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Protocol

Detection of rotavirus A in sewage samples using multiplex qPCR and an evaluation of the ultracentrifugation and adsorption-elution methods for virus concentration

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Group A rotaviruses (RV-A) are the most common agents of viral gastroenteritis in children worldwide. The goal of this study was to compare two different methods to concentrate RV-A from sewage samples and to improve the detection and quantification of RV-A using a multiplex quantitative PCR assay with an internal control. Both RV-A and the internal control virus, bacteriophage PP7, were seeded into wastewater and then concentrated using either an ultrafiltration-based adsorption-elution protocol or an ultracentrifugation-based protocol. Real time multiplex quantitative PCR was used to quantify the purified RV-A and PP7, and the results of the multiplex assay were compared with the results of the monoplex assays. The ultracentrifugation-based method had a mean recovery rate of 47% (range: 34–60%), while the ultrafiltration-based adsorption-elution method had a mean recovery rate of 3.5% (range: 1.5–5.5%). These results demonstrate that ultracentrifugation is a more appropriate method for recovering RV-A from wastewater. This method together with the multiplex qPCR assay may be suitable for routine laboratory use.

1. Introduction

More than 140 virus types that cause a broad variety of illnesses in humans can be found in water as a result of contamination with waste (Gerba et al., 1996; Kukkula et al., 1997). Sewage influent contains pathogens shed by infected individuals, and the molecular detection and characterization of human viruses in urban sewage has been used extensively to derive information on the viruses circulating in a given population (Bofill-Mas et al., 2000; Ferreira et al., 2009; Fumian et al., 2010; Pintó et al., 2007; Rodríguez-Díaz et al., 2009; Victoria et al., 2010; Villena et al., 2003). Several of these viruses have been implicated in waterborne outbreaks of gastroenteritis including rotavirus A (RV-A) (Gerba et al., 1996; Hewitt et al., 2007; Kukkula et al., 1997; Villena et al., 2003). Despite the difficulty in determining the proportion of gastroenteritis cases resulting from waterborne infection, a significant percentage of the cases can be associated with poor water quality (Bosch et al., 2008). RV-A has been detected in environmental samples using different methods for virus concentration and detection (Ferreira et al., 2009; Kittigul et al., 2005; Mehnert et al., 1997; Villena et al., 2003).Natural inhibitors present in environmental samples and/or reagents used to concentrate viruses can be an obstacle for detecting viruses from these samples by interfering mainly in the molecular biology-based methods used for virus detection (Ijzerman et al., 1997; Schwab et al., 1995). A relatively low initial cost method based on adsorption-elution/ultrafiltration and elution with inorganic reagents has been shown to minimize this problem (Katayama et al., 2002). While this method favors the detection of viruses using molecular methods, it has the disadvantage of requiring two steps of virus concentration. This increases the cost per analysis, and purifying viruses from samples with a high amount material in suspension can be difficult. However, using this method, several authors have recovered viruses from different water matrices, including wastewater samples, at a low rate (Guimarães et al., 2008; Haramoto et al., 2006; Katayama et al., 2008; Miagostovich et al., 2008; Victoria et al., 2009, 2010; Villar et al., 2007).The main objective of this study was to compare the percentage of RV-A that could be recovered from

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wastewater using the either the adsorption-elution of the virus from negatively charged membranes or the ultracentrifugation-based concentration method that has been used largely to detect enteric viruses in wastewater (Bofill-Mas et al., 2006; Pina et al., 1998; Rodríguez-Díaz et al., 2009). The initial cost to obtain an ultracentrifuge and the ability to analyze only small sample volumes are two disadvantages of this method. However, the advantages of this method include the low cost per sample (all of the consumables used in this assay can be autoclaved and reused) and the lack of a second concentration step (Pina et al., 1998; Wyn-Jones and Sellwood, 2001).

