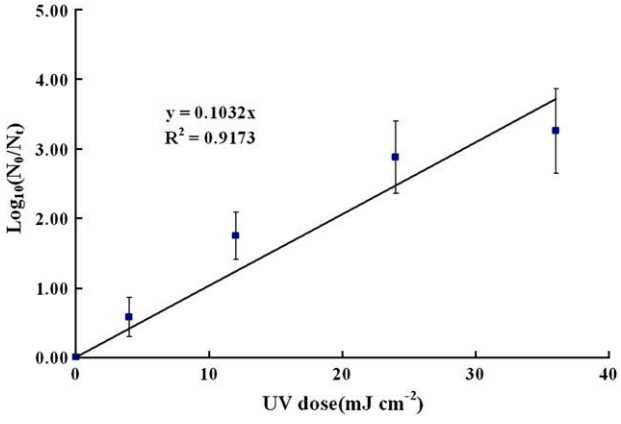

Fig. 2 – UV dose–response curve for rotavirus SA11 activity detected by cell culture assay based on CPE in MA-104 cells. X-axis: the UV dose. Y-axis: the $\log_{10}$ inactivation detected by CPE method. Solid line is the linear regression fitted line with R2 values shown in figure, and dotted line shows the connection of data points.

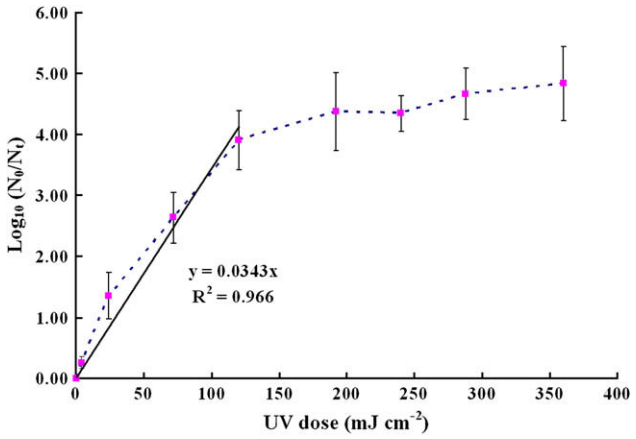

Fig. 3 – UV dose–response curve for rotavirus SA11 activity detected by ICC real-time RT-PCR assay. X-axis: the UV dose. Y-axis: the $\log_{10}$ inactivation detected by ICC real-time RT-PCR. The linear line connecting the first five data points is linear regression fitted line, and the remaining line is simply connecting the other data points to show the trend.

The ICC real-time RT-PCR method was also applied to evaluate the UV inactivation and it allowed for UV inactivation assessment of SA11 with much wider UV dose range than that by the traditional cell culture assay based on CPE. As shown in Fig. 3, the higher sensitivity of the method allows for inactivation assessment with UV dosed up to 360 mJ cm^{-2}. Initially, a first-order dose–response curve was observed, as indicated by the linear relationship between the logarithmic rotaviruses inactivation and the UV dosage ranging from 0 to 120 mJ cm^{-2}, and the inactivation rate constant was estimated to be 0.0343 cm^2 mJ^{-1} ($R^2 = 0.966$). The UV dose–response curve showed a saturation pattern when the UV dose increased from 120 to 360 mJ cm^{-2}, a region where the slope of the dose–response decreases with the increased UV dose.

Note that the values of log inactivation ($\log_{10} N_0/N_t$) of SA11 at the same UV dose, measured by cell culture PFU assay and ICC Real-time RT-PCR assay, were different (in Figs. 2 and 3). For example, at the UV dose of 24 mJ cm^{-2}, approximate 2.8 $\log_{10}$ reductions were measured by cell culture assay based on the formation of CPE (Fig. 2). However, at the same UV dose and with the same initial virus concentration, the reduction detected by ICC real-time RT-PCR was about 1 $\log_{10}$ (Y-axis). This is because the ICC real-time RT-PCR detects the RNA replicated in host cells after incubation, while the cell culture counts the CPE produced in host cells. And, the non-linear relationship, as shown in Fig. 1, between the inoculated SA11 dose (by PFU) and VP7 copies (by ICC real-time RT-PCT) in host cells caused the different inactivation rates observed.

