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Comparison of Concentration Methods for Quantitative Detection of Sewage-Associated Viral Markers in Environmental Waters

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Pathogenic human viruses cause over half of gastroenteritis cases associated with recreational water use worldwide. They are relatively difficult to concentrate from environmental waters due to typically low concentrations and their small size. Although rapid enumeration of viruses by quantitative PCR (qPCR) has the potential to greatly improve water quality analysis and risk assessment, the upstream steps of capturing and recovering viruses from environmental water sources along with removing PCR inhibitors from extracted nucleic acids remain formidable barriers to routine use. Here, we compared the efficiency of virus recovery for three rapid methods of concentrating two microbial source tracking (MST) viral markers human adenoviruses (HAdVs) and polyomaviruses (HPyVs) from one liter tap water and river water samples on HA membranes (90 mm in diameter). Samples were spiked with raw sewage, and viral adsorption to membranes was promoted by acidification (method A) or addition of $MgCl_2$ (methods B and C). Viral nucleic acid was extracted directly from membranes (method A), or viruses were eluted with NaOH and concentrated by centrifugal ultrafiltration (methods B and C). No inhibition of qPCR was observed for samples processed by method A, but inhibition occurred in river samples processed by B and C. Recovery efficiencies of HAdVs and HPyVs were ~10-fold greater for method A (31 to 78%) than for methods B and C (2.4 to 12%). Further analysis of membranes from method B revealed that the majority of viruses were not eluted from the membrane, resulting in poor recovery. The modification of the originally published method A to include a larger diameter membrane and a nucleic acid extraction kit that could accommodate the membrane resulted in a rapid virus concentration method with good recovery and lack of inhibitory compounds. The frequently used strategy of viral absorption with added cations (Mg^{2+}) and elution with acid were inefficient and more prone to inhibition, and will result in underestimation of the prevalence and concentrations of HAdVs and HPyVs markers in environmental waters.

Discharges from sewage treatment plants (STPs), storm water drains, improperly designed septic systems, and fecal contamination from livestock and wildlife are known to degrade environmental water quality in terms of elevating fecal indicator bacteria and possibly pathogen concentrations (1–5). Fecal indicator bacteria such as *Escherichia coli* and *Enterococcus* spp. have been widely used as an indirect measure of microbial risk associated with environmental waters. However, identifying the health risks associated with enteric viruses and protozoa by monitoring fecal indicator bacteria has been questioned (5–8).

The risk of infectious disease associated with recreational water use may well be of viral etiology (9). The transmission of these viruses occurs via the fecal-oral route, nasal mucosa or the conjunctiva and the infected individual may shed up to 10^{11} viral particles/gram of feces (10). Some of these viruses are more resistant to unfavorable environmental conditions and treatment processes (chlorination, UV radiation, and filtration) than bacterial and protozoa pathogens (11). Moreover, these viruses often have extremely low infectious doses (12) and can remain infective for extended period of time in environmental waters (13). It has been reported that the risk of viral infection via drinking water can be 10- to 10,000-fold higher than pathogenic bacteria at similar rates of exposure (14).

Among enteric viruses, human adenoviruses (HAdVs) and human polyomaviruses (HPyVs) are responsible for a wide array of diseases in healthy and immunocompromised individuals (15–17). The concentrations of HAdVs and HPyVs are reported to be high (10^6 to 10^7 viral particles/liter) in raw sewage (18, 19), therefore these viruses have been considered as useful microbial source tracking (MST) markers to detect sewage pollution in environmental waters (6, 20–24).

Detection and quantification of enteric viruses by PCR in most environmental water samples require concentration of the viruses. A wide range of virus concentration methods have been developed and used to concentrate viruses from various types of environmental waters (25–31). Among these methods, an adsorption/elution-based protocol with negatively charged membrane has been shown to recover high concentration of viruses from small volume of water samples with minimal PCR inhibitory effects (27). This method involves adding $MgCl_2$ to a sample, and then the viruses are absorbed on negatively charged membranes, followed by elution of viruses with NaOH and further concentrated by centrifugal ultrafiltration. Katayama et al. (27) reported up to 33 to 90% recovery rate of polioviruses from purified water and seawater. In contrast, low recoveries of

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noroviruses (0.8%) in seawater and HAdVs (0.92 to 1.03%) in river water have been reported (32, 33).

From the limited published studies, it appears that the recovery efficiencies of these methods are highly variable and can be quite poor (19, 34–36). Therefore, improved, rapid, efficient (high recovery), and cost-effective virus concentration methods are needed for routine monitoring of these sewage-associated viral markers in environmental waters to ensure microbial safety worldwide. It has been suggested that capturing viruses on membranes, followed by direct nucleic acid extraction, may result in higher recoveries compared to protocols that require viral elution from membranes (37).