The use of a bacteriophage PP7 as an internal control (IC) was also evaluated in both concentration methods. An IC is an important tool for molecular diagnostic assays to avoid the false negative results associated with enzymatic inhibition (Hoorfar et al., 2004). A negative IC result suggests failure during the concentration, extraction and/or PCR procedures. Additionally, a multiplex qPCR-based protocol was compared to using monoplex assays that have been used previously to detect RV-A and PP7 in order to further reduce the cost of the assay (Rajal et al., 2007; Zeng et al., 2008).

2. Materials and methods

2.1. Viruses and sewage samples

RV-A, G1P8 Wa prototype, was used for the construction of the standard curve.

For the spiking experiments a RV-A G1P8 isolate, isolated from an acute case of gastroenteritis using molecular techniques and confirmed by sequencing, was used (Genbank accession no. GU831596).

PP7 bacteriophage was provided kindly by Dr. Verónica Rajal (Salta University, Argentina), and a large amount was obtained by culture in the host *Pseudomonas aeruginosa* (ATCC 15692) using a protocol described previously (Rajal et al., 2007).

Raw sewage samples were collected from a sewage treatment plant in Rio de Janeiro, Brazil in order to perform the studies to determine the efficiency of the adsorption-elution and ultracentrifugation methods. Fourteen additional samples were collected at the same plant and stored in glass bottles at 4 °C until processed.

2.2. Virus concentration methods

RV-A and PP7 were concentrated using either an adsorption-elution/ultrafiltration method or an ultracentrifugation-based method. The adsorption-elution/ultrafiltration method was based on negatively charged membranes and included the addition of an acid rinse step for the removal of cations, as described previously by Katayama et al. (2002). The ultracentrifugation-based method was based on the protocol of Pina et al. (1998). In both cases, 42 mL of raw sewage was used as the starting material.

For the ultrafiltration-based method, the starting volume of sewage was diluted with 2 L of distilled water to facilitate the filtration process. Briefly, prior to filtration, $MgCl_2$ was added to a final concentration of 25 mM and the pH was adjusted to 5.0. The samples were filtered through a type HA negatively charged membrane (Nihon Millipore, Tokyo, Japan) with a 0.45 μm pore size using a vacuum pump system. The membrane was rinsed with 350 mL of 0.5 mM H_2SO_4 (pH 3.0), and 15 mL of 1 mM NaOH (pH 10.8) was used to release the viruses from the membrane. To neutralize the solution, 50 μL of 50 mM H_2SO_4 and TE buffer (100×; pH 8.0) was added. The eluate was filtered using a Centriprep Concentrator 50 (Nihon Millipore, Tokyo, Japan) and centrifuged at 1500 × *g* for 10 min at 4 °C to obtain a final volume of 2 mL. The filtration system was soaked briefly in a 10% bleach solution and rinsed with deionized H_2O prior each use.

For the ultracentrifugation-based method, 42 mL of sewage was ultracentrifuged at 100,000 × *g* for 1 h at 4 °C using a Beckman ultracentrifuge equipped with a type 35 rotor. Viral particles were resuspended in 3.5 mL of 0.25 N glycine buffer (pH 9.5) and incubated on ice for 30 min. The solution was neutralized by the addition of 3.5 mL of 2× phosphate-buffered saline (PBS, pH 7.2). The supernatant was clarified by centrifugation (12,000 × *g* for 15 min), and the viruses were finally recovered by ultracentrifugation at 100,000 × *g* for 1 h at 4 °C in an SW41 rotor. Viral particles were resuspended in 200 μL of 1× PBS pH 7.2 and processed immediately for nucleic acid extraction or stored at −80 °C until use.

2.3. Spiking experiments

Two experiments performed in triplicate, on different days, were carried out to evaluate the recovery rate of both methods. One mL of a RV-A-containing fecal sample was spiked into the sewage samples, and one unseeded sample was used as negative control. The total amounts of both RV-A and PP7 were determined using both monoplex and multiplex qPCR. Part of the stool sample used to spike the sewage samples was used for RNA extraction and quantification together with the concentrated sewage samples. The number of viral particles was determined by adjusting the values according to the volumes used for each step of the procedure (extraction, cDNA synthesis and qPCR reaction). The estimated virus recoveries were calculated as the percentage of the number of copies of the inoculated RV-A recovered.

