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A one-step centrifugal ultrafiltration method to concentrate enteric viruses from wastewater

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A B S T R A C T

A one-step centrifugal ultrafiltration method was developed to enhance rapid detection of human enteric viruses and co-occurring viruses in wastewater. Samples were collected pre- and post-UV treatment at two full-scale tertiary municipal wastewater treatment plants in Calgary, Canada. Viruses were concentrated from 100 mL wastewater samples through direct centrifugation using the Centricon Plus-70 ultrafilter. Seven viruses, including norovirus, rotavirus, sapovirus, astrovirus, enterovirus, adenovirus and JC virus, were tested using real-time quantitative PCR (rt-qPCR) and cell culture. All of the viruses were detected in pre- and post-UV samples by rt-qPCR, with rotavirus the most numerous (6.6 $\log_{10}$ GE copies/L). Infectious viruses, by cell culture, were found in all tested pre-UV samples but only in one post-UV sample. The results were comparable and consistent to that obtained using virus adsorption-elution method, indicating that the centrifugal ultrafiltration method is adequate to retain the viruses and maintain their infectivity during processing. As a simple, rapid and cost-effective method to screen wastewater viruses, this one-step centrifugal ultrafiltration method may serve as an effective approach to assess virus removal and gain knowledge of human virus activity during wastewater treatment.

Human viruses shed via the gastrointestinal and urinary tracts are frequently detected in raw sewage and treated wastewater. Some of these viruses are major waterborne pathogens (Bosch et al., 2008; Qiu et al., 2015). Classic enteric viruses include norovirus (NoV), rotavirus (RV), sapovirus (SaV), astrovirus (AsV), and adenovirus (AdV) (Fong and Lipp, 2005). Due to their low infectious dose, these viruses are very contagious, especially NoV, which is a major cause of global gastroenteritis (Ahmed et al., 2014). Furthermore, the U.S. EPA (U.S. Environmental Protection Agency, 2012) has recognized enteric viruses as the likely major hazard group in sewage-polluted receiving waters, and is seeking appropriate methods to assess the efficacy of virus removal during wastewater treatment to reduce environmental pollution.

Due to the typically low virus levels in water matrices, the first and most critical step to detect virus in environmental water is to concentrate virus to enhance detection. Currently, the most common concentration method is virus adsorption-elution (VIRADEL) using electropositive or electronegative microporous membrane filters, such as Virosorb 1MDS and NanoCeram filters (Ikner et al., 2012; Pang et al., 2012; Cashdollar and Wymer, 2013). The process involves the absorption of viral particles to the membrane through ionic interaction followed by elution with pH adjustment. However, VIRADEL is time consuming, labor intensive and needs large sample volume that may require a secondary concentration procedure to reduce the volume of the eluate to enhance the sensitivity for detection. Ultracentrifugation is another concentration technique to pellet macromolecules and viruses from water (Ammersbach and Bienzle, 2011). It has been used to recover RV and AdV in wastewater and recreational water (Prata et al., 2012). As an alternative approach for the concentration of microbes from water, ultrafiltration was first reported for environmental samples in Australia (Grohmann et al., 1993). This method, which is based on the size exclusion, has since been increasingly used in the last decade (Rhodes et al., 2016). The pore size of the ultrafiltration ranges from 5 nm to 0.1 μm, which can retain a broad range of virus

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particles, including enteric viruses (Ikner et al., 2012; Cashdollar and Wymer, 2013). Typically, the water sample passes through the capillaries or hollow fibres using tangential flow. Many viruses, bacteria and parasites have been concentrated and detected in environmental waters using ultrafiltration (Hill et al., 2007, 2009). In addition to the traditional hollow-fibre ultrafilters, there are small centrifugal ultrafilters that have been mainly used for secondary concentration of viruses. Centricon Plus-70 is one of these centrifugal ultrafilters with a molecular weight cut off (MWCO) ranging from 5 to 100 kDa. The Centricon Plus-70 device has been used as a secondary concentration method for viruses, bacteria and parasites in several studies (Hill et al., 2007; Ikner et al., 2011; Kahler et al., 2015; Kim et al., 2015). However, the possibility of using centrifugal ultrafilters as the primary concentration method in water matrices, especially in wastewater, appears to be unexplored.

Pang et al. reported a virus concentration method that used NanoCeram disc filtration, beef extract elution and flocculation based on VIRADEL (Pang et al., 2012). This method has been used to concentrate viruses in both wastewater (Qiu et al., 2015) and surface water (Pang et al., 2012). However, there are limitations with this method, such as a large sample volume to be processed, the loss of virions due to multiple processing steps, long processing time and limited sample throughput. These limitations have prompted us to look for an alternative approach for virus concentration. In this study, a simple and rapid one-step centrifugal ultrafiltration method was developed using the Centricon Plus-70 centrifugal ultrafilter (30-kDa MWCO, Millipore) to concentrate human enteric viruses from wastewater. In order to determine the effectiveness of the one-step ultrafiltration method, VIRADEL developed by Pang et al. (2012) was used in parallel to concentrate virus from the same water samples. Real-time quantitative PCR (rt-qPCR) and cell culture were used to quantify the amount of virus/free viral nucleic acids and infectious virions respectively after each concentration method.