The main aim of the present study was to compare the performance of three virus concentration methods: (i) direct nucleic acid extraction from a negatively charged membrane, (ii) an adsorption/elution-based protocol with a negatively charged membrane, and (iii) a modified adsorption/elution-based protocol with a negatively charged membrane. Tap and river water samples were spiked with a known volume of raw sewage and the viral markers were concentrated with the above methods. Quantitative PCR (qPCR) assays were used to measure the concentrations of HAdVs and HPyVs in sewage spiked tap and river water samples to identify the best performing method in terms of recovery efficiency and freedom from inhibition of the PCR.

MATERIALS AND METHODS

Sample preparation. A 1-liter raw sewage sample was collected from a metropolitan STP in Brisbane, Australia. The STP has a flow capacity of 54 ML/day. A 40-liter tap water sample was collected from the Ecosciences Precinct Laboratory at Dutton Park, Queensland, Australia, whereas 40-liter River water samples were collected from the upstream of Brisbane River. This site receives overflow of water from the Wivenhoe Reservoir after precipitation. The suspected sources of fecal pollution include wildlife. The site is used for swimming and fishing by local residents.

Sewage, tap, and river water samples were stored at 4°C for no more than 3 h before processing. For each separate trial ($n = 3$), 10 ml of sewage sample was added to 990 ml of tap water or river water. Each sample was tested in triplicate. The pH and turbidity of the tap and river water samples were 7.3 ± 0.4 and 0.5 ± 0.1 nephelometric turbidity units (NTU) for tap water and 8.0 ± 0.1 and 5.2 ± 0.3 NTU for river water. The concentrations of *E. coli* and *Enterococcus* spp. in sewage, tap, and river water samples were enumerated using the U.S. Environmental Protection Agency's standard membrane filtration methods (38, 39). In brief, sample serial dilutions were made and filtered through 0.45-μm-pore size (47-mm-diameter) nitrocellulose membranes (Millipore, Tokyo, Japan) and then placed on modified membrane-thermotolerant *E. coli* agar (modified mTEC agar; Difco, Detroit, MI) and membrane-*Enterococcus* indoxyl-D-glucoside (mEI) agar (Difco) for the isolation of *E. coli* and *Enterococcus* spp. Modified mTEC agar plates were incubated at 35°C for 2 h to recover stressed cells, followed by incubation at 44°C for 22 h, whereas the mEI agar plates were incubated at 41°C for 48 h. The concentrations of HAdVs and HPyVs in sewage, tap, and river water samples were determined using qPCR assays (see below for methodological details).

Viral markers concentration. Viruses were concentrated using previously published methods, with modifications as noted. A method flow chart is provided in Fig. 1. These methods are referred to as method A (direct nucleic acid extraction from negatively charged membranes [19]), method B (an adsorption/elution-based protocol with negatively charged membranes [27]), and method C (a modified version of an adsorption/elution-based protocol with negatively charged membranes [33]). Method A began with adjustment of the sample pH to 3.5 using 2.0 N HCl. The samples were then passed through 0.45-μm-pore-size, 90-mm-diameter negatively charged HA membranes (HAWP09000; Merck Millipore, Ltd., Sydney, Australia) via a glass funnel and base (Merck Millipore). The membranes were then placed into 50-ml PowerMax bead solution tubes. Nucleic acid was extracted directly from the membranes using a Mo Bio PowerMax soil DNA isolation kit. Extracted viral nucleic acid was eluted through spin filter membranes by adding 2 ml of solution C6 and stored at −20°C until processed.

Method B began with the addition of $MgCl_2$ to a final concentration of 2.5 mM to each sample. Samples were filtered through 0.45-μm-pore-size, 90-mm-diameter HA membranes as described above. Subsequently, 200 ml of 0.5 mM H_2SO_4 (pH 3.0) was passed through the membranes to remove magnesium ions and other positively charged substances, followed by passage through 10 ml of 1 mM NaOH (pH 11) for the elution of viruses from the membranes. The eluates were recovered in sterile 50-ml polycarbonate tubes containing 50 μl of 100 mM H_2SO_4 (pH 1.0) and 100 μl of 100× Tris-EDTA buffer (pH 8.0) for neutralization. All 10-ml eluates were stored at −80°C until further processing. The samples were then further purified, concentrated, and desalted with Amicon Ultra-15 (30 K) centrifugal filter devices (Merck Millipore) by centrifugation at 4,750 × *g* for 10 to 15 min. Concentrated samples (180 to 200 μl) were collected from the filter device sample reservoir by using a pipette. Viral nucleic acid was extracted from each concentrated sample using a DNeasy blood and tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Extracted nucleic acid was resuspended in 200 μl of AE buffer and stored at −20°C until processed.

