Recovery rate of multiple enteric viruses artificially seeded in water and concentrated by adsorption–elution with negatively charged membranes: interaction and interference between different virus species

Andréia Dalla Vecchia, Caroline Rigotto, Mayra Cristina Soliman, Fernanda Gil de Souza, Isabel Cristina Giehl and Fernando Rosado Spilki

ABSTRACT

Viral concentration method by adsorption–elution with negative membranes has been widely employed for concentrating viruses from environmental samples. In order to provide an adequate assessment of its recovery efficiency, this study was conducted to assess viral recovery rates for viral species commonly found in water (HAdV-5, EV, RV, BAdV and CAV-2), quantifying viral genomes at the end of the five different steps of the process. Recovery rates were analyzed for several viruses combined in a single water sample and for each virus assayed separately. Ultrapure water samples were artificially contaminated and analyzed by real-time quantitative polymerase chain reaction (qPCR). High recovery rates were found after the final stage when assessed individually (89 to 125%) and combined in the same sample (23 to > 164%). HAdV-5 exhibited > 100% recovery when assayed with human viruses and other AdVs, whereas BAdV and CAV-2 were not detected. These data suggest that recovery efficiency could be related to viral structural characteristics, their electric charges and other interactions, so that they are retained with greater or lesser efficiency when coupled. This protocol could be applied to environmental samples, since high recovery rates were observed and infectious viruses were detected at the end of the concentration process.

Key words | adsorption–elution method, enteric viruses, recovery rate, viral viability

INTRODUCTION

Pathogenic enteric viruses such as enterovirus (EV), rotavirus (RV) and adenovirus (AdV) are present in the gastrointestinal tract and are frequently eliminated in feces, both by symptomatic individuals and those running subclinical infections. These agents are excreted in high titres, the transmission occurs via the fecal–oral route in food and water and the infections may trigger a range of different conditions, with emphasis on disorders affecting the gastrointestinal tract (Bosch et al. 2008; Hamza et al. 2009), thus inducing an impact on public health (Fong & Lipp 2005). These agents are commonly found contaminating surface waters in urban areas because of continuous discharge of domestic sewage without adequate treatment (Donovan et al. 2008; Sinclair et al. 2009). Adenoviruses are classified as members of the Adenoviridae family and the Mastadenovirus genus. They are icosahedral, they have a double-stranded DNA genome with 26 to 45 kb and a 70–100 nm viral particle (Berk 2007). Enteroviruses have a single-stranded positive RNA genome, are small, at approximately 28 to 30 nm, and belong to the Picornaviridae family (Pallansch & Roos 2007; Racaniello 2007). The RV genome is a double-stranded segmented RNA, it is 65–75 nm in diameter, and these viruses belong to the genus Rotavirus and the Reoviridae family (Estes & Kapikian 2007). All AdV, EV and RV are nonenveloped and as such are highly resistant to adverse environmental factors such as pH, heat, dehydration and disinfectant compounds (Racaniello 2007; Bosch et al. 2008).

It is difficult to detect the presence of these viral particles in water and it is necessary to employ viral concentration methods and molecular techniques such as real-time quantitative polymerase chain reaction (qPCR),

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which is a viable tool that is widely used for detecting viruses in environmental samples because of its sensitivity, specificity and the speed of processing (Griffin et al. 2003; Bosch et al. 2008). A number of different methods exist for concentrating viruses in water samples, including adsorption-elution with a positively charged membrane, adsorption-elution with a negative membrane, flocculation, and ultrafiltration (Grabow 2001; Katayama et al. 2002). Recently the ultracentrifugation method has been shown to offer high recovery rates (Fumian et al. 2010; Prata et al. 2012). In practice, the best processing method for viral concentration from environmental matrixes has not yet been defined. However, the low number of studies designed to adequately assess the efficiency of viral recovery from water samples when adsorption-elution methods for viral concentration are used always raises questions and doubts with regard to undesirable under estimation or overestimation, whether of detection rates or of the viral loads found (Di Pasquale et al. 2010; Cashdollar & Wymer 2013). Additionally, it is unknown what types of interferences may arise from different viruses in a single sample when the method used is based on adsorption or adhesion of particles to a solid substrate.

