Journal of Virological Methods 193 (2013) 140–146

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Evaluation of methods using celite to concentrate norovirus, adenovirus and enterovirus from wastewater

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Article history:
Received 14 December 2012
Received in revised form 10 May 2013
Accepted 16 May 2013
Available online 31 May 2013

Keywords:
Norovirus
Adenovirus
Enterovirus
Wastewater
Celite

A B S T R A C T

Enteroviruses, noroviruses and adenoviruses are among the most common viruses infecting humans worldwide. These viruses are shed in the feces of infected individuals and can accumulate in wastewater, making wastewater a source of a potentially diverse group of enteric viruses. In this study, two procedures were evaluated to concentrate noroviruses, adenoviruses and enteroviruses from primary effluent of wastewater. In the first procedure, indigenous enteroviruses, noroviruses and adenoviruses were concentrated using celite (diatomaceous earth) followed by centrifugation through a 30K MWCO filter and nucleic acid extraction. The second procedure used celite concentration followed by nucleic acid extraction only. Virus quantities were measured using qPCR. A second set of primary effluent samples were seeded with Coxsackievirus A7, Coxsackievirus B1, poliovirus 1 or enterovirus 70 before concentration and processed through both procedures for recovery evaluation of enterovirus species representatives. The pairing of the single step extraction procedure with the celite concentration process resulted in 47–98% recovery of examined viruses, while the celite concentration process plus additional centrifugal concentration before nucleic acid extraction showed reduced recovery (14–47%). The celite concentration process followed by a large volume nucleic acid extraction technique proved to be an effective procedure for recovering these important human pathogens from wastewater.

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1. Introduction

Enteric viruses, such as the noroviruses (NoV; family *Caliciviridae*, genus *Norovirus*), adenoviruses (AdV; family *Adenoviridae*, genus *Mastadenovirus*) and enteroviruses (EV; order *Picornavirales*, family *Picornaviridae*, genus *Enterovirus*) enter the human host through ingestion, and replicate in the epithelium of the small intestine. As a result, these viruses are excreted in high concentrations (up to 10^{10} particles/g) in feces of infected individuals (Okoh et al., 2010). Typically, human waste is transported through the sewer system and is collected at wastewater treatment plants for removal of chemical and biological contaminants prior to discharge into our waterways. Thus, enteric viruses have been reported in many wastewater products including raw sewage (Cantalupo et al., 2011; Flannery et al., 2012; Fong et al., 2010; Kuo et al., 2010; Yang et al., 2012), primary effluents (Dong et al., 2010; Fong et al., 2010; Kuo et al., 2010; Rodriguez et al., 2008), secondary effluents (Fong et al., 2010; Kuo et al., 2010) and tertiary effluents (Flannery et al., 2012; Fong et al., 2010; Rodriguez et al., 2008; Schlindwein et al., 2010).

Viruses present in wastewater likely represent wild-type viruses currently being transmitted throughout a community. Therefore, they are a rich resource for studying many aspects of environmental virology. For example, viruses isolated from wastewater can be quantified to determine the occurrence of a particular virus group during a specific season. Additionally, viruses isolated from wastewater could be used as an exogenous source of viral material for spiking experiments to evaluate the performance of a particular method or treatment technology. However, to obtain the rich virus source, a method capable of concentrating and isolating enteric viruses from wastewater with high efficiency is necessary.

A variety of techniques have been utilized to concentrate and recover viruses from wastewater. These include the methodologies typically employed for concentration of large volumes (>100 l) of water (groundwater, surface water and tap water), such as electronegative filtration and elution, with and without additional concentration using low molecular weigh cut-off ultrafilters (Ahmed et al., 2010; Fong et al., 2010; Schlindwein et al., 2010), electropositive filtration and elution followed by organic

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0166-0934/$ – see front matter. Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.jviromet.2013.05.014

flocculation procedure (Kuo et al., 2010; Simmons et al., 2011), and hollow fiber ultrafiltration followed by polyethylene glycol precipitation (Dong et al., 2010). In addition, abbreviated versions of the large-scale methods are also used. For example, the organic flocculation procedure has been used as an isolated process to concentrate and recover viruses from wastewater (Cantalupo et al., 2011; Rodriguez et al., 2008). Previously, Dahling and Wright (1988) showed that celite, a diatomaceous silicate, could effectively and efficiently adsorb viruses from wastewater. Recently, Rhodes et al. (2011) showed that the celite concentration procedure could recover 89.5% of poliovirus seeded into the retentate obtained after hollow fiber ultrafiltration. Furthermore, McMinn et al. (2012) reported efficient recovery of AdV 40 and 41 with calcinated, small and medium sized particle celite preparations.

In this study, the celite concentration process was paired with two additional concentration and extraction schemas in order to have more sample representation when assayed by molecular techniques. The first scheme employed a Vivaspin 30K MWCO centrifugal concentrator followed by nucleic acid extraction using the QIAamp DNA Blood Mini Extraction kit (Celite + Vivaspin + Mini Extraction), which is described in US EPA Method 1615 (Fout et al., 2010). The second scheme utilized a relatively large volume of celite concentrate (10 ml) to further concentrate and extract nucleic acids in one step using the QIAamp DNA Blood Maxi Extraction kit (Celite + Maxi Extraction). The goals of this research were to (1) investigate the ability of the celite procedure to concentrate a broader range of viruses that have not been previously tested and (2) examine which of two secondary schemas is more efficient at further concentrating and recovering NoV, AdV and EV from primary effluent of wastewater. The procedure with the optimal recovery efficiency for NoV, AdV and EV was then used to determine the concentration of these viruses in a composite primary effluent sample.