HA membranes from method B were also checked for retention of viruses. Filters were placed in 50-ml PowerMax bead solution tubes. Nucleic acid was directly extracted from the membranes using a Mo Bio PowerMax soil DNA isolation kit as described above for method A.

Method C is the modified version of method B. Briefly, 200 ml of 0.5 mM H_2SO_4 (pH 3.0) was passed through the HA negatively charged membranes. The membranes were then placed into sterile 50-ml polycarbonate tubes containing 10 ml of 1 mM NaOH (pH 11) and vortexed for 5 min at room temperature to release the membrane-adsorbed viruses. For neutralization, 50 μl of 100 mM H_2SO_4 (pH 1) and 100 μl of 100× Tris-EDTA buffer (pH 8) was added into the eluates. The samples were further purified, concentrated, and desalted with Amicon Ultra-15 centrifugal filter devices. Viral nucleic acid was extracted from each concentrated sample using a DNeasy blood and tissue kit. Extracted nucleic acid was resuspended in 200 μl of AE buffer and stored at −20°C until processed. The nucleic acid concentrations in each of the sample obtained using methods A, B, and C were quantified using a NanoDrop spectrophotometer (ND-1000; NanoDrop Technology, Wilmington, DE).

PCR inhibition. To obtain information on the level of PCR inhibition, purified nucleic acid samples extracted from sewage spiked tap and river water samples were spiked with a known amount (10 pg) of *Oncorhynchus keta* DNA (Sigma Chemical Co., St. Louis, MO). The threshold cycle (C_T) values for *O. keta* DNA (10 pg) were determined in PCRs with DNase- and RNase-free water. The *O. keta* C_T values in DNase- and RNase-free water were compared to the spiked *O. keta* in tap and river water samples to obtain information on the PCR inhibition level. The primer sequences and amplification condition for the *O. keta* assay are shown in Table 1.

Preparation of qPCR standards. HAdV and HPyV positive controls were isolated from adenovirus strain 41 (ATCC VR-930) and sewage, respectively. The PCR amplified products were purified by using a QIAquick PCR purification kit (Qiagen), cloned into a pGEM-T Easy vector system II (Promega, Madison, WI), transferred into *E. coli* JM109 competent cells, and plated on Luria-Bertani agar plates containing ampicillin, IPTG (isopropyl-β-D-thiogalactopyranoside), and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), as recommended by the manufacturer. Recombinant plasmids with corresponding inserts were purified by using a plasmid minikit (Qiagen). Standards for qPCR assays of HAdVs and HPyVs were prepared from plasmid DNA, ranging from 3×10^5 to 3×10^1 (for HAdVs) and 5×10^5 to 5×10^0 (for HPyVs). The