Natural contamination (i.e., pre-existing RV-A in the sewage sample) was quantified from the unseeded controls, and these values were subtracted from the total amounts to correct the results of the seeded samples.

2.4. Extraction of viral RNA and reverse transcription (RT)

Viral RNA was extracted from 200 μL of concentrated virus using the glass powder method (Boom et al., 1990). cDNA synthesis was carried out using reverse transcription with random primers (PdN6 – 50 A260 units – Amersham Biosciences, Chalfont St. Giles, Buckinghamshire, UK), as described previously (Ferreira et al., 2008). cDNA was prepared using RNA and a 1:10 dilution of RNA to investigate the presence of inhibitors in the sewage samples.

2.5. Real time multiplex quantitative PCR (qPCR)

The primers and probes used to detect RV-A and PP7 have been described previously by Zeng et al. (2008) and Rajal et al. (2007), respectively. The VIC-labeled RV-A probe, designed to target a highly conserved region of the non-structural protein 3 (NSP3), was synthesized by Applied Biosystems (CA, USA), and the PP7 primers were designed to amplify a region of the PP7 replicase gene (Table 1). The generation of the plasmids and the construction of the standard curve were performed as described previously by Fumian et al. (2009).

Multiplex qPCR was carried out using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, CA, USA). A standard curve (SC; 10^7, 10^5, 10^3 and 10^1 copies per reaction) was generated using tenfold serial dilutions of pCR2.1 vectors (Invitrogen, USA) containing either the RV-A NSP3 gene or the PP7 replicase gene. The optimal concentration of the primers and probe for RV-A was 400 nM and 200 nM, respectively; the optimal concentration of the primers and probe for PP7 was 500 nM and 120 nM, respectively. The multiplex qPCR reaction was performed in a 25 μL volume containing 12.5 μL of Universal PCR Master Mix (Applied Biosystems, CA, USA), 2.2 μL DNase/RNase-free water, 5 μL cDNA

Table 1
Primers and probes used in the multiplex qPCR assay for the detection of RV-A and PP7.

Virus	Primer and probe	Sequence (5′ → 3′)	Size amplicon (bp)	Location
RV	NSP3 f	ACCATCTWCACRTRACCCTCTATGAG[a]		963–988
	NSP3 r	GGTCACATAACGCCCCTATAGC	86	1028–1049
	NSP3 probe	VIC-AGTTAAAAGCTAACACTGTCAAA-MGB		995–1017
PP7	247 f	GTTATGAACCAATGTGGCCGTTAT		247–270
	320 r	CGGGATGCCTCTGAAAAAAG	73	320–339
	274 probe	FAM-TCGGTGGTCAACGAGGAACTGGAAC-TAMRA		274–298

[a] IUB code: W = A/T, R = A/G.

and 1.25 μL each primer and 0.3 μL probe for PP7 or 1 μL each primer and 0.5 μL probe for RV-A. The initial concentration of all primers and probes used in the multiplex qPCR mix was 10 μM. After initial incubations at 50 °C for 2 min to activate the uracil-N-glycosylase and at 95 °C for 10 min for denaturation, two-step PCR amplification was performed with 40 cycles of 94 °C for 15 s and 56 °C for 1 min. Amplification data were collected and analyzed using Sequence Detection Software version 1.0 (Applied Biosystems, CA, USA). The sensitivity was evaluated using tenfold serial dilution of viral RNA and performing single real time RT-PCR versus multiplex real time RT-PCR for RV-A and PP7.

For all molecular procedures, four separate rooms were used to avoid cross-contamination of samples. Milli-Q water (Invitrogen, USA) was used as a negative control, in all procedures.

