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ORIGINAL PAPER

Methods Evaluation for Rapid Concentration and Quantification of SARS-CoV-2 in Raw Wastewater Using Droplet Digital and Quantitative RT-PCR

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Abstract
Wastewater surveillance of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an emerging public health tool to understand the spread of Coronavirus Disease 2019 (COVID-19) in communities. The performance of different virus concentration methods and PCR methods needs to be evaluated to ascertain their suitability for use in the detection of SARS-CoV-2 in wastewater. We evaluated ultrafiltration and polyethylene glycol (PEG) precipitation methods to concentrate SARS-CoV-2 from sewage in wastewater treatment plants and upstream in the wastewater network (e.g., manholes, lift stations). Recovery of viruses by different concentration methods was determined using Phi6 bacteriophage as a surrogate for enveloped viruses. Additionally, the presence of SARS-CoV-2 in all wastewater samples was determined using reverse transcription quantitative PCR (RT-qPCR) and reverse transcription droplet digital PCR (RT-ddPCR), targeting three genetic markers (N1, N2 and E). Using spiked samples, the Phi6 recoveries were estimated at 2.6–11.6% using ultrafiltration-based methods and 22.2–51.5% using PEG precipitation. There was no significant difference in recovery efficiencies ($p < 0.05$) between the PEG procedure with and without a 16 h overnight incubation, demonstrating the feasibility of obtaining same day results. The SARS-CoV-2 genetic markers were more often detected by RT-ddPCR than RT-qPCR with higher sensitivity and precision. While all three SARS-CoV-2 genetic markers were detected using RT-ddPCR, the levels of E gene were almost below the limit of detection using RT-qPCR. Collectively, our study suggested PEG precipitation is an effective low-cost procedure which allows a large number of samples to be processed simultaneously in a routine wastewater monitoring for SARS-CoV-2. RT-ddPCR can be implemented for the absolute quantification of SARS-CoV-2 genetic markers in different wastewater matrices.

Keywords SARS-CoV-2 · Coronavirus · PEG precipitation · RT-ddPCR · RT-qPCR · Wastewater-based epidemiology

Introduction

Since the emergence and spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus that causes coronavirus disease 2019 (COVID-19), many cities around the world have rapidly expanded their viral surveillance systems, including wastewater monitoring for SARS-CoV-2. This is because SARS-CoV-2 can be shed in the feces of infected individuals from both symptomatic and asymptomatic cases (Park et al., 2020; Wu et al., 2020a, 2020b).

Coronaviruses are positive-strand RNA enveloped viruses with the largest viral genomes of all RNA viruses (27 to 32 kb). They have a spherical virion of about 120 nm in diameter surrounded by a lipid envelope with pronounced spiked glycoproteins (S) embedded. The vast majority of studies on the presence of viruses in human excreta and municipal wastewater have been focused on non-enveloped enteric viruses. There are a number of established methods for the detection of non-enveloped enteric viruses in wastewater, but only fewer evaluated protocols for human enveloped viruses such as SARS-CoV-2 (Haramoto et al., 2018). Analysis of environmental matrices for human viruses often

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require concentration steps due to the low ambient concentrations of the viruses. Therefore, laboratory methods for the detection of SARS-CoV-2 in wastewater need to examine both sample concentration and RNA quantification methods along with optimizing limits of detection.