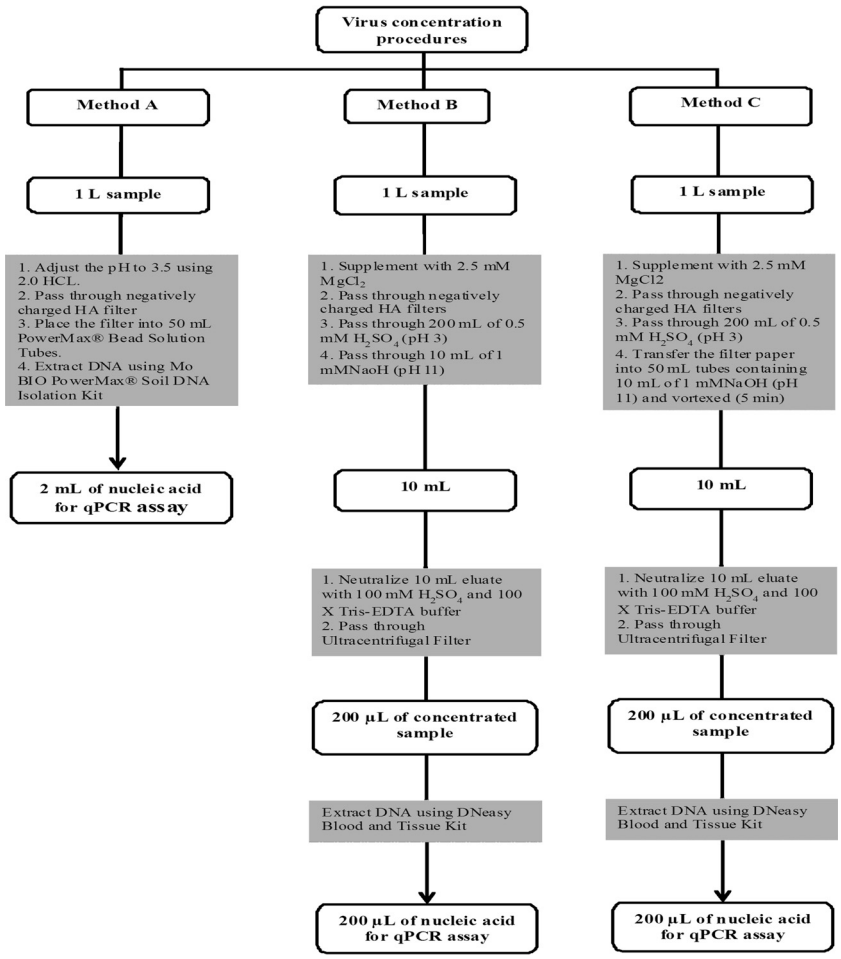

FIG 1 Procedures for virus concentration methods in sewage spiked tap and river water samples. Method A is direct nucleic acid extraction from negatively charged membranes, method B includes an adsorption/elution-based protocols with negatively charged membranes, and method C is a modified version of adsorption/elution-based protocols with negatively charged membranes.

amplification efficiency (E) was determined by analysis of the standards and was estimated from the slope of the standard curve as $E = 10^{-1/\text{slope}}$.

qPCR assays. qPCR assays were performed using previously published primers, probes, and cycling parameters (Table 1) (19, 54, 55). HAdVs qPCR amplifications were performed in 20-µl reaction mixtures using SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Richmond, CA). The qPCR mixtures contained 10 µl of Supermix, 250 nM concentrations of each primer, and 3 µl of template nucleic acid.

To separate the specific product from nonspecific products, including primer dimers, melting-curve analysis was performed. During melting-curve analysis, the temperature was increased from 65 to 95°C in 0.5°C increments. HPyVs qPCR amplifications were performed in 50-µl reaction mixtures using TaqMan Universal PCR master mix (Applied Biosystems, Foster City, CA). The qPCR mixtures contained 25 µl of TaqMan Universal PCR master mix, no AmpErase UNG, 500 nM concentrations of each primer, a 400 nM concentration of probe, and 5 µl of template nucleic acid. The qPCR assays were performed using a CFX96 thermal cycler (Bio-Rad Laboratories). All qPCRs were performed in triplicate.

TABLE 1 Target, primer/probe sequences, and amplification conditions for endpoint PCR and qPCR assays used in this study

Assays	Target gene	Primer or probe sequence (5′–3′)[a]	Amplification conditions	Reference
Sketa22 endpoint PCR	ITS region 2	F, GGT TTC CGC AGC TGG G R, CCG AGC CGT CCT GGT CTA P, FAM-AGT CGC AGG CGG CCA CCG T-TAMRA	10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 45 s at 63°C	54
HAdV qPCR	Hexon gene	F, GCC ACG GTG GGG TTT CTA AAC TT R: GCC CCA GTG GTC TTA CAT GCA CAT C	10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 20 s at 60°C, and 20 s at 95°C	55
HPyV qPCR	Homologous T antigen	F, AGT CTT TAG GGT CTT CTA CCT TT R, GGT GCC AAC CTA TGG AAC AG P, FAM-AGT CGC AGG CGG CCA CCG T-MGBNFQ	10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 15 s at 55°C, and 60 s at 60°C	19

[a] F, forward primer; R, reverse primer; P, probe; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; MGBNFQ, molecular-groove binding nonfluorescence quencher.

Endpoint Sketa22 PCR amplification (for *O. keta*) was performed in 25-μl reaction mixtures using iQ Supermixes (Bio-Rad Laboratories). The PCR assay mixtures contained 12.5 μl of Supermixes, 300 nM concentrations of each primer, 400 nM probe, 3 μl of template DNA, and 2 μl (10 pg) of *O. keta* DNA.

qPCR assays LLOQ. The lower limit of quantification (LLOQ) provides a C_T value where a quantitative number can be determined (40). To determine the qPCR LLOQ, 10-fold serial dilutions of standards (3×10^5 to 3×10^0 gene copies for HAdVs and 5×10^5 to 5×10^0 gene copies for HPyVs) were tested in triplicates. The smallest amount of diluted standards detected in 100% of triplicate assays was considered the qPCR LLOQ.

Recovery efficiency. The recovery efficiencies of HAdVs and HPyVs for sewage spiked tap and river water for all three virus concentration methods were calculated as follows: recovery efficiency (%) = (concentration recovered/concentration spiked) × 100.

Quality control. To minimize qPCR contamination, nucleic acid extraction, and qPCR set up were performed in separate laboratories. A method blank was included for each batch of tap and river water samples. A reagent blank was also included during nucleic acid extraction to account for any contamination during extraction. For each qPCR experiment, corresponding positive (standards) and negative controls (DNase and RNase free water) were included.

Statistical analysis. Significant differences between C_T values for *O. keta* DNA spiked into DNase and RNase free water versus tap and river water were determined by one-way analysis of variance (ANOVA) using GraphPad Prism 6 software. ANOVA was also used to assess investigate whether the concentrations of HAdV and HPyV gene copies obtained through methods A, B, and C significantly differed from each other. Statistical significance was determined at $P < 0.05$.