2.6. Semi-nested RT-PCR

Semi-nested RT-PCR for RV-A VP4 and VP7 detection has been used for molecular classification of RV-A into the G (VP7) and P (VP4) genotypes. The primers and protocols for this detection have been described previously (Das et al., 1994; Gentsch et al., 1992).

3. Results

3.1. Establishment of the multiplex quantitative PCR assay

In this study a multiplex qPCR assay was designed to detect RV-A and PP7 simultaneously. The sensitivity of the multiplex qPCR assay was evaluated in parallel experiments comparing the SC generated using purified DNA in a monoplex qPCR assay with the SC generated using the same cDNA in the multiplex qPCR assay and with results obtained from the sewage concentration tests. Table 2 lists the Ct values obtained for the standard curve using purified plasmid DNA to quantify RV-A and PP7 using the monoplex and multiplex strategies. No differences were observed over the range of DNA concentrations tested using these methods.

3.2. Comparison of the concentration methods for rotavirus A recovery from residual water

Using the ultracentrifugation-based method, natural contamination was observed in two unseeded samples used as negative controls (see Section 2). After concentration of the sample using this method, the qPCR assay detected a mean of 1.9×10^6 RV-A genomes per L of raw sewage and demonstrated a positive result until the tenfold dilution. In contrast, following concentration with the adsorption-elution method, naturally occurring RV-A was detected in just one sample, and the number of RV-A copies recovered was ten times less (1.8×10^5 cDNA copies per L of raw sewage) compared with the ultracentrifugation-based method. These values for the pre-existing RV-A level in the samples were used to correct the total copy number determined following either concentration strategy (see Section 2).

In this way, the corrected recovery rate following the ultracentrifugation method was 47% (range: 34–60%), while the corrected recovery rate following the adsorption-elution method was 3.5% (range: 1.5–5.5%; Fig. 1).

3.3. Natural occurrence of Rotavirus A

All fourteen raw sewage samples concentrated using the ultracentrifugation method and tested by qPCR tested positive for RV-A and PP7. The number of RV-A genomes per L of raw sewage ranged from 2.5×10^4 to 1.6×10^7. The VP4 and VP7 genes were genotyped in all positive samples, and 14 samples were G2, five samples were P4 and five samples were P6. In four samples, the P genotype could not be determined.

4. Discussion

In the present study, the efficiency of two methods used to concentrate viruses from raw sewage samples spiked with RV-A and PP7 was evaluated. The multiplex qPCR assay detected as few as 30 RV-A and 10 PP7 genomes per reaction with Ct values of 34.82 and 37.51, which is comparable to the monoplex qPCR used in this study and the original published protocol (Rajal et al., 2007; Zeng et al., 2008).

The advantages of using the multiplex protocol for the simultaneous amplification and quantification of the target and IC sequences include reduced procedure time and reagent cost. Environmental sewage samples can potentially contain a large quantity of PCR inhibitors, which could cause false negative results (Ijzerman et al., 1997). Therefore, the IC may assist in the detection of inhibitors. The successful amplification of the IC confirms that a negative result obtained for the tested virus is not a false negative (Rajal et al., 2007; Rolfe et al., 2007). The usefulness of an IC to avoid false negative results has been recognized, and the development

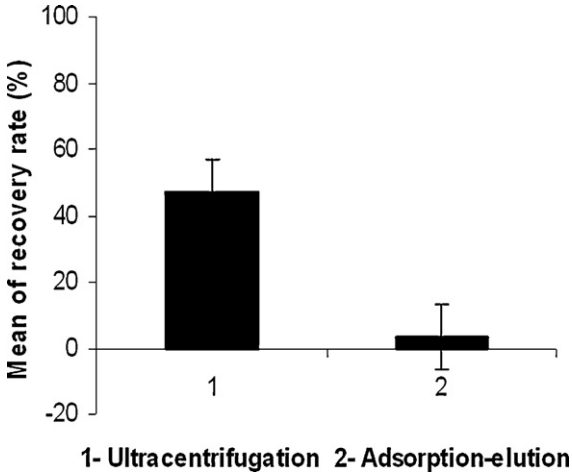

Fig. 1. The mean recovery rate of RV-A from sewage water following concentration using either the ultracentrifugation or adsorption-elution method ($n = 6$).