Globally, there have been over forty reports on the molecular detection of SARS-CoV-2 in wastewater (e.g., Ahmed et al., 2020a; Ahmed et al., 2020b; Ampuero et al., 2020; Arora et al., 2020; Balboa et al., 2020; Barcelo, 2020; Chavarria-Miró et al., 2020; Curtis et al., 2020; Döhla et al., 2020; Fernández de Mera et al., 2020; Fongaro et al., 2020; Green et al., 2020; Guerrero-Latorre et al., 2020; Haramoto et al., 2020; Hata et al., 2020; Jorgensen et al., 2020; Kocamemi et al., 2020a; Kocamemi et al., 2020b; Kumar et al., 2020; La Rosa et al., 2020a, 2020b, 2020c; Medema et al., 2020; Miyani et al., 2020; Nemudryi et al., 2020; Bar-Or et al., 2020; Peccia et al., 2020; Prado et al., 2020; Randazzo et al., 2020a; Randazzo et al., 2020b; Rimoldi et al., 2020; Sharif et al., 2020; Sherchan et al., 2020; Trottier et al., 2020; Vallejo et al., 2020; Wang et al., 2020; Weidhaas et al., 2020; Westhaus et al., 2021; Wu et al., 2020a; Wu et al., 2020b; Wurtzer et al., 2020; Zhang et al., 2020a; Zhang et al., 2020b; Zhou et al., 2020). These studies have had large variability in the numbers of samples from as few as 10 samples collected to over 120 with SARS-CoV-2 RNA being detected at concentrations ranging from 10^2 to 10^6 copies per liter. These SARS-CoV-2 surveillance studies analyzed volumes of raw sewage, treated wastewater and sewage sludge ranging from 2.5 to 2000 ml, using various concentration methods such as adsorption-elution-based membrane filtration, precipitation (using polyethylene glycol, aluminum hydroxide), ultracentrifugation and ultrafiltration prior to RNA extraction in order to recover the virus. The majority of studies quantified the viral RNA in wastewater using quantitative reverse transcription polymerase chain reaction (RT-qPCR) with external standard curves. Several gene targets specific to the SARS-CoV-2 have been used in wastewater surveillance, including the RNA-dependent polymerase (RdRP), nucleocapsid (N1, N2), envelope protein (E), spike glycoprotein (S), membrane glycoprotein (M) and ORF1ab genes (e.g., Corman et al., 2020; Lu et al., 2020).

Currently, cell culture for SARS-CoV-2 requires a Biosafety Level 3 laboratory and specially trained personnel. Therefore, surrogate viruses have been used to mimic SARS-CoV-2 to evaluate virus concentration methods for wastewater. These surrogate viruses include F-specific RNA phages (Balboa et al., 2020; Hata et al., 2020; Medema et al., 2020), mengovirus (Randazzo et al., 2020a), avian coronavirus of infectious bronchitis virus (Kocamemi et al., 2020a), Alphacoronavirus HCoV 229E (La Rosa et al., 2020b), bovine coronavirus BCoV (LaTurner et al., 2021), porcine epidemic diarrhea virus (PEDV) (Randazzo et al., 2020b), bovine respiratory syncytial virus (BRSV) (Gonzalez et al., 2020), and murine hepatitis virus (Ahmed et al., 2020c). Estimated mean recovery efficiencies for these surrogate viruses ranged from 1 to 73% using different concentration methods originally developed for the detection of enteric viruses in environmental samples (Medema et al., 2020; Randazzo et al., 2020a). *Pseudomonas* phage Phi6 has also been used as a model enveloped virus in recovery and persistence studies (Gendron et al., 2010; Aquino de Carvalho et al., 2017; Ye et al., 2016). Similar to coronaviruses, Phi6 is an enveloped RNA virus, with a segmented genome and glycerophospholipids in its envelope (Vidaver et al., 1973). Since Phi6 is not pathogenic to humans, it is easier to work with than other enveloped animal viruses and no special laboratory biosafety is required.

Rapid, cost-effective, and efficient methods are needed to provide precise data to support public health decision making. This is so that changes in concentrations of SARS-CoV-2 gene markers in wastewater provide meaningful data to inform COVID-19 surveillance. Therefore, the objectives of this study were to (i) evaluate the efficiencies of polyethylene glycol (PEG) precipitation and ultrafiltration methods to recover *Pseudomonas* phage Phi6, coronavirus OC43, and SARS-CoV-2 from different wastewater matrices; (ii) compare two PCR-based methods, reverse transcription quantitative PCR (RT-qPCR) and reverse transcription droplet digital PCR (RT-ddPCR) for the detection of SARS-CoV-2 in different wastewater matrices; and (iii) develop a rapid, cost-effective, and precise quantification workflow for SARS-CoV-2 in wastewater.

Materials and Methods

Wastewater Samples and Sampling Sites

Wastewater samples (500–1000 ml) for this study were collected from 11 sanitary sewer sites and four wastewater treatment plant (WWTP) influent streams (after grit removal) (Supplemental materials Table A1 and A2). A total of twenty sanitary sewer samples were collected as grab samples from the 11 manholes or lift stations. Sanitary sewer samples consisted of wastewater flowing from university dormitories, local communities, and hospital. Influent samples (n = 11) from four WWTPs were collected as 24-h composite samples. Samples used for the comparison of the SARS-CoV-2 surrogates Phi6 and human coronavirus OC43 were collected from two California wastewater treatment plant influents as previously described by Pecson et al. (2021). All samples were kept at 4 °C for up to 72 h. If samples were unable to be processed

within 72 h of collection, then they were frozen at − 80 °C until analysis.