In this view, the aim of this study was to evaluate the efficiency of a method for viral concentration by adsorption-elution that employs a negatively charged membrane, in order to determine recovery rates for the human enteric viruses HAdV-5 (adenovirus), EV (enterovirus) and RV (rotavirus), and a selection of species of adenovirus, HAdV-5 (human adenovirus serotype 5), BAdV (bovine) and CAV-2 (canine adenovirus serotype 2), and detect possible interference with recovery when multiple viral species are present in the same sample. More specifically, this study evaluates the five different stages of the concentration process, in tests with each different virus in a separate sample and when multiple viruses are combined into a single water sample, in order to identify possible interactions between viruses and the negative membrane, between viral particles of different species and, consequently, to determine the recovery rate after concentration and relative recovery rates when more than one type of virus is present.

**MATERIALS AND METHODS**

**Virus recovery experiments**

In order to determine the viral recovery efficiency of the adsorption–elution concentration method that utilizes a negatively charged membrane (Katayama et al. 2002), as slightly modified (Dalla Vecchia et al. 2012a, b), known viral titers of HAdV-5 $6.85 \times 10^{10}$ GC/ml (GC: genomic copies), EV $1.0 \times 10^8$ ID/ml (ID: infectious dose) and RV $1 \times 10^7$ ID/ml were inoculated into 500 ml of MilliQ water (purified), that had been autoclaved in advance to remove any prior contamination by the viruses under investigation. These experiments consist of evaluations of the viral recovery rate at different stages of the concentration process for three species of human virus artificially seeded into MilliQ water, individually or in combination. A second series of experiments was then conducted to test the efficiency of recovery of species of human adenovirus and of adenoviruses from animal hosts. Samples of MilliQ water were inoculated with HAdV-5 ($6.85 \times 10^{10}$ GC/ml), BAdV-3 ($1 \times 10^8$ ID/ml) and (CAV-2 $8.7 \times 10^7$ GC/ml) individually into separate flasks of MilliQ water and all three viruses, HAdV-5, BAdV-3 and CAV-2, were also seeded into the same flask containing 500 ml of ultrapure water. For each experiment, 1 ml samples were collected at each stage of the viral concentration process (Figure 1). The process comprises five stages, as follows: stage 1- after contamination of samples, but before starting the concentration protocol proper; 2- after adjusting pH and after addition of 25 mM MgCl$_2$; 3- after the water has passed through the membrane; 4- after 0.5 mM H$_2$SO$_4$ has passed through the membrane; 5- final sample collection, after adding 1 mM NaOH and neutralizing the sample with 50 mM H$_2$SO$_4$ and TE 100×. For each experiment, one aliquot was drawn before inoculation of viruses, to provide a negative control for each test, and also at each stage: in stage 2 after insertion of salts and pH adjustment, in stage 5 in the collection cup, in stage 4 after the passage of H$_2$SO$_4$, and in stage 5 after the addition of NaOH and neutralization with H$_2$SO$_4$ and TE 100×. The concentration product was aliquoted, stored in a freezer at −80°C until extraction; synthesis of cDNA

**Figure 1** | Stages of the process for concentration of viruses in water samples, based on a protocol described by Katayama et al. (2002), with minor adaptations.
for RNA viruses and then qPCR was performed for all samples. For all viral species, recovery rates were calculated by comparing the quantification according to the qPCR reaction at each stage with the quantification at 5 μl detected in stage 1 (unconcentrated sample).