Table 2
The mean Ct value obtained from the standard curve using either a monoplex or multiplex assay to detect rotavirus A (RV-A) and bacteriophage PP7 ($n = 6$).

Virus	PCR type	Quantity[a]/Ct value (sd[b])			
		10^7	10^5	10^3	10^1
RV-A	Monoplex	14.86 (0.030)	21.34 (0.10)	27.95 (0.121)	34.69 (0.55)
	Multiplex	15.15 (0.085)	21.87 (0.036)	28.50 (0.135)	34.82 (0.238)
PP7	Monoplex	16.62 (0.079)	23.13 (0.094)	29.49 (0.022)	36.28 (0.789)
	Multiplex	17.41 (0.086)	23.01 (0.030)	30.52 (0.079)	37.51 (0.392)

[a] Presented as the genome copy number per reaction.
[b] Standard deviation.

of a multiplex qPCR assay to detect RV-A and an IC simultaneously would meet the conditions for a suitable method for routine laboratory use: sensitivity, reproducibility, consistency and cost-effectiveness. There are no reports of PP7 occurring naturally in natural waters, and it was used previously to validate hollow fiber ultrafiltration because of its similarity in size (25 nm) and physico-chemical properties to poliovirus (Rajal et al., 2007).

The ultracentrifugation-based method resulted in a higher recovery rate of RV-A compared to the adsorption-elution method. Similar results were not obtained for PP7, demonstrating that the recovery efficiency depends on the physical and chemical properties of each virus, including specific density, morphology and membrane attachment patterns (Lewis and Metcalf, 1988; Nupen and Bateman, 1985). The high initial cost to acquire an ultracentrifuge has been a drawback preventing the widespread use of this technique. However, in laboratories with access to an ultracentrifuge, this method is an excellent alternative since it is robust and simple, and the tubes can be autoclaved and reused (Wyn-Jones and Sellwood, 2001).

The filters used in the adsorption-elution method can be clogged by the large quantity of debris that may be present in the sample, reducing the recovery rate (Guimarães et al., 2008; Victoria et al., 2010). Therefore, to prevent clogging, the sewage samples were diluted in 2 L of distilled water.

Both methods have been described as a useful tools to recover enteric viruses from sewage samples, allowing for detection using molecular methods, such as regular PCR and qPCR (Bofill-Mas et al., 2006; Guimarães et al., 2008; Ferreira et al., 2009; Fumian et al., 2010; Haramoto et al., 2006; Miagostovich et al., 2008; Pina et al., 1998; Rodríguez-Díaz et al., 2009; da Silva et al., 2007; Victoria et al., 2009; Villar et al., 2007).

To demonstrate the usefulness of the combination of the ultracentrifugation method and the NSP3 multiplex qPCR for investigating the natural occurrence of RV-A in wastewater, fourteen raw samples were analyzed. The results demonstrated the environmental dissemination of different G and P genotypes. As described previously, NSP3 qPCR provide a rapid and sensitive method for detection of RV-A and can detect different rotavirus G types, demonstrating its use for the detection of RV-A in environmental samples (Pang et al., 2004; Zeng et al., 2008). Moreover, according to Matthijnssens et al. (2008) NSP3 is the second-most conserved gene between the eleven genes of RV.

The multiplex qPCR assay evaluated in this study demonstrated a sensitivity similar to the monoplex assay and reduced the time and cost of detecting viruses in environmental samples. The availability of a sensitive method for viral quantification that includes an internal control, along with knowledge of the recovery efficiency of the concentration method, allows for a more accurate assessment of the potential presence of viruses in the environment.

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