The results of ICC real-time RT-PCR in our study estimated that 1, 2, 3, 4 $\log_{10}$ reductions of SA11 required UV dose of approximately 29.1, 58.3, 87.5 and 116.6 mJ cm^{-2}, respectively, which were interpolated from the linear regression lines (Fig. 3). SA11 inactivation of 3 $\log_{10}$ was achieved at UV dose of 28–42 mJ cm^{-2} by MA-104 cell culture assay in Chang's study (Chang et al., 1985), which is comparable to 31 mJ cm^{-2} obtained by cell culture assay in our study. However, in contrast, 87.5 mJ cm^{-2} will be required for 3 $\log_{10}$ reduction as detected by ICC real-time RT-PCR method in our study. This is because that the relatively high detection limit of traditional culture assay would yield "false" non-detectable end point and lead to higher log reduction value than what would be detected with ICC real-time RT-PCR method. Furthermore, there is an apparent difference in the shape of UV dose–response curve obtained with the two different detection methods. The UV inactivation was well described by a first-order model summarized by Hijnen et al. (2006) while the shape of UV dose–response curve detected by ICC real-time RT-PCR typically showed first-order inactivation at lower UV doses and a trailing region at higher UV dose. It is also noted that the curve starts to show a little tailing when the UV dose was more than 24 mJ cm^{-2} in our study detected by cell culture assay. Saturation/tailing effects with other virus such as poliovirus have been reported by Chang et al. (1985) using cell culture assay. The results demonstrated the UV-persistence phenomena of rotavirus, which otherwise could not be clearly observed with cell culture method due to detection limits. The mechanism of persistence effect is still not clear and it has been related to viral aggregation or population heterogeneity (Simonet and Gantzer, 2006). Tailing may be attributed to the presence of UV-resistant sub-populations of the microorganism and the presence of particulate-associated and clumped microorganisms (U.S. Environmental Protection Agency Office of Water, 2006). The results of ICC real-time RT-PCR indicated that a small fraction of SA11 remained active with UV dose as high as 360 mJ cm^{-2} (Fig. 3).

The experimental conditions may affect the response of virus to UV inactivation (Ko et al., 2005; Linden et al., 2002; Thurston-Enriquez et al., 2003). For example, different suspending media including sterilized water, tap water, seawater, secondary effluent, and PBS were used and compared in previous studies. The Ito^r P13 of group A human rotavirus suspending in freshwater needed higher UV dose than in seawater, which needed 200 and 220 mJ cm^{-2} for 4 $\log_{10}$ reductions, respectively (Santiago et al., 2004). PBS (pH 7.2) was applied in our study, the same as which used in the study of Chang et al. (1985) and Ko et al. (2005). In addition, the UV dose–response is generally independent on UV intensity of UV disinfection apparatus, whereas the UV absorbance, temperature and pH of suspending media (U.S. Environmental Protection Agency Office of Water, 2006), the reliability of UV dosimetry apparatus and UV dose dosimetry measurement may vary with different manufacturers. Furthermore, many previous studies had proved that the ironic strength, coagulation and particles of suspending media could have affected the UV dose–response of virus while others showed those factors had little impact of UV inactivation (U.S. Environmental Protection Agency Office of Water, 2006). In order to understand the impact of different factors and compare the results well and truly, UV dose–response of virus should be characterized simultaneously using both conventional cell culture detection and ICC real-time RT-PCR assay under the same experimental conditions. The results of cell culture in our study were similar to those previously reported in the literature. Previous studies also pointed out that repeated freeze-thawing were presumed to be the reason for the inconsistent results (Hijnen et al., 2006).