### Molecular detection

Viral genomes recovered using the concentration protocol were then extracted using a commercial kit (RTP DNA/RNA Virus Mini Kit, Stratagene®, Carlsbad, California, USA) and reverse primers (High Capacity cDNA Reverse Transcription, Applied Biosystems®, Germany) according to the manufacturer’s instructions, using an initial volume of 400 μl of concentrated material and producing 60 μl of elution product at the end of the extraction process. Next, the reverse transcription stage was conducted using a kit (High Capacity cDNA Reverse Transcription, Applied Biosystems®, Carlsbad, California, USA) and reverse primers specific for each virus with an RNA genome. The next step was to perform the qPCR assay, which comprised a final reaction of 25 μl using a commercial kit (Platinum Syber Green qPCR SuperMix-UDG, Invitrogen, California, USA) and 5 μl of the sample diluted 10x in water free from DNase and RNase in order to reduce inhibitors and increase the efficiency of recovery. Table 1 lists the primers employed for each virus tested in the recovery experiments. Reactions for detection of EV comprised 40 cycles at an annealing temperature of 56 °C and with a detection sensitivity of 1.65 × 10^1 ID/5 μl. The qPCR reactions for HAdV-5 (VTB2 primer) comprised 40 cycles with an annealing temperature of 55 °C and sensitivity of 6.2 × 10^1 GC/5 μl, whereas for non-human AdVs (F1 and R1 primers) reactions comprised 50 cycles with an annealing temperature of 58 °C and sensitivity of 6.88 × 10^1 GC/5 μl. Finally, the qPCR reactions for RV comprised 40 cycles, with an annealing temperature of 54 °C and analytical sensitivity of up to 1.62 × 10^1 ID/5 μl. For all reactions standard curves were used as positive controls. The curves were produced by serial base 10 dilutions of the viral suspensions in known concentrations of ID/5 μl for EV and RV and in CG/5 μl for the AdVs. The positive controls used for quantification of HAdV in GC by qPCR were obtained from known titers of virus suspensions expressed in PFU (plaque forming units) and calculated on the basis of quantification of a commercially available AdV (AmpliRun® ADENOVIRUS DNA CONTROL – Vircell, Granada, Spain). The different species of AdV were distinguished from one another by observation of specific melting temperatures for each virus, which were 85.5 °C for BAdV and 82 °C for CAV, and confirmation was obtained on the basis of a positive sample of each virus in the assay. The positive controls for EV and RV employed in the molecular assays were viral suspensions with known titers obtained by cell culture. For all assays ‘no template controls’ (NTC) and negative controls were used to rule out cross contamination in the qPCR reactions. All qPCR assays for virus recovery were conducted in three independent experiments for each virus studied and all reactions were conducted with the aid of StepOne™ Software v2.2.2 – Applied Biosystems and Bio-Rad iQ5, Optical System Software – version 2.1.

### Analysis of viral viability by ICC-qPCR

The samples collected during the process of filtration through the negatively charged membrane (Figure 1) were also assessed to test the viability of the viruses that had been inoculated artificially, using HAdV-5 as the model for DNA viruses and EV (Polio 1) as the model for RNA viruses and employing the same cellular model for co-infection for both viruses (A549). Viability was tested using an integrated cell culture quantitative PCR (ICC-qPCR) technique adapted from Ko et al. (2005) and Rigotto et al. (2010), with certain modifications.

Before the viral viability of the concentrated samples was analyzed using ICC-qPCR analysis, a pilot study was conducted in order to determine the best incubation period for the viral fluids, which were subjected to ten serial base 10 dilutions and then immediately inoculated.
into cells. Viral fluids at concentrations of HAdV-5 $6.85 \times 10^{10}$ GC/ml ($1.5 \times 10^6$ PFU/ml) and EV $1 \times 10^8$ ID/ml were used to infect A549 cells (permissive to both viruses), which were then incubated for periods of 24, 48, 72 and 120 h. After these periods had elapsed, supernatant was removed and viral nucleic acids were extracted from the cellular lysate with a commercial kit (RTP DNA/RNA Virus Mini Kit, Stratec®, Germany) according to the manufacturer’s instructions. Infectious viruses were quantified by ICC-qPCR. This pilot study demonstrated that 48 h was the ideal duration of the incubation period for quantifying the two viruses under investigation (HAdV-5 and EV), since samples were positive up to the $10^{-7}$ dilution, equating to concentrations of $6.96 \times 10^5$ GC/μl and $7.97 \times 10^5$ ID/5 μl for HAdV-5 and EV, respectively.

For the analysis of the viral infectivity of the concentrated samples, A549 cells (cell density: $2.5 \times 10^5$ cells/well) were cultivated in 24-well plates and inoculated with 200 μl of concentrate pre-diluted in MEM 1× (dilution factor established on the basis of the results of the cytotoxicity test). The inocula had been filtered in advance through a 0.22 m membrane. The inoculum was placed into contact with the cells for 1 h, at 37 °C in a 5% CO₂ atmosphere and agitated uniformly every 15 min. Next, the inoculum was removed and 1 ml of MEM containing 1% PSA was added to the cells. Two cell controls were also prepared containing only the cells and the maintenance medium. Plates were then incubated at 37 °C for 48 h, in a 5% CO₂ atmosphere. After 48 h, total nucleic acids were extracted, as mentioned above. A reverse transcription (RT) was needed to obtain the EV cDNA, followed by qPCR to quantify the number of genome copies created by infectious viruses for AdV and to quantify the infectious dosage for EV.