RESULTS

Ambient concentrations of fecal indicator bacteria and viral markers in sewage, tap and environmental waters. The mean concentrations and standard deviations of *E. coli*, *Enterococcus* spp., HAdVs, and HPyVs in sewage sample were $3.1 \times 10^5 \pm 1.6 \times 10^4$ CFU/10 ml, $2.0 \times 10^5 \pm 1.6 \times 10^4$ CFU/10 ml, $3.9 \times 10^4 \pm 7.9 \times 10^3$ gene copies/10 ml, and $2.5 \times 10^4 \pm 3.9 \times 10^3$ gene copies/10 ml, respectively. The tap water samples did not contain detectable concentrations of fecal indicator bacteria or viruses. The river water samples had lower concentrations of *E. coli* ($2.5 \times 10^1 \pm 1.0 \times 10^1$ CFU/100 ml of water) and *Enterococcus* spp. ($1.2 \times 10^1 \pm 0.4 \times 10^1$ CFU/100 ml). None of the viruses were detected in 1 liter of unspiked river water samples.

qPCR standards and the LLOQ. qPCR standards were analyzed in order to determine the reaction efficiencies. The standards had a linear range of quantification from 3×10^5 to 3×10^1 (for HAdVs) and from 5×10^5 to 5×10^0 (for HPyVs) gene copies/μl of nucleic acid extract. The slope of the standards ranged from −3.30 to −3.42 (for HAdVs) and −3.23 to −3.38 (for HPyVs). The amplification efficiencies ranged from 96 to 101% (for HAdVs) and from 102 to 103% (for HPyVs) and the correlation coefficient (r^2) ranged from 0.98 to 0.99 (for both HAdVs and HPyVs). The LLOQs of the qPCR assays were determined using the standards. The qPCR LLOQs were 30 and 5 gene copies for HAdVs and HPyVs, respectively, for all triplicate samples.

PCR inhibition assessment. The mean C_T value and standard deviation for the *O. keta* spiked DNase and RNase free water was 29 ± 0.1 (Table 2). C_T values for *O. keta* spiked water samples were comparable for tap water samples processed by all methods and for river water processed by method A (Table 2). However, *O. keta* DNA did not amplify in DNA from river water samples obtained via methods B and C, indicating the presence of PCR inhibitors in samples processed by these methods. These nucleic acid samples were then serially diluted to relieve PCR inhibitors and reanalyzed by spiking *O. keta* DNA in the serially diluted DNA samples. The mean C_T values and standard deviations of *O. keta* for the 10-fold diluted river water samples were 30 ± 0.6 (method B) and 40 ± 0.7 (method C). ANOVA of the C_T values indicated that the tap and river water (method A), tap water (methods B and C), and 10-fold-diluted river water (methods B and C) did not differ significantly from the C_T value obtained for the *O. keta* spiked DNase- and RNase-free water. Based on the results, all of the samples without PCR inhibition (undiluted and 10-fold diluted samples) were used for qPCR assays of HAdVs and HPyVs.

TABLE 2 *O. keta* endpoint PCR assay for the evaluation of inhibition in raw sewage spiked into tap ($n = 3$) and river ($n = 3$) water nucleic acid samples as opposed to DNase- and RNase-free water samples[a]

Method[b]	Sample type	ng of DNA/μl of extract (range)	Mean C_T ± SD for *O. keta* endpoint PCR assay	
			Undiluted nucleic acid	10-fold-diluted nucleic acid
A	Tap water	1.1–2.1	30 ± 0.4	
	River water	3.3–5.8	29 ± 0.5	
B	Tap water	0.4–1.7	30 ± 0.3	
	River water	6.5–17	NA[c]	30 ± 0.6
C	Tap water	3.3–9.7	29 ± 0.3	
	River water	4.6–30	NA	30 ± 0.7
	DNase- and RNase-free water		29 ± 0.1	

[a] DNase- and RNase-free water samples and diluted and undiluted DNA samples were spiked with 10 pg of *O. keta*.
[b] A, the Mo Bio PowerMax soil DNA isolation kit was used to extract nucleic acid; B, a Qiagen DNeasy blood and tissue kit was used to extract nucleic acid; C, not tested.
[c] NA, no amplification.

Recovery efficiency of HAdVs and HPyVs. The mean concentration of HAdVs (1.3×10^4) in concentrated tap and river water samples obtained through method A was 10-fold higher than those obtained through methods B and C (Fig. 2a). Similar results were also obtained for HPyVs. The mean concentration of HPyVs in tap (1.3×10^4) and river (2.0×10^4) water samples obtained using method A were also 10-fold higher than those obtained using methods B and C, respectively (Fig. 2b). ANOVA indicated that the concentrations of HAdVs and HPyVs obtained by method A significantly differed ($P < 0.001$) from the concentrations obtained by methods B and C. The concentrations of HAdVs and HPyVs in tap and river water samples within each method slightly differed from each other, but the differences were not statistically significant.