It is possible that multiple freezing–thawing procedures damage the viral particle, making it more susceptible to disinfection (Gerba et al., 2002; Ko et al., 2005). In our study, SA11 were prepared by repeated freezing and thawing at least four times. However, our data suggested that repeated freeze-thawing did not seem to affect the UV susceptibility of SA11 since our results was similar to that for adenovirus 41 reported by Ko et al., in which they repeated at least three times freeze-thawing (Ko et al., 2005).

3.3. *High sensitivity and effectiveness of the ICC real-time RT-PCR methods for evaluation of UV disinfection*

The ICC real-time RT-PCR assay is more sensitive than the cell culture assay based on CPE, because the cell culture assay relies on the formation of CPE, while ICC real-time RT-PCR combines the replication of viruses on a suitable cell line and subsequent detection of replicated virus in host cells with quantitative PCR. Integrated cell culture combined with high-sensitive PCR method greatly increases the sensitivity for detecting infectious virus. Previous studies also showed that ICC RT-PCR assay is more sensitive than the cell culture assay (Blackmer et al., 2000; Ko et al., 2003, 2005). The higher sensitivity of the ICC real-time RT-PCR assay allowed for detection of residual infective rotaviruses below the detection limit of the conventional cell culture assay and led to more detailed evaluation of UV disinfection with a wide dose range.

Our results demonstrated that detection method plays an important role in assessing UV resistance. The results indicated that the observed UV dose required to achieve a certain level virus activity reduction can be interpreted differently depending on the virus detection methods applied (Figs. 2 and 3). Employing ICC real-time RT-PCR method showed that a much higher UV dose (87.5 mJ cm^{-2}) is needed to achieve 3 log_{10} reductions, than the previously reported values determined by the cell culture assay (this study and Chang et al., 1985). The difference of using ICC real-time RT-PCR and traditional cell culture to characterize inactivation of adenovirus and poliovirus also has been reported in other studies. Meng and Gerba (1996) reported that adenovirus Ad41 required approximately 222 mJ cm^{-2} of UV dose for a 4 log_{10} inactivation, which was significantly higher than the value of 112 mJ cm^{-2} for 4 logs reductions determined using a conventional cell culture infectivity assay. In another study on investigating chlorine disinfection using CPE method, poliovirus was completely inactivated by a 0.5 mg L^{-1} dose of free chlorine after 2 min, whereas, integrating cell culture and PCR detected chlorinated polioviruses for up to 8 min of the same dose exposure and 10 min were required for complete inactivation (Blackmer et al., 2000). Chlorine disinfection efficiency for poliovirus evaluated by ICC RT-PCR indicated a required contact time that is 5-fold longer than that reported based on detecting visible CPE. Therefore, assessment of virus UV inactivation based on CPE may be a general problem due to its underestimation of the required UV dose and therefore demands further investigation by other methods such as integrated cell culture in combination with molecular methods.

More importantly, our results indicated that there were UV-persistent rotaviruses that can enter and replicate in MA-104 cells, which cannot be detected by CPE. Those rotaviruses can survive UV irradiation but may be need repair during cell incubation. Rotavirus with double-stranded RNA may able to use the host cell enzymes to repair damages in the RNA caused by UV irradiation. Those results seriously imply that previous research may have underestimated the resistance of enteric virus to disinfection process. This could help to explain why infectious viruses have been detected in drinking water after receiving disinfection believed to be adequate (Gratacap-Cavallier et al., 2000; Brassard et al., 2005). This new application of ICC real-time RT-PCR assay is proven to be more effective and sensitive for determining the potency of UV disinfection, minimizing the chance for false-negative results with cell culture alone, especially for rotaviruses which failed to produce clear CPE.