**Calculation of viral recovery rate**

Viral recovery rates were calculated by taking the values observed for qPCR during the first stage of the process as baseline, and defining this level as 100% recovery, since we are not evaluating losses during stages prior to extraction and cDNA. The values recovered from 5 μl by qPCR after stages 2 through 5 were calculated by dividing the results observed by the quantity recovered in the first stage and then multiplying by 100, as follows: $X = b/a \times 100$, where $X =$ recovery rate (%), $a =$ quantification result for 5 μl by qPCR after stage 1, and $b =$ result for 5 μl by qPCR after each stage 2 through 5.

**RESULTS**

**Viral infectivity after concentration steps**

The analysis of EV viral infectivity detected positive samples at stage 5 (end of concentration) at a concentration of $3.17 \times 10^6$ ID/l. The HAdV-5 analyses were positive after stage 2 (after adjusting pH and adding MgCl₂) and after stage 5, at concentrations of $4.0 \times 10^7$ GC/l and $7.2 \times 10^6$ GC/l, respectively.

**Efficiency of recovery of different viruses**

Table 3 lists the viral recovery rates achieved for the three different species of AdVs, showing that it was not possible to determine the recovery efficiency for BAdV or for CAV-2 when we evaluated all three AdVs in a single sample. The presence of three different adenoviruses in the same flask and the use of qPCR primers that simultaneously detect all of the viral species, meant that it was not possible to quantify the concentration of each virus specifically and this is a limitation of the technique. Notwithstanding, it was possible to determine whether the results were positive for each species using the disassociation curve. The HAdV-5 virus exhibited the highest recovery rate after the last stage (10270%) and the greatest loss (17.4%) after stage 3 and was the only adenovirus detected at the end of concentration when all AdVs were tested in the same sample. The BAdV virus was detected after stages 2, 3 and 4, but was not detected at stage 5 and all stages were negative for CAV-2 when it was tested in conjunction with the other AdVs. When assessed individually, BAdV exhibited high recovery rates after stages 2 and 5 and did not suffer viral losses during stages 3 or 4, while CAV was only detected at the end of concentration ($1.03 \times 10^{10}$ GC/l) and could not be detected at any other stage in the process.