The estimated mean recovery efficiencies of HAdVs in tap and river water samples through method A were 31 and 32%, respectively (Table 3). However, the recovery efficiencies of tap and river water samples obtained using methods B and C ranged from 2.4 to 5.3%, indicating that these methods did not recover HAdVs as effectively as did method A. Similar trends were also observed for HPyV recovery. The estimated recovery efficiencies of HPyVs in tap and river water samples by method A were 40 and 78%, respectively, thus outperforming methods B and C.

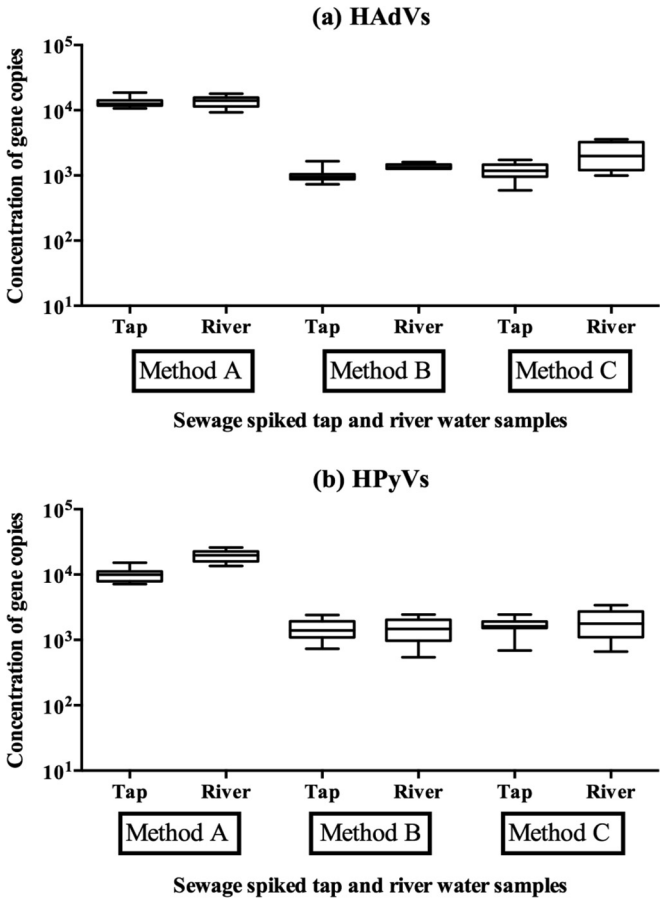

FIG 2 Box-and-whisker plots of the concentration of gene copies of HAdVs (a) and HPyVs (b) in sewage spiked tap and river water samples. The inner box lines represent the medians, while the outer box lines represent the 25th and 75th percentiles.

DISCUSSION

Measuring pathogenic viruses in environmental waters has historically been problematic due to their low concentrations in water and uneven distribution in human populations. Filtration on the scale of 100-liter samples is difficult to accomplish in the field, and many methods require expensive, expendable filters that cannot be reused. A number of recent studies have taken the optional approach of concentrating 1- to 2-liter volumes of surface water by membrane filtration to test for human viruses (6, 20, 21, 27, 33, 44–48); however, few comparisons of method effectiveness for virus recovery have been carried out. Hence, we compared the performance of three relatively simple virus concentration methods to determine their efficiency in recovering two viral MST markers (HAdVs and HPyVs) in tap and river water samples spiked with raw sewage. The strategy of spiking with sewage rather than with cultured viruses (27, 41, 42) was used to better mimic a natural scenario that includes viruses in various states of intactness and disruption.

Little has been published on the recovery efficiency of these MST viral markers through the processes of concentration, nucleic acid extraction, and purification. It has been suggested that nucleic acid extraction directly from the membranes, as performed in method A, may result in higher recoveries compared to protocols that require viral elution from membranes (37). An important step in method A is adjusting the pH of the water sample to 3.5 (below the isoelectric point of the viruses), which imparts a positive charge to the viruses and allows them to bind reversibly to the negatively charged HA membrane. The prototype version of method A used 500-ml sample volumes and a 47-mm-diameter membrane (19).