2. Materials and methods

2.1. Virus stocks and cell lines

Coxsackievirus A7 (CVA7; order *Picornavirales*, family *Picornaviridae*, genus *Enterovirus*, species *Enterovirus A*; AB-IV Russian, ATCC# VR-1012), Coxsackievirus B1 (CVB1; order *Picornavirales*, family *Picornaviridae*, genus *Enterovirus*, species *Enterovirus B*; Conn-5, ATCC# VR-1032) and enterovirus 70 (EV70; order *Picornavirales*, family *Picornaviridae*, genus *Enterovirus*, species *Enterovirus D*; J670/71, ATCC# VR-836) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Additionally, poliovirus 1 (PV1; order *Picornavirales*, family *Picornaviridae*, genus *Enterovirus*, species *Enterovirus C*; Mahoney) (Dahling and Wright, 1986a) was used in this study. Each virus serotype was propagated in Buffalo Green Monkey Kidney (BGM) cells to make high titer virus stocks. Stock cultures of BGM were propagated as previously described (Dahling and Wright, 1986a).

To prepare high titer virus stocks, 1 ml of cell culture supernatant obtained from ATCC was introduced to a 25 cm^2 cell culture flask, and all flasks were rocked gently for a minimum of 80 min before addition of the maintenance media containing equal parts (50:50) of Minimum Essential Medium Eagle (Sigma–Aldrich, St. Louis, MO, USA) and Leibovitz's L-15 Media (Sigma–Aldrich), supplemented with 0.67% sodium bicarbonate, 2% calf serum (Hyclone, Pittsburg, PA, USA) and antibiotic–antimycotic liquid (Invitrogen, Carlsbad, CA, USA) was added. Cultures were incubated up to 7 days at 37 °C and were checked at least twice weekly for cytopathic effects (CPE). Those flasks which exhibited 75–100% CPE were immediately frozen at −70 °C. Those that did not exhibit CPE were frozen at −70 °C after 7 days of incubation. All cell culture lysates were prepared by a series of 2 freeze–thaw cycles, and then centrifuged at 3000 × *g* for 10 min to pellet cell debris. The lysates were then sterilized by passing through a 0.2 μm Steriflip filter (Millipore Corporation, Bedford, MA, USA). Lysates that showed CPE after incubation on the 25 cm^2 cell culture flask were consecutively passaged onto larger cell culture flasks (75 cm^2) and then perforated roller bottles (2100 cm^2) to obtain high titers of virus. Those lysates that did not show CPE were again passaged onto a fresh monolayer in a 25 cm^2 cell culture flask in an attempt to acclimate the viruses to the cell line. PV1 was incubated directly in the 2100 cm^2 bottle since this strain was previously cultured in our laboratory. Once harvested from the 2100 cm^2 bottle, all virus stocks were treated to remove cell debris by centrifugation at 3000 × *g* for 10 min, followed by centrifugation at 10,000 × *g* for 10 min. The supernatants were sterilized using a 0.2 μm Steriflip filter and the filtrate was diluted 1:1 in 1× Dulbecco's PBS (without $CaCl_2$ and $MgCl_2$, US Biologicals, Swampscott, MA, USA), pH 7.0. Titers of EV stocks were determined by plaque assay on the BGM cell line as described (Dahling and Wright, 1986a). Virus stocks were stored at −70 °C. Identities of stock viruses were confirmed by amplification and sequencing of the VP4 gene.

2.2. Collection of samples for analysis of indigenous NoV, AdV and EV

To examine recovery of indigenous NoV, AdV and EV, four-one liter (approximate volume) samples of primary effluent were collected from a local wastewater treatment plant and transported back to the lab on ice. Primary effluent is a product of the first step in wastewater treatment and is the aqueous portion obtained after settling by gravity. Sterile stir bars were added to each sample, the sample was mixed and a 10 ml subsample was removed and transferred to a 50 ml polypropylene tube so that the quantities of indigenous NoV, AdV and EV present in the sample before concentration could be determined. The 10 ml subsamples from each of the 4 the primary effluent samples were extracted using the QIAamp DNA Blood Maxi Extraction Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions with modifications. AVL buffer was used instead of the AL buffer supplied with the kit. Manufacturer's instructions were followed and nucleic acids were eluted with 1 ml of AE buffer supplemented with 400 units of Recombinant RNasin Ribonuclease Inhibitor (Promega, Madison, WI, USA). The eluted nucleic acid was collected and reloaded onto the column for a second elution to increase yield. Nucleic acid extracts (approximately 1 ml) were stored at −70 °C until quantities could be determined using RT-qPCR or qPCR as described in Sections 2.8 and 2.9.

From the volume remaining in each of the 4 replicate primary effluent samples, 0.5 l was concentrated with celite as described in Section 2.4.

2.3. Collection of samples for analysis of seeded EV

Additional primary effluent samples were collected at various times during the winter season (when the EV background is expected to be low) from two local wastewater treatment plants and transported back to the lab on ice packs then stored at −70 °C until further processing. For each of 4 processing events, 3 l of frozen primary effluent were thawed, combined into a 4 l beaker and mixed to make a composite sample. The composite primary effluent sample was then separated into 5–0.5 l subsamples. Four of the 0.5 l subsamples were seeded with approximately 1000 plaque forming units (PFU) of CVA7, CVB1, PV1 or EV70, made by dilutions based on plaque titers, and were mixed for at least 10 min. The last 0.5 l subsample remained unseeded so that background levels of EV, if any, could be measured. The 0.5 l samples were processed with