**DISCUSSION**

This study evaluated the efficiency of virus recovery for a selection of species of viruses found in humans and other animals after each of five steps of a process of concentration by adsorption–elution with a negatively charged membrane. The results revealed high viral recovery rates before starting to pass the water through the membrane (stage 2), which was expected, since at this stage only pH adjustment and addition of MgCl₂ have so far occurred, leading to recovery rates varying from 57.7% to greater than 100% (Tables 2 and 3).
<table>
<thead>
<tr>
<th>Concentration process steps</th>
<th>HAdV-5 % mean* (min max)</th>
<th>HAdV-5 mean GC/5 μl* (min max)</th>
<th>EV % mean* (min max)</th>
<th>EV mean ID/5 μl* (min max)</th>
<th>RV % mean* (min max)</th>
<th>RV mean ID/5 μl* (min max)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MilliQ Water, just 1 virus</td>
<td>100%</td>
<td>5.78 x 10^6 (3.55 x 10^6–7.10 x 10^6)</td>
<td>100%</td>
<td>2.47 x 10^3 (2.24 x 10^3–2.66 x 10^3)</td>
<td>100%</td>
<td>4.05 x 10^2 (1.96 x 10^2–5.73 x 10^2)</td>
</tr>
<tr>
<td>1-after concentration MgCl₂ and adjusting pH</td>
<td>94.6 (66.4–129.1)</td>
<td>4.73 x 10^6 (4.45 x 10^6–5.15 x 10^6)</td>
<td>126.3 (106.5–166.4)</td>
<td>3.12 x 10^3 (2.67 x 10^3–3.72 x 10^3)</td>
<td>57.7 (0.0–132.3)</td>
<td>2.34 x 10^2 (1.31 x 10^2–5.89 x 10^2)</td>
</tr>
<tr>
<td>2-after MgCl₂ and adjusting pH</td>
<td>0.08 (0.06–0.09)</td>
<td>4.08 x 10^3 (2.12 x 10^3–5.80 x 10^3)</td>
<td>89.0 (85.0–93.9)</td>
<td>2.20 x 10^3 (1.96 x 10^3–2.50 x 10^3)</td>
<td>0.8 (0.0–0.8)</td>
<td>3.10 x 10^2 (2.24 x 10^2–3.95 x 10^2)</td>
</tr>
<tr>
<td>3-after filtering water through membrane</td>
<td>1.05 (0.70–1.14)</td>
<td>5.28 x 10^4 (3.28 x 10^4–7.61 x 10^4)</td>
<td>32.0 (28.5–35.2)</td>
<td>7.91 x 10^2 (7.15 x 10^2–9.38 x 10^2)</td>
<td>1.9 (0.0–2.7)</td>
<td>7.88 x 10^2 (3.65 x 10^2–1.21 x 10^3)</td>
</tr>
<tr>
<td>4-after passing H₂SO₄ through membrane</td>
<td>30.4 (29.8–30.9)</td>
<td>2.00 x 10^5 (1.37 x 10^5–3.06 x 10^5)</td>
<td>55.6 (54.5–56.9)</td>
<td>7.17 x 10^2 (6.33 x 10^2–8.21 x 10^2)</td>
<td>45.6 (3.9–2135.7)</td>
<td>8.58 x 10^1 (9.47 x 10^1–2.25 x 10^2)</td>
</tr>
<tr>
<td>5-end of concentration</td>
<td>91.8 (56.1–105.7)</td>
<td>4.59 x 10^6 (3.75 x 10^6–6.04 x 10^6)</td>
<td>89.0 (83.4–96.2)</td>
<td>2.20 x 10^3 (2.09 x 10^3–2.35 x 10^3)</td>
<td>125.4 (0.0–134.8)</td>
<td>5.08 x 10^2 (4.15 x 10^2–6.00 x 10^2)</td>
</tr>
<tr>
<td>MilliQ Water, all three viruses</td>
<td>100%</td>
<td>6.57 x 10^6 (4.53 x 10^6–9.88 x 10^6)</td>
<td>100%</td>
<td>1.29 x 10^3 (1.11 x 10^3–1.51 x 10^3)</td>
<td>100%</td>
<td>1.88 x 10^2 (1.08 x 10^2–3.21 x 10^2)</td>
</tr>
<tr>
<td>1-after concentration MgCl₂ and adjusting pH</td>
<td>123.6 (116–147.4)</td>
<td>8.12 x 10^6 (6.19 x 10^6–1.15 x 10^6)</td>
<td>178.3 (121.6–199.2)</td>
<td>2.30 x 10^3 (1.53 x 10^3–3.00 x 10^3)</td>
<td>177.1 (51.5–8656.9)</td>
<td>3.33 x 10^3 (9.33 x 10^3–7.80 x 10^3)</td>
</tr>
<tr>
<td>2-after MgCl₂ and adjusting pH</td>
<td>30.4 (29.8–30.9)</td>
<td>2.00 x 10^5 (1.37 x 10^5–3.06 x 10^5)</td>
<td>55.6 (54.5–56.9)</td>
<td>7.17 x 10^2 (6.33 x 10^2–8.21 x 10^2)</td>
<td>45.6 (3.9–2135.7)</td>
<td>8.58 x 10^1 (9.47 x 10^1–2.25 x 10^2)</td>
</tr>
<tr>
<td>3-after filtering water through membrane</td>
<td>7.4 (5.9–8.2)</td>
<td>4.89 x 10^2 (2.69 x 10^2–8.12 x 10^2)</td>
<td>1.8 (1.6–2.07)</td>
<td>2.39 x 10^1 (2.06 x 10^1–2.60 x 10^1)</td>
<td>162.7 (1.3–284.7)</td>
<td>3.06 x 10^2 (2.23 x 10^2–9.14 x 10^2)</td>
</tr>
<tr>
<td>4-after passing H₂SO₄ through membrane</td>
<td>164.3 (149.4–180.9)</td>
<td>1.08 x 10^7 (8.09 x 10^6–1.48 x 10^7)</td>
<td>23.8 (16.0–34.2)</td>
<td>3.08 x 10^2 (2.01 x 10^2–3.81 x 10^2)</td>
<td>32.7 (8.4–553.4)</td>
<td>6.15 x 10^1 (5.97 x 10^1–1.58 x 10^2)</td>
</tr>
</tbody>
</table>