One limitation of using smaller diameter membranes (compared to our version at 90 mm diameter) is that they tend to clog, particularly when water samples are higher in turbidity, and are generally not suitable for processing >500 ml of environmental water samples. In addition, nucleic acid extraction using the Mo Bio PowerSoil DNA isolation kit does not utilize the entire sample, which may influence the recovery efficiency of captured viruses. In view of these limitations, we processed 1-liter tap and river water samples through 0.45-μm-pore-size, 90-mm-diameter negatively charged membranes. The larger diameter membrane provides much larger net area (4.5 times more than 47-mm membranes), which allowed us to process up to 1 liter of water sample. For nucleic acid extraction, we used the Mo Bio PowerMax soil DNA isolation kit, which can easily accommodate a 90-mm-diameter membrane. Unlike the smaller-scale kit, the PowerMax soil kit utilizes the entire sample, contributing to better recovery of nucleic acid.

A limitation of all direct filtration methods for concentrating viruses and recovering nucleic acids is the potential for concentrating PCR inhibitors on the membranes. However, we found no evidence of inhibition in samples processed by method A. This was supported by the Sketa22 PCR assay undertaken here, which indicated the absence of PCR inhibitors in samples processed by method A but indicated inhibition in river water samples processed by methods B and C. Method A was able to effectively concentrate and quantify more HAdVs and HPyVs in both tap and river water samples, which was not the case for methods B and C. The mean recovery efficiencies of 32% (HAdVs) and 78% (HPyVs) of method A in the present study can be considered highly sensitive for simultaneous detection/quantification of these two viral markers in environmental waters compared to other methods (32, 33, 43).

The method B used here was originally developed to detect enteroviruses and noroviruses from coastal seawater samples (27). This approach has been used to concentrate and detect a wide range of DNA/RNA viruses from small volumes (500 ml to 2 liters) of environmental water samples using PCR/qPCR assays (6, 20, 21, 32, 42,

TABLE 3 Recovery efficiency of HAdVs and HPyVs from tap and river water samples

Method	Sample type	Mean recovery efficiency (%) ± SD[a]	
		HAdVs[b]	HPyVs[c]
A	Tap water	31 ± 5.2	40 ± 3.9
	River water	32 ± 5.0	78 ± 16
B	Tap water	2.4 ± 0.2	5.9 ± 0.9
	River water	3.1 ± 0.2	6.9 ± 0.8
C	Tap water	2.8 ± 0.5	6.8 ± 1.9
	River water	5.3 ± 0.2	12 ± 6.7

[a] The recovery efficiency (%) was calculated as the (concentration recovered/concentration spiked) × 100.
[b] $3.9 \times 10^4 \pm 7.9 \times 10^3$ gene copies of HAdVs were spiked.
[c] $2.5 \times 10^4 \pm 3.9 \times 10^3$ gene copies of HPyVs were spiked.

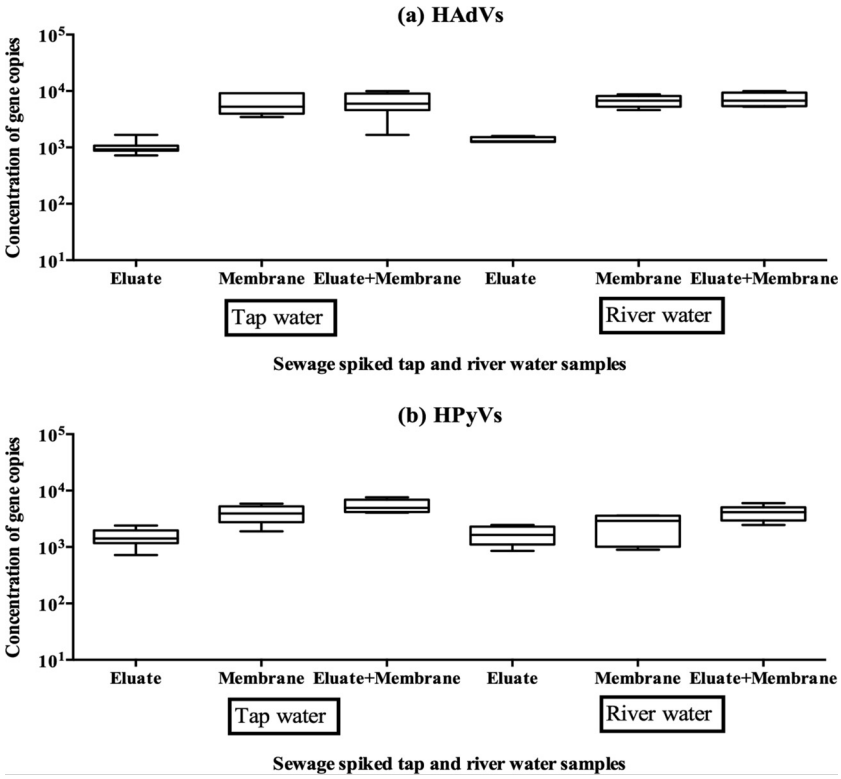

FIG 3 Box-and-whisker plots of the concentration of gene copies of HAdVs (a) and HPyVs (b) in nucleic acid samples extracted from eluates and membranes using method B. The inner box lines represent the medians, while the outer box lines represent the 25th and 75th percentiles.