Virus Stocks

Bacteriophage Phi6 and its bacterial host *Pseudomonas syringae* were kindly provided by Dr. Krista Wigginton's lab at University of Michigan. To propagate Phi6, *P. syringae* was grown in King's B medium at 24 ℃ for 6 h in stationary culture. Phi6 was added to the host and incubated under the same conditions for 16 to 18 h. After incubation and observed clearing of cell suspension due to lysis, cells and debris were removed from the Phi6 suspension by filtration using 0.22 µm membranes. The Phi6 stocks were stored at 4 °C and titered using an overlay method. For the overlay process, 2 ml of host was added to the overlay tube containing King's B agar and 0.5 ml of virus suspension, mixed, and poured onto a plate containing King's B agar. Plates were incubated at 24 ℃ for 16–24 h and plaques were counted. Virus titers of approximately 10^9 plaque forming unit (PFU) per ml were routinely obtained.

Virus Concentration Methods and Experiments

Four distinct comparisons were performed in this study. First, three viral concentration methods were tested for their efficiency in recovering Phi6 phages and SARS-CoV-2 in different types of wastewater. Methods 1 (CEN1) and 2 (CEN2) are based on the ultrafiltration principle and used centrifugal filters. Method 3 is a precipitation using polyethylene glycol (PEG). The second comparison was between RT-ddPCR and qPCR using the three viral concentration methods. The third comparison was determining if a rapid PEG precipitation approach (without an overnight incubation) would be able to perform as well or better than PEG precipitation with a 16 h overnight incubation. Lastly, Phi6 was compared against the human coronavirus OC43 using RT-ddPCR to determine if recovery efficiencies between the two SARS-CoV-2 surrogates were equivalent.

For each experiment, 350 ml of wastewater sample was inoculated with 1 ml of 10^6 plaque forming units (PFU)/ml of Phi6 and homogenized for 10 min at 4 °C. SARS-CoV-2 was not added to the sample. After homogenization, the sample was subdivided into three 101 ml of aliquots in 250 ml centrifuge bottles for processing with each concentration method. One milliliter of sample was removed from each 250 ml bottle containing the subsample for use in determining the seeded virus level for recovery efficiency of each method. Recovery efficiencies were determined by comparing the concentration of the spiked Phi6 bacteriophage in each subsample prior to processing with the concentration of Phi6 in their final concentrate using RT-ddPCR. All viral concentration experiments, for each method and each type of wastewater, were conducted in triplicate.

Method 1 (CEN1) was adapted from Ye et al. (2016) but modified to include virus recovery steps from wastewater solids. Briefly, 100 ml of wastewater sample was first centrifuged at 2500×*g* for 5 min at 4 °C in order to pellet any solids present in the sample. The supernatant was then collected without disturbing the pellet and filtered through a 0.22 µm polyethersulfone (PES) membrane filter (MilliporeSigma, St. Louis, MO). The sample was then concentrated using a 10 kDa Centricon Plus-70 centrifugal filter unit (MilliporeSigma, St. Louis, MO) according to the manufacturer's protocol. A 1:1 volume of 0.25 N glycine buffer was added to the pellet and remaining liquid. The pellet was vortexed every 10 min for 30 min while on ice to dislodge the viruses from suspended solids. After the 30 min incubation the glycine-processed sample was neutralized 1:1 with 2×PBS. The sample was then centrifuged at 10,000×*g* for 30 min at 4 °C. The supernatant was processed with the same centrifugal filter and the resulting concentrates were combined.

Method 2 (CEN2) involved the use of the same centrifugal filter but without a pre-filtration step (Medema et al., 2020). In this method, 100 ml of sample was centrifuged at 4654×*g* for 30 min at 4 °C without brake. The supernatant was then collected and directly filtered through a 10 kDa Centricon Plus-70 centrifugal filter unit (MilliporeSigma, St. Louis, MO) according to the manufacturer's protocol. The pellet was processed using the same protocol as described in the Method 1 (CEN1).