*Average calculated based on the values of the three experiments.
This stage is essential to the concentration process, since addition of salts alters the charges on the viral particles, turning them positive and charged with cations, making it more likely the viruses will be retained by the negatively charged membrane (Katayama et al. 2002). When only human viruses were tested, the greatest recovery efficiency was achieved at the end of concentration (stage 5) for HAdV-5 164.3% (149.4 to 180.9%) and for RV 32.7% (8.4 to 553.4%), contrasting with EV at 23.8% (16.0 to 34.2%) when combined with other viruses in the sample (Table 2). In the tests of the different AdV species, HAdV-5 exhibited a high recovery rate at the end of the process (10270%), whereas BAdV and CAV-2 could not be detected after the last stage (Table 3). These data suggest that recovery efficiency could be related to structural characteristics that are peculiar to each virus and impact on adsorption through their charges and through interactions between each other when in the same sample, so that they are retained with greater or lesser efficiency, as loss through the membrane pores is avoided or increased (Cashdollar & Wymer 2013). These data are similar to results found by Haramoto et al. (2002), who used the electronegative membrane-vortex (EMV) method using an electronegative membrane and addition of cations to concentrate both viruses and protozoans simultaneously, but observed variable recovery rates (27.7 to 86.5%) for poliovirus (28.0–60.0%) and for oocysts of Cryptosporidium, combined with high detection rates for HAdV and Norovirus GI and GII, when mixed in the same sample. The recovery efficiency at stage 5 (after concentration) for HAdV-5 was extremely high (10270%), but BAdV and CAV were not detected, suggesting that there is inhibition of adsorption of AdVs from other animals in the presence of human adenovirus, since only HAdV-5 exhibited efficient recovery at the end of concentration when we analyzed all three AdV species in the same samples. When assayed in isolation, HAdV-5 was detected (91.8%) and BAdV exhibited an extremely high (10270%), 2 log greater than the value detected after stage 1, but the presence of CAV-2 was only detected at the last stage of concentration (1.03 × 10^{10} GC/l), and was not detected when analyzed together with other AdVs (Table 3).

When the three species of adenovirus were assayed, no losses were observed during intermediate stages (3 and 4) when BAdV and CAV-2 were tested alone. However, when the human viruses were assayed, viral losses were observed after steps 3 and 4, varying from 0.08% for HAdV-5 to >100% for RV, when assessed in isolation or in conjunction, which was not expected, since the

### Table 3 | Detection, quantification (GC/5 μl) and mean rate (%) of virus recovery for the adenoviruses BAdV, CAV-2 and HAdV-5 after the 5 steps of a concentration process in ultrapure water

<table>
<thead>
<tr>
<th>Concentration stages</th>
<th>BAdV % mean* (min max)</th>
<th>CAV-2 mean* GC/5 μl</th>
<th>HAdV-5% mean* (min max)</th>
<th>HAdV-5 mean GC/5 μl* (min max)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MilliQ Water, just 1 virus</td>
<td>100%</td>
<td>ND</td>
<td>100%</td>
<td>5.78 × 10^{6} (3.55 × 10^{6}–7.10 × 10^{6})</td>
</tr>
<tr>
<td>1-before concentration</td>
<td>2-after MgCl2 and pH adjustment</td>
<td>129 (70.0–136.7)</td>
<td>ND</td>
<td>94.6 (66.4–129.1)</td>
</tr>
<tr>
<td>3-after filtering water through membrane</td>
<td>0.0</td>
<td>ND</td>
<td>0.08 (0.06–0.09)</td>
<td>4.08 × 10^{5} (2.12 × 10^{5}–5.80 × 10^{5})</td>
</tr>
<tr>
<td>4-after passing H2SO4 through membrane</td>
<td>0.0</td>
<td>ND</td>
<td>1.05 (0.7–1.1)</td>
<td>5.28 × 10^{4} (3.28 × 10^{4}–7.61 × 10^{4})</td>
</tr>
<tr>
<td>5-end of concentration</td>
<td>12170 (6902.5–15204.8)</td>
<td>3.46 × 10^{5}</td>
<td>91.8 (56.1–105.7)</td>
<td>4.59 × 10^{6} (3.75 × 10^{6}–6.04 × 10^{6})</td>
</tr>
<tr>
<td>MilliQ Water, all three viruses</td>
<td>Positive*</td>
<td>ND</td>
<td>100%</td>
<td>1.45 × 10^{7} (1.02 × 10^{7}–1.98 × 10^{7})</td>
</tr>
<tr>
<td>1-before concentration</td>
<td>2-after MgCl2 and pH adjustment</td>
<td>Positive*</td>
<td>ND</td>
<td>105.5 (90.4–115.6)</td>
</tr>
<tr>
<td>3-after filtering water through membrane</td>
<td>Positive*</td>
<td>ND</td>
<td>17.4 (9.9–19.8)</td>
<td>2.53 × 10^{6} (1.02 × 10^{6}–3.83 × 10^{6})</td>
</tr>
<tr>
<td>4-after passing H2SO4 through membrane</td>
<td>Positive*</td>
<td>ND</td>
<td>0.3 (0.2–0.4)</td>
<td>4.93 × 10^{4} (4.16 × 10^{4}–5.38 × 10^{4})</td>
</tr>
<tr>
<td>5-end of concentration</td>
<td>ND</td>
<td>ND</td>
<td>10270 (5382.1–15979.0)</td>
<td>1.49 × 10^{9} (5.48 × 10^{8}–3.1 × 10^{9})</td>
</tr>
</tbody>
</table>