3.4. *Genome persistence of SA11 after UV disinfection detected by real-time RT-PCR assay*

During the inactivation of rotavirus SA11 by exposure to UV, the stability of genomes (inferred from the detection by VP7-specific real-time RT-PCR) was also evaluated using direct RNA extraction followed by the RT-PCR method. The inactivation curve of SA11 genomes detected by direct VP7-specific real-time RT-PCR was presented in Fig. 4. Unlike the detection of infectivity, the maximum reduction of VP7 gene of rotavirus is only 1.5 log_{10}. The reduction of rotavirus genome was approximately first-order, with no evidence of tailing or flattening of inactivation curve, and an inactivation rate constant was estimated to be 0.0043 cm^2 mJ^{-1} ($R^2 = 0.905$).

Although the mechanism by which UV irradiation inactivates rotavirus has not been completely elucidated, UV is known to cause pyrimidine dimer formation, i.e., thymine dimers and, in addition, intense irradiation can disrupt the virion structure (Linden et al., 2002). Genome degradation seems to be the main phenomenon explaining UV inactivation of RNA virus (Simonet and Gantzer, 2006). The application of molecular technique such as real-time RT-PCR has favored the relatively rapid, sensitive and specific detection of viral

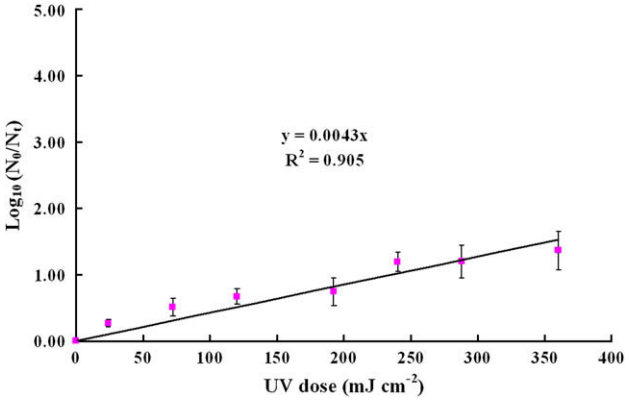

Fig. 4 – UV dose–response curve for the fragment genome of SA11 detected by direct real-time RT-PCR assay. X-axis: the UV dose. Y-axis: the log_{10} inactivation detected by real-time RT-PCR. The line shown is linear regression fitted line with R^2 value shown in figure.

genome sequence (Espinosa et al., 2008) without differentiating its association with virus infectious activity. In this study, the real-time RT-PCR assay was used to evaluate the stability of rotavirus genomes by VP7-specific real-time RT-PCR. The results showed that a 318-base of rotavirus VP7 genomes was degraded for 1.5 $\log_{10}$ reduction disinfected with more than 300 mJ cm^{-2} UV dose, whereas the infectious rotavirus was inactivated for 4 $\log_{10}$ reductions treated with 116.6 mJ cm^{-2} UV dose (obtained by the ICC real-time PCR method). This clearly demonstrated that the PCR method alone would be biased towards all virus genome detection, in contrast to the ICC real-time RT-PCT method, which would detect infectious activity-associated virus genome. In previous studies, Leveque et al. (1995) investigated the effect of UV light on HAV in artificial seawater. They found that the infectious virus was no longer detectable after 15 min irradiation of 3 L experimentally contaminated water, whereas genomic amplification by PCR still allowed the detection of viral RNA in all the samples even after 60 min irradiation.

Another aspect worthy of mentioning is that the region and length of the virus nucleic acid segment that is targeted for evaluating the activity and infectivity of virus is very important. It was found that different positions of the virus nucleic acid showed different levels of resistance to chlorine (Li et al., 2002). Therefore, the primers and PCR products location in genome may affect the results of activity assessment. In this study, the primers were designed on the region of segment 9 (1062 bp), which codes outer capsid layers VP7 of rotaviruses and plays an important role in terms of the mechanisms of cell attachment and membrane penetration (Jayaram et al., 2004), and we believe that it indicates the infectious status of the virus. However, further investigation is desired to make an assessment of the damage to the viral RNA over its entire length by UV radiation, containing 11 segments with about 18 kbp in total, using multiple primers targeting various regions.