Method 3 (PEG) was adapted from Borchardt et al. (2017) for the detection of avian influenza virus RNA in groundwater. The samples were mixed with 8% (w/vol) molecular biology grade PEG 8000 (Promega Corporation, Madison WI) and 0.2 M NaCl (w/v). The samples were mixed slowly on magnetic stirrer at 4 °C for 2 h and then held at 4 °C for 16 h. Following the overnight incubation, samples were centrifuged at 4700×*g* for 45 min at 4 °C. The supernatant was then removed, and the pellet resuspend in the remaining liquid. All sample concentrates were aliquoted and stored at − 80 °C until further processing.

After the initial comparison of two ultrafiltration methods and PEG precipitation, a rapid PEG precipitation approach (without an overnight incubation) was evaluated with 19 wastewater samples. Each sample was inoculated with Phi6 and homogenized as described above. After mixing the sample with 8% (w/vol) PEG 8000 and 0.2 M NaCl for 2 h at 4 °C, the sample was immediately centrifuged at 4700×*g* for 45 min at 4 °C.

Finally, a comparison between Phi6 and OC43 was performed using wastewater from two California wastewater treatment plants split into 5 subsamples each and processed with the overnight PEG precipitation method.

RNA Extraction and Quantification by RT-ddPCR and RT-qPCR

Viral ribonucleic acid (RNA) was extracted from wastewater concentrates using the Qiagen QIAmp Viral RNA Minikit according to the manufacturers protocol with modifications (Qiagen, Germany). In this study, a total of 200 µl of concentrate was used for RNA extraction resulting in a final elution volume of 80 µl. Extracted RNA was stored at − 80 °C until analysis.

Detection of SARS-CoV-2, Phi6, and Coronavirus OC43 Using RT-ddPCR

One-step RT-ddPCR approach was used to quantify the Phi6 RNA to determine the recovery efficiencies for each concentration method. All the primers and probes used in this study are listed in Table A3. Droplet digital PCR was performed using Bio-Rad's 1-Step RT-ddPCR Advanced kit with a QX200 ddPCR system (Bio-Rad, CA, USA). Each reaction contained a final concentration of 1 × Supermix (Bio-Rad, CA, USA), 20 U ul-1 reverse transcriptase (RT) (Bio-Rad, CA, USA), 15 mM DTT, 900 nmol l^{-1} of each primer, 250 nmol l^{-1} of each probe, 1 µl of molecular grade RNAse-free water, and 5.5 µl of template RNA for a final reaction volume of 22 µl. Droplet generation was performed by microfluidic mixing of 20 µl of each reaction mixture with 70 µl of droplet generation oil in a droplet generator (Bio-Rad, CA, USA) resulting in a final volume of 40 µl of reaction mixture-oil emulsions containing up to 20,000 droplets with a minimum droplet count of > 9000. The resulting droplets were then transferred to a 96-well PCR plate which was heat-sealed with foil and placed into a C1000 96-deep-well thermocycler (Bio-Rad, CA, USA) for PCR amplification using the following parameters: 25 °C for 3 min, 50 °C for 1 h, 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min with ramp rate of 2 °C s 1 followed by a final cycle of 98 °C for 10 min. Following PCR thermocycling, each 96-well plate was transferred to a QX200 Droplet Reader (Bio-Rad, CA, USA) for the concentration determination through the detection of positive droplets containing each gene target by spectrophotometric detection of the fluorescent probe signal.

SARS-CoV-2 RNA and OC43 in wastewater samples were also quantified using the same one-step RT-ddPCR approach except the annealing temperature was set at 55 °C. Three SARS-CoV-2 markers were chosen for analysis, the nucleocapsid 1 (N1) and nucleocapsid 2 (N2) gene targets designed by the US Centers for Disease Control and Prevention (CDC) (Lu et al., 2020), the envelope (E) gene from Corman et al. (2020), and OC43 (Table A3). The N1 and N2 gene targets were analyzed in a duplex assay. All analyses were run in triplicate for each marker. Quality controls were run with every plate including positive and non-template controls, extraction controls, and processing blanks for each batch of samples.