*Not quantified in qPCR reaction. ND: Not detected.
*Average calculated based on the values of the three experiments.
concentration process should tend to increase detection efficiency and should not allow viral losses as the process progresses, because the majority of the viral particles should be retained by the affinity of their electrostatic charges to the negatively charged membrane (Di Pasquale et al. 2010). Passing the acid solution (H₂SO₄ 0.5 mM) through the membrane (stage 4) is intended to remove residual cations and other inhibitors, without removing the viral particles that have adhered to the membrane (Katayama et al. 2002). Although the results show that there were losses during intermediate stages, the level of virus recovery obtained at the end of the process (stage 5) demonstrated viability, since infectivity rates of 7.2 × 10⁶ GC/l for HAdV-5 and 3.17 × 10⁵ ID/l for EV were detected by ICC-qPCR. This finding adds further weight to evidence reported by Miagostovich et al. (2014) and Rigotto et al. (2010) who observed infectious HAdV in samples of superficial water concentrated using the same method. This demonstrates that even under the varying and extreme pH conditions (ranging from 5 to 10) to which they were subjects during the concentration process, viral particles remained infectious.

For HAdV-5 and RV, virus recovery efficiency was greater than baseline (before concentration): (1) when the sample analyzed contained all of the viruses in the same flask, (2) after the stage in which ionic charges are adjusted by adding MgCl₂ and pH is adjusted, and (3) at the end of concentration. The concentration of EV was greater than baseline when it was assayed in isolation and in stage 2 of the assays of samples containing multiple viruses (Table 2). The greater than 100% increases in recovery rates after addition of salts confirm their importance and their potential effect on adsorption of the virus by the membrane, and also demonstrate the viability of the method for detecting different viral species in the same water sample (Katayama et al. 2002; Fong & Lipp 2005).

These data show that the method of viral adsorption–elution using a negatively charged membrane employed in this study produced high viral recovery rates, particularly for recovery of the human viruses, since at the end point it detected 23.8%, 164.3% and 32.7% of the viruses EV, HAdV-5 and RV, respectively, when assayed in the same sample. High recovery rates at the end of the process are not surprising and reaffirm the method’s viability, considering that at this stage of concentration it is expected that there will be a greater yield than at the non-concentrated stage. High recovery rates of 89.0%, 91.8%, 125.4% and 12170% were also observed for EV, HAdV-5, RV and BAdV, respectively, when assayed individually in ultrapure water. These data corroborate the findings of other investigators who have reported high recovery rates for human viruses in waters of this type, such as, for example, Haramoto et al. (2009a, b) who showed a mean recovery >100% for HuNoV and Poliovirus and Rigotto et al. (2009) who observed 100% recovery efficiency for HAdV in MilliQ water.