44–48). The recovery efficiency of method B varied from 33 to 95% (purified water), 38 to 89% (seawater), 28 to 46% (river water), and 32 to 87% (tap water) using poliovirus as a model (27). Information is also available on the recovery efficiencies of hepatitis A virus (12%), adenovirus type 41 (35 to 58%), and adenovirus type 5 (4.0 to 36%) in various types of water (41, 48, 49).

The mean recovery efficiencies of HAdVs and HPyVs determined in the present study using methods B and C were similar to each other, ranging from 2.4 to 12% for both tap and river water samples, which is comparable to a previous HAdVs spiking study in Milli-Q (0.2 to 7.0%) and river (0.3 and 1.5%) water sample (32). Similar values of recovery efficiency were reported in another study that used method B for norovirus (3.3 and 18%) and astrovirus (2.3 and 43%) in tap and river water samples at an $MgCl_2$ concentration of 5 mM (33). Keuckelaere et al. (43) reported the recovery of murine noroviris 1 (4.8 to 22%) and MS2 bacteriophage (3.4 to 5.6%) in various water types using a modified adsorption-elution method originally developed by Katayama et al. (27). The results are also comparable to the recovery efficiency obtained in the present study. Caution should be exercised when comparing published studies on recovery efficiency of virus concentration methods since variations in several factors, such as adsorption of viruses to membranes, membrane type, elution buffer, seeding materials and concentrations, sample type and volume, and the sensitivity of qPCR assays, can influence recovery efficiency (18, 34, 41, 50).

Binding of viruses to HA membranes contributed to the low recovery efficiency of methods B and C that we observed, since a considerable fraction of the total nucleic acid could be extracted directly from method B membranes. The concentrations of both HAdVs and HPyVs were higher in the nucleic acid extracts from the membranes than those obtained from the eluate, although in theory all of the viruses should have been eluted from the membranes (Fig. 3). The combined concentrations (eluate and membrane) of HAdVs and HPyVs in tap and river water samples, ranging from 3.9×10^3 to 7.9×10^3 gene copies, were still less than those obtained using method A.

Viruses in the elution buffers of methods B and C underwent an additional concentration step in an Amicon filter devices before nucleic acid extraction. Reconcentration methods are commonly used for analysis of viruses in environmental waters due to their low concentrations. Reconcentration methods such as organic flocculation (51) and polyethylene glycol precipitation (52) have some disadvantages, e.g., these methods do not produce consistent recovery efficiency for different viruses, and the sample processing time can be lengthy (52). Alternatively, specifically designed ultrafilters, which retain viruses based on molecular weight cutoff, can be used as a secondary concentration step. In a previous study, Centriprep filter concentrators provided high and stable recovery yields (74%) of polioviruses (53). Another study reported the 35% recovery of adenovirus 41 through Centricon filters (49). These findings clearly suggest that a reconcentration step may result in the loss of viral particles.

It has been suggested that the acid rinse step in methods B and C may reduce PCR inhibitors in the eluate from environmental waters samples (27). This was not in accordance with our findings. The nucleic acid extracted from river water samples using methods B and C both contained PCR inhibitors and required 10-fold dilutions. To extract nucleic acid from samples obtained through methods B and C, a DNeasy blood and tissue kit was used, which

does not have PCR inhibitors removal technology. Based on the results, it is recommended that if method B or C is used for virus concentration, perhaps a commercial kit possessing PCR inhibitory technology would be more suitable for nucleic acid extraction. In the present study, we simply assumed that the nucleic acid extraction efficiency of the DNeasy blood and tissue kit and Mo Bio PowerSoil DNA isolation kit is 100% in order to calculate the concentrations of viruses in sewage and sewage spiked water samples. Considering the potential loss of viral particles during nucleic acid extraction, the actual numbers of HAdVs and HPyVs in sewage and spiked water samples could be higher than those reported here.

This comparative study of three virus concentration methods indicates that direct nucleic acid extraction from viruses captured on membranes (method A) provide better recovery for HAdVs and HPyVs in tap and river water samples compared to an adsorption/elution-based protocol with negatively charged membranes (method B) and a modified version of adsorption/elution protocols with negatively charged membranes (method C). The recovery efficiencies of HAdVs and HPyVs were ~10-fold greater for method A than for methods B and C. Further analysis of membranes from method B revealed that the majority of viruses were not eluted from the membrane, resulting in poor recovery. The advantages of method A for the virus concentration is that efficient recovery allows processing of smaller volumes, with concomitantly fewer issues with inhibition, lower cost, and less time and labor. We anticipate that many enteric viruses will be coconcentrated with high recovery efficiencies. Further investigation is required to obtain information on the recovery of sewage-associated RNA viral markers such as pepper mild mottle viruses and noroviruses.

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