Detection of SARS-CoV-2 Using RT-qPCR

RT-qPCR approach was also used to quantify SARS-CoV-2 gene markers in wastewater samples. All RT-qPCR reactions were performed using a StepOne Plus™ real-time PCR sequence detector (Applied Biosystems, Foster City, CA). For each assay, a tenfold diluted standard curve of at least five points, a non-template control, and samples were tested in triplicate. The quantitative synthetic SARS-CoV-2 RNA includes fragments from nucleocapsid and envelope regions (ATCC VR-3276SD) was used to generate standard curves. Amplification reaction mixtures (final total volume of 20 µl) contained 5 µl template RNA, 10 µl of 2 × qScript one-step RT-qPCR ToughMix (QuantaBio), 300 nM, 500 nM and 400 nM of forward primer for N1, N2, and E gene, respectively, 500 nM, 800 nM, and 800 nM of reverse primer for N1, N2, and E gene, respectively, and 200 nM of probe. The thermal cycling protocol was as follows: 10 min at 50 °C for cDNA synthesis, 3 min at 95 °C for initial denaturation, followed by 45 cycles of two steps consisting of 3 s at 95 °C and 30 s at 55 °C. qPCR amplification efficiencies for the quantification of the N1, N2 and E gene assays were 92.6 ± 4.3%, 95.1 ± 3.4%, and 91.6 ± 2.2%, respectively, and the correlation coefficients (R^2) of the standard curves were 0.968 ± 0.002, 0.982 ± 0.004, and 0.988 ± 0.0006, respectively.

Data Analysis

All SARS-CoV-2, Phi6, and OC43 gene data were converted from gene copies (GC) per reaction to GC per 100 ml before analysis. Non-detects (ND) were assigned their individual sample's limit of detection. The limit of detection was calculated for each individual sample based on both the molecular assays' theoretical detection limits (i.e., 3 positive droplets for RT-ddPCR; the lowest standard curve concentration for RT-qPCR) and the concentration factor of each processing method examined.

$$\text{Virus GC per100 ml} = \frac{\frac{\text{GC per reaction}}{V_r} \times V_e \times \frac{V_f}{V_c}}{V_i} \times 100$$

where: V_i = Initial volume of sample concentration in ml, V_f = Final volume of sample after concentration in ml, V_r = Volume of RNA template used per PCR reaction in µl, V_e = Final volume of RNA eluted from RNA extraction in µl,

V_c = Volume of concentrated sample used for RNA extraction in ml.

Recovery efficiency was calculated by dividing the total gene copies (GC) / 100 ml concentration of the Phi6 bacteriophage measured in each methods' final concentrate by the concentration (GC/ 100 ml) of Phi6 in each sample before concentration and then multiplying by 100.

Statistics and data visualization were performed using Graphpad Prism 8 (Graphpad Software, CA, USA). Results for the three methods comparison were analyzed with a two-way ANOVA with a Tukey's multiple comparisons test to determine method significance (p-value < 0.05). A two-way ANOVA (p < 0.05) and a paired t test (p < 0.05) were performed for the comparison of "normal" (16 h hold) vs "rapid" (no hold) PEG precipitation methods.

Results

Wastewater Characteristics

Wastewater samples from both sanitary sewer systems and treatment plants were evaluated in this study. All site-specific details including physiochemical data and sampling dates for each sanitary sewer and WWTP site are shown in Table A1 and Table A2, respectively. Wastewater collected from sanitary sewer locations had more variations in each parameter than wastewater collected from WWTP. For example, while sanitary sewer sites showed a wide range of turbidities ranging from 1.87 up to 191 NTU, WWTP influent samples showed less variation (e.g., 20.2 to 111 NTU). Sanitary sewer sites showed little variation in pH and temperature with each ranging from 6.57–8.58 and 13–26.4 °C, respectively (Table A1). Influent samples collected from WWTPs had a smaller degree of variation in pH (7.33–7.8) than sanitary sewer sites but had greater variation in temperatures which ranged from 1.40 to 21.67 °C (Table A2). Total suspended solids (TSS) and daily flows for each WWTP were also measured. Specifically, samples collected from facility W had the largest range of TSS (48–920 mg l^{-1}) and the highest daily flows ranging from 14.6–27.6 million gallons per day (mgd). Facility E had the smallest range of TSS (164–208 mg l^{-1}) and the lowest daily flow of 2.87 mgd, but facility M had the smallest range of daily flows (3.24–3.86 mgd).

Recovery of Phi6 from Wastewater Samples Using Ultrafiltration and PEG Methods

Prior to seeding experiments, ambient concentrations of *Pseudomonas* phage Phi6 were determined using RT-ddPCR. All wastewater samples were negative for Phi6.