This study demonstrated that the adsorption–elution protocol for detection of viruses in ultrapure water is valid and offers a margin of detection such that it is expected that we will be able to apply it to environmental samples, with a clearer idea of the process and the losses that can be expected during intermediate stages. Recovery rates observed after the final stage varied from 89 to 125% when viruses were assayed individually and rates ranged from 23 to greater than 100% when the three human viruses were present in the same sample (Table 2). Recovery rates when assayed in the same sample were 164%, 23% and 32% for HAdV-5, EV and RV, respectively, and when the same viruses were assayed separately we achieved 91%, 89% and 125%. Therefore, EV and RV had lesser recovery rates in the presence of other viruses, and higher rates of the three viruses assayed when analyzed separately. HAdV-5 had high recovery rates whether analyzed isolated or in conjunction with other viruses, at 91% and 164%, respectively, demonstrating that this protocol is suitable for applications in which viruses are monitored in environmental matrices. Notwithstanding, other studies have reported inferior recovery rates when the protocol has been applied to environmental samples. Miagostovich et al. (2014) achieved recovery rates ranging from 0.5 to 6.2% for RVA and 3.3 to 6.7% for NoVGII in polluted water samples from different parts of Rio de Janeiro. Fumian et al. (2010) observed low recovery efficiency for RVA, with a mean of 3.5% (1.5 to 5.5%) in wastewater. Victoria et al. (2009) achieved rates varying from 3% to 64% in several different environmental sources. Haramoto et al. (2009a) observed a wide variation (15 to 100%) in recovery rates in a range of environmental matrices. Rigotto et al. (2009) also reported lower rates (10%) for HAdV in a number of different environmental matrices. Additionally, Prado et al. (2014) obtained a viral recovery rate lower than 7.5%, both for a concentration method employing meat extract and for ultracentrifugation, but 90% for HAdV, 50% for RVA and 8% for NoV were achieved in sewage samples using a method based on elution with meat extract (recovery efficiency <4.3%).

It is extremely important to design a highly sensitive method with high recovery efficiency that can guarantee viral detection in environmental samples. In the present study, the adsorption–elution method with negative
membrane demonstrated recovery efficiency and although the rates were different for different viruses they are suitable for application to environmental matrices, since a rate of 54% was achieved for HAdV in superficial waters in other studies that have attempted to detect different viral species (Dalla Vecchia et al. (2022a, b), study ongoing, data not shown). Additionally, Corrêa & Miagostovich (2013) employed a negative membrane in their concentration process and achieved recovery rates for murine and human norovirus varying from 66 to 32% in lettuce samples. Notwithstanding, the ultracentrifugation technique has also exhibited higher recovery rates when applied to a range of different environmental matrices. Prata et al. (2022) observed a mean rate of 69 (66–72%) in residual waters and 71 (66–76%) in recreational waters, Fumian et al. (2010) observed 47 (34–60%) recovery efficiency for RVA in wastewater, which is better than the adsorption–elution method based on ultrafiltration which detected 3.5% positivity. On the other hand, Prado et al. (2014) observed low recovery rates using the ultracentrifugation method with wastewater (lower than 7.3%) and the detection rate was very much lower than that achieved using the meat extract method.

Different viruses may have different survival times in different environments, however in several studies, enteric viruses have been reported to remain intact longer and occur more frequently at lower temperatures in different natural environments such as sea water, river water and groundwater (Green & Lewis 1999; Lipp et al. 2001, 2002; Wetz et al. 2004). In a survey for a period of a year in clean water in South Africa showed higher adenovirus detection in the winter months when 30% and 60% of the samples of treated water and raw water were positive for adenovirus, respectively (Van Heerden et al. 2003). Lipp et al. (2001) detected enterovirus in an estuary in southwest Florida only when the water temperature was below 23 °C. Another study in vitro showed that there is an increase of survival and detection of poliovirus in sea water in temperatures over 22 °C in relation to temperatures of 30 °C (Wetz et al. 2004). In temperatures of 4 °C poliovirus it took 671 to be inactivated 90% while at 25 °C was inactivated in only 25 days (Gantzer et al. 1998). Thus Fong et al. (2005) demonstrated that both bovine and human enteric viruses correlate with cold water.

**CONCLUSION**

This study has revealed important data with relation to recovery of different species of virus in ultrapure water at different stages of a method for viral concentration using a negative membrane and has provided information with respect to the reliability that the method offers, since there were infectious viral particles in the viruses recovered at the end of the concentration process. Nevertheless, future studies to evaluate these and other methods of concentration in environmental samples are needed in order to allow us to assess which is the best technique to be used for simultaneous detection of the greatest number of viral species and also other microorganisms in the many different environmental matrices.

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