Wastewater samples were collected monthly from two municipal wastewater treatment plants (WWTP), Pine Creek (PC) and Bonnybrook (BB), located in the City of Calgary, Canada from November, 2014 to April, 2015. A total of 12 samples were collected by directly grabbing from the wastewater flow before UV treatment (pre-UV) and post-UV treatment respectively during the study. Ten liters of grab water samples were processed using VIRADEL as previously described (Pang et al., 2012; Qiu et al., 2015). The centrifugal ultrafiltration was used to concentrate virus from 100 mL wastewater sample using the Centricon Plus-70 filter according to the Manufacturer's instructions, except for the pre-rinse step. Briefly, 70 mL of water sample were added into the filter cup and centrifuged at $1900 \times g$ for 10 min at room temperature. The filtrate was discarded and the same procedure was repeated for the rest of the sample. The filtrate collection cup was then removed and the concentration cup was placed on top of the filter cup. The whole device was inverted carefully and centrifuged at $800 \times g$ for 2 min. The concentrated sample was collected from the concentration cup and the volume was measured. The processed concentrate was stored at −70 °C until use.

The volume of the concentrated sample after centrifugal ultrafiltration ranged from 230 μL to 955 μL with a median of 327 μL. Each concentrated sample was made up to a final volume of 2 mL with PBS. Two hundred microliters and 1 mL of the concentrate were used for nucleic acid extraction and cell culture, respectively. Virus mixture containing NoV GII and AdV isolated from clinical stool samples and confirmed by in-house rt-qPCR assay, as well as cultured coxsackie B virus (CoV, 4.68×10^4 infectious unit/mL) was used for recovery test (Pang et al., 2012). The recovery of virus was determined by spiking 1 mL of the virus mixture into 99 mL and 10 L of the same pre-UV samples for centrifugal ultrafiltration and VIRADEL, respectively. One mL of the same aliquot of virus mixture was mixed with 1 mL (total of 2 mL) and 14 mL (total of 15 mL) of PBS to test in parallel with the spiked wastewater samples as the baseline control for centrifugal ultrafiltration and VIRADEL, respectively. The recovery rate (%) was expressed as a ratio of viral load detected in the respective processed concentrates as compared to the baseline control (Qiu et al., 2015). Nucleic acid extraction, reverse transcription and cell culture were performed as previously described (Qiu et al., 2015). The qPCR reaction was performed in a total volume of 10 μL containing 2 × TaqMan Fast Universal Master Mix (Applied Biosystems), 900 nM of each primer, 250 nM of specific probe, and 2.5 μL cDNA. Amplification consists of initial incubation at 95 °C for 20 s followed by 45 cycles of 3 s at 95 °C, 30 s at 60 °C. An external standard curve was established for quantification using the 875 bp DNA fragment of NoV GII by 10-fold dilution from 100 copies to 1.0×10^6 copies. Seven types of viruses including NoV GI/GII, RV, SaV, AsV, AdV, enterovirus (EV) and JC virus (JCV) were tested by rt-qPCR. The viral load was expressed as $\log_{10}$ genome equivalent (GE) copies/L after correction of the dilution steps and original volume of the wastewater sample.

The median% recovery (range) was 3% (1–8%) for NoV, 50% (4–75%) for CoV and 3% (2–4%) for AdV using the centrifugal ultrafiltration; and 18% (5–60%) for NoV, 24% (16–28%) for CoV and 5% (4–12%) for AdV using the VIRADEL. CoV showed better recovery in both methods compared to NoV and AdV, which may be due to the source of viruses since NoV and AdV isolated from stool samples may contain a proportion of free nucleic acids. Previous studies demonstrated that viral nucleic acids had much lower recovery rate compared to infectious virions (Haramoto et al., 2007; Li et al., 2010). The virus level spiked into the water may also affect recovery efficiency. Our recent studies showed higher input of the spiked NoV in wastewater can increase the recovery (data not shown). The wide range of recovery for each virus may be attributed to the variation of wastewater sample matrix. The limit of detection (LOD) for centrifugal ultrafiltration was 8 GE copies/mL using rt-qPCR. The concentration of various viruses in pre- and post-UV samples using both methods are showed in Tables 1 and 2. Four viruses (NoV GI/GII, RV, AsV and AdV) were detected in all 12 pre- and post-UV samples, whereas SaV, EV and JCV were occasionally undetectable by both methods. SaV, EV and JCV were detected in a fewer number of samples by the one-step centrifugal ultrafiltration compared to VIRADEL. Several factors might have contributed to this result, such as low level of EV and JCV present in the wastewater samples, the smaller sample volume used for centrifugal ultrafiltration, and sampling variations for grab samples.

Using centrifugal ultrafiltration, the mean viral load ranged from 3.6 (JCV) to 6.5 (RV) $\log_{10}$ GE copies/L in pre-UV samples (Table 1), and 4.2 (JCV) to 6.6 (RV) $\log_{10}$ GE copies/L in post-UV samples (Table 2). Using VIRADEL, the mean viral load ranged from 3.5 (EV) to 6.0 (RV) $\log_{10}$ GE copies/L in pre-UV samples and 3.1 (SaV) to 5.8 (RV) $\log_{10}$ GE copies/L in post-UV samples. It appears that the viral load detected using the centrifugal ultrafiltration was slightly higher than VIRADEL. The lower level of detected virus using VIRADEL could be due to the multi-step procedures including the preconditioning step. The results from this study suggested that the one-step centrifugal ultrafiltration has at least as effective virus recovery and quantification from wastewater to that of the more commonly used VIRADEL approach.

Among the 24 pre- and post-UV samples, ten concentrated samples (5 pre-UV and 5 post-UV) were used in cell culture to examine the infectivity of the viruses in the processed concentrate. Clinical strains of coxsackievirus B obtained from the Provincial Laboratory for Public Health, Calgary site (courtesy of Dr. Julie Fox) was used as positive control. Strong cytopathic effect (CPE, define as low, medium and strong using symbols: +, ++ and +++), were observed in all of the pre-UV samples for both methods, indicating that the infectivity of the viruses was retained during the concentration