The mean recovery efficiencies of the two ultrafiltration-based and PEG precipitation methods for the detection of Phi6 using RT-ddPCR in different types of wastewater are summarized in Table 1. For the various wastewater matrices, mean recoveries of ultrafiltration-based Method 1 ranged from 2.6 to 10.6% and Method 2 ranged from 2.7 to 11.6%. The Phi6 virus recovery was statistically higher (p < 0.0001) for both sanitary sewers and WWTP influent samples using the PEG method compared to the ultrafiltration methods, with mean recoveries ranging from 22.19 to 51.47% (Table 1).

The source of wastewater had no significant impact (two-way anova, n = 18, p-value = 0.4736) on the recovery efficiency of Phi6, regardless of the virus concentration method yet more variability was seen when testing sanitary sewer samples using PEG (Table A4).

Detection of SARS-CoV-2 in Wastewater Samples Using Ultrafiltration and PEG methods

All wastewater samples using the three concentration methods were also analyzed for SARS-CoV-2 using RT-ddPCR and RT-qPCR. The N1 and N2 gene targets showed similar results between the two PCR methods (Table 2). While the

Table 1 Recovery efficiencies of ultrafiltration and PEG methods for the detection of Phi6 in seeded wastewater samples

Wastewater Type	Sampling site (n = x)	Phi6 phage recovery as measured by RT-ddPCR Mean ± SD % (range)		
		Method 1/CEN1	Method 2/ CEN2	Method 3/PEG
Sanitary Sewer	Hospital Lift Station (3)	9.59 ± 1.14 (8.90–10.91)	4.99 ± 0.04 (4.95–5.02)	51.47 ± 26.08 (26.52–78.55)
	Community manhole (6)	10.60 ± 14.58 (1.98–39.9)	11.64 ± 6.05 (5.77–22.07)	25.49 ± 18.46 (3.93–47.49)
Wastewater Treatment Plant Influent	WWTP A (3)	6.05 ± 4.89 (0.48–9.64)	2.73 ± 2.04 (1.23–5.05)	36.01 ± 19.41 (23.03–58.33)
	WWTP E (3)	9.25 ± 15.72 (0.05–27.41)	9.21 ± 15.37 (0.10–26.95)	31.98 ± 7.52 (23.57–38.07)
	WWTP M (3)	2.60 ± 1.39 (1.03–3.64)	10.37 ± 12.61 (0.87–24.68)	22.19 ± 15.72 (4.67–35.04)

Table 2 The detection of SARS-CoV-2 genes (N1, N2, E) using ultrafiltration and PEG precipitation (with 16-h incubation) concentration methods

Concentration method	Sample Type	% Positive (Mean GC per 100 ml ± SD)					
		N1 gene		N2 gene	E gene		
		RT-ddPCR	RT-qPCR	RT-ddPCR	RT-qPCR	RT-ddPCR	RT-qPCR
CEN1	Sanitary Sewer (n = 9)	55.6 ($2.44 \pm 2.98 \times 10^3$)	11.1 ($8.74 \times 10^3 \pm 1.86 \times 10^4$)	66.7 ($3.27 \pm 3.20 \times 10^3$)	77.8 ($2.48 \pm 3.86 \times 10^4$)	88.9 ($5.06 \pm 6.32 \times 10^3$)	11.1
	WWTP Influent (n = 9)	55.6 ($1.57 \pm 2.71 \times 10^3$)	ND[a]	88.9 ($1.48 \pm 1.63 \times 10^3$)	44.4 ($1.38 \pm 1.45 \times 10^3$)	55.6 ($1.93 \pm 2.68 \times 10^3$)	ND
CEN2	Sanitary Sewer (n = 9)	55.6 ($3.82 \pm 5.21 \times 10^3$)	11.1 ($1.37 \pm 1.56 \times 10^3$)	55.6 ($1.48 \pm 3.63 \times 10^4$)	100 ($4.68 \pm 5.89 \times 10^3$)	66.7 ($3.16 \pm 4.95 \times 10^3$)	11.1
	WWTP Influent (n = 9)	55.6 ($6.55 \pm 8.33 \times 10^2$)	ND	88.9 ($2.01 \pm 1.94 \times 10^3$)	77.8 ($3.92 \pm 8.62 \times 10^3$)	44.4 ($5.01 \pm 3.81 \times 10^2$)	ND
PEG (with 16-h incubation)	Sanitary Sewer (n = 9)	44.4 ($1.27 \pm 1.98 \times 10^4$)	33.3 ($1.85 \pm 4.50 \times 10^4$)	33.3 ($1.22 \pm 2.08 \times 10^4$)	66.7 ($2.67 \pm 4.32 \times 10^4$)	44.4 ($9.27 \times 10^3 \pm 1.31 \times 10^4$)	11.1
	WWTP Influent (n = 9)	77.8 ($6.62 \pm 7.15 \times 10^3$)	22.2 ($7.55 \times 10^3 \pm 1.64 \times 10^4$)	66.7 ($6.76 \pm 7.14 \times 10^3$)	66.7 ($4.17 \pm 9.02 \times 10^4$)	66.7 ($5.87 \pm 8.10 \times 10^3$)	ND
All Methods	Sanitary Sewer (n = 27)	51.9 ($6.33 \times 10^3 \pm 1.24 \times 10^4$)	25.9 ($9.53 \times 10^3 \pm 2.80 \times 10^4$)	51.9 ($1.01 \pm 2.38 \times 10^4$)	81.5 ($1.87 \times 10^4 \pm 3.39 \times 10^4$)	66.7 ($5.83 \pm 8.90 \times 10^3$)	11.1
	WWTP Influent (n = 27)	63 ($2.95 \pm 5.03 \times 10^3$)	7.4 ($2.93 \pm 9.73 \times 10^3$)	81.5 ($3.42 \pm 4.85 \times 10^3$)	63 ($1.57 \pm 5.37 \times 10^4$)	55.6 ($2.77 \pm 5.27 \times 10^3$)	ND