3.5. *The resistance of rotavirus to UV disinfection*

The resistance of rotavirus to UV disinfection has been reported previously and the extent of UV resistance seems to be organism-specific. The resistance of Ito^r P13 of group A human rotavirus to UV irradiation estimated by Santiago et al. (2004) was significantly higher than that of SA11 summarized by Hijnen et al. (2006). In order to obtain a 3 $\log_{10}$ inactivation by 254 nm UV irradiation, doses of 28–42 mJ cm^{-2} were demanded for SA11 in Chang' study (Chang et al., 1985), while a dose of 50 mJ cm^{-2} was needed for Rotaviruses Wa (Hijnen et al., 2006), and doses of 140–160 mJ cm^{-2} were required for Ito^r P13 of group A human rotavirus in Santiago' study (Santiago et al., 2004). The critical factor of the resistance of different virus may be the structure of virus, the size and nature of the genome, or the way of transcription and replication in host cells. Previous studies also showed that different virus serotypes had considerably different UV susceptibilities (Ko et al., 2005). To achieve a 4 $\log_{10}$ reductions by 254 nm UV, a dose of 160 mJ cm^{-2} was required for adenovirus serotype 2 (Ad 2), while doses of 203–226 mJ cm^{-2} were required for adenovirus serotype 40 (Ad 40) in one study and doses of 112–124 mJ cm^{-2} were required for Ad 40 and adenovirus serotype 41(Ad 41) in another study (Ko et al., 2005).

Rotavirus, which belongs to the *Reoviridae* family, is a non-enveloped, icosahedral, double-stranded RNA (dsRNA) virus and has a three-layer capsid protein. The greater resistance of rotavirus may also have been due to the fact that it has triple-layered structure with projected structural protein 4 (VP4) (Hariharan et al., 2004); this projected protein may cause a shadowing effect or a disruption in the absorbance by the viral nucleic acids. However, the structure of rotavirus may affect the resistance to UV irradiation. It has been experienced that viruses with double-stranded genome exhibit higher UV resistance than those single-stranded viruses, such as poliovirus, echovirus and Coxsackievirus (Gerba et al., 2002; Duizer et al., 2004). This is because even though these double-stranded viruses lack the necessary enzymes for repairing the damage, they are able to use the host cell enzymes to repair UV imposed damages in the genome (Gerba et al., 2002). It should be noted that rotaviruses may use the host cell enzymes system to repair their own RNA damage caused by UV irradiation. Furthermore, it has also been proved that different cell lines may provide different UV resistance, for instance, the greater availability of repair enzymes may result in a greater number of viral particles surviving UV irradiation (Liltved et al., 2006).

4. Conclusion

In this study, the resistances of rotavirus to UV disinfection were characterized by conventional cell culture assay, ICC real-time RT-PCR and direct real-time RT-PCR assay. Our study indicated that:

- The ICC real-time RT-PCR assay provided a more sensitive, rapid and reliable method for detection of infectious rotavirus. The results demonstrated that ICC real-time RT-PCR revealed more detailed assessment of virus resistance to UV disinfection at much wider dosage range compared to conventional cell culture assay.
- UV disinfection evaluation performed using ICC real-time RT-PCR method revealed that rotaviruses are more resistant to UV disinfection than previous observed using conventional cell culture assay. As a result, previous studies using CPF method likely underestimated the UV dosage required to achieve certain level virus inactivation.
- Rotavirus genomes were found to be quite persistent under UV disinfection and the detection of virus genome with direct real-time RT-PCR method without cell culture incubation does not directly correlate with the infectivity of virus. Virus genome may persist longer in the water and contribute to false-positive detection of high level of virus.
- Our results suggest that re-evaluation of resistance of other waterborne viruses to UV irradiation disinfection may be necessary, using more sensitive infectivity detection method (such as ICC real-time RT-PCR), in order to determine the appropriate UV doses required for the control of waterborne viruses. Further studies are also needed to determine the exact mechanism of resistance and if other types of enteric viruses exhibit similar phenomena.

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