ND Non-detect

[a]The detection limit of N1 gene for qPCR is 4.7 GC per reaction. The lower detection limit of the E gene for qPCR was 47 gene copies per reaction

E gene target performed satisfactorily on the RT-ddPCR platform, it showed poor results on the RT-qPCR platform with nearly all samples being identified as non-detects with no detected samples above the lower limit of quantification (LLOQ) (Table 2). The N2 gene target performed the best overall for the RT-qPCR assay. Using RT-ddPCR, the N1, N2, and E gene performed similarly with coefficients of variation for their detection of SARS-CoV-2 of 0.03 and 0.20 for sanitary sewer and WWTP influent samples, respectively (Table 2). Across three concentration methods RT-ddPCR showed fairly consistent patterns of SARS-CoV-2 detection, while the RT-qPCR assays relied heavily on the N2 gene target for SARS-CoV-2 detection (Table 2). Overall RT-ddPCR performed better at detecting SARS-CoV-2 gene targets than RT-qPCR in the wastewater samples tested with the exception of the N2 gene target in sanitary sewer samples which performed better with RT-qPCR (Table 2).

The overall concentrations of SARS-CoV-2 measured by RT-ddPCR for the three gene targets (N1, N2, E) ranged from < LLOD—5.71×10^4 GC/100 ml, < LLOD—1.11×10^5 GC/100 ml, and < LLOD—3.94×10^4 GC/100 ml, respectively (Table A3–A5). The overall concentrations of SARS-CoV-2 measured by RT-qPCR for the three gene targets (N1, N2, E) ranged from < LLOD—1.38×10^5 GC/100 ml, < LLOD—2.80×10^5 GC/100 ml, and < LLOQ, respectively (Table A3–A5). Slightly higher concentrations of N2 gene target in sanitary sewer and WWTP influent samples were obtained using RT-qPCR as compared to RT-ddPCR (Table 2).

Higher precision between gene targets was observed in the sanitary sewer samples versus the WWTP influent samples for both RT-ddPCR and RT-qPCR (Fig. 1). RT-ddPCR showed significantly lower coefficients of variations for every combination of concentration method and sample type with the exception of WWTP influents processed by CEN2 and PEG (Fig. 1).

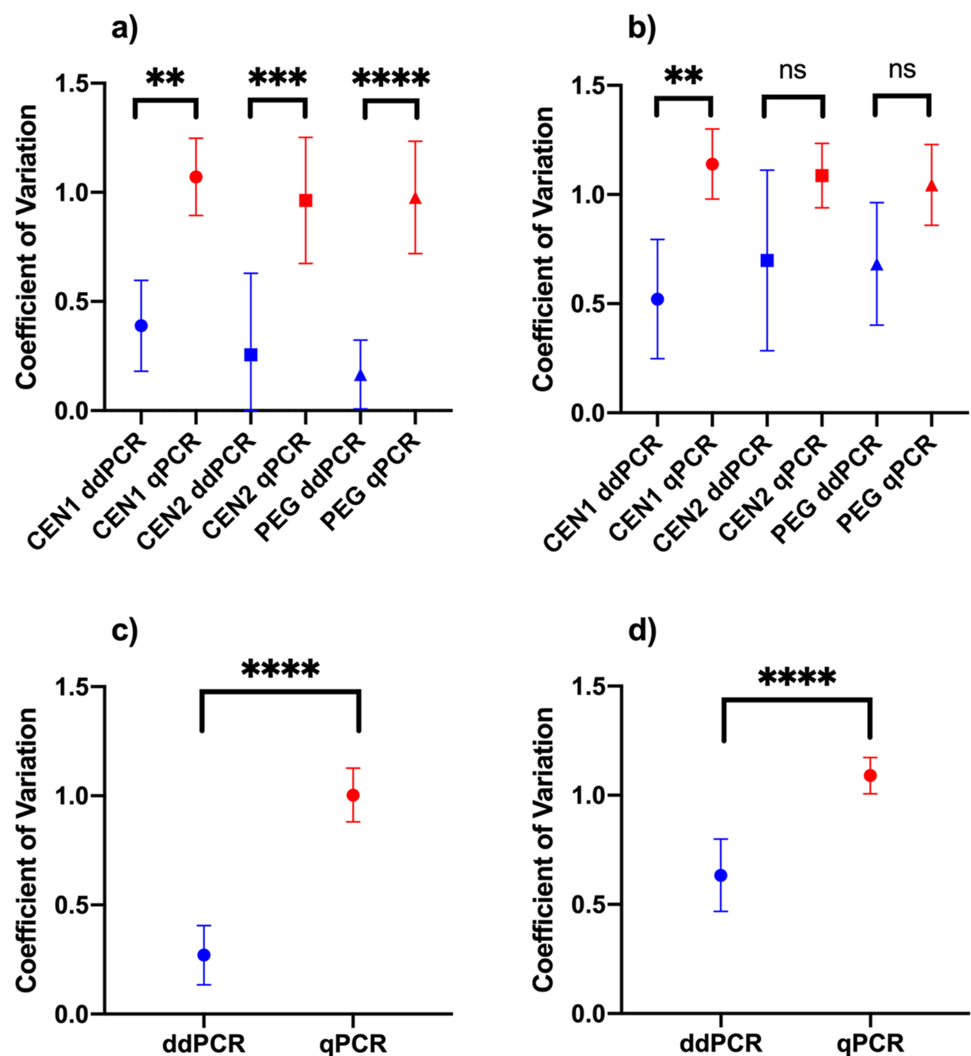

Fig. 1 Coefficients of variations for SARS-CoV-2 gene targets; **a** sanitary sewer samples, **b** WWTP influent samples, **c** sanitary sewer samples with all concentration methods, and **d** WWTP influent samples with all concentration methods. Two-way ANOVA analysis results shown above each graph; *ns* Not significant, *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001, ****p-value < 0.0001

Evaluation of Rapid PEG Approach for the Detection of Phi6 and SARS-CoV-2 in Wastewater

Based on the comparison between ultrafiltration and PEG methods for wastewater, the PEG precipitation was evaluated further for the detection of Phi6 and SARS-CoV-2 with and without 16 h of incubation. As shown in Table 3, without an overnight incubation, the PEG method showed an average recovery efficiency of 18.8% for sanitary sewer and of 35% for WWTP influent samples. In general, this approach produced lower recovery for Phi6 as compared with the PEG with overnight incubation, regardless of the type of wastewater samples. However, the difference was not statistically significant (Table 3).

For the detection of SARS-CoV-2 using RT-ddPCR, comparable results were obtained for the N1 and N2 gene targets with and without overnight incubation (Table 4). However, the rapid PEG method produced a lower percentage of WWTP influent samples positive for the E gene. Average SARS-CoV-2 concentrations varied little between sample types, PEG methods (i.e., with and without overnight incubation), and gene targets using RT-ddPCR, ranging from $1.82 \pm 4.55 \times 10^3$ GC/100 ml to $8.57 \times 10^3 \pm 1.12 \times 10^4$ GC/100 ml (Table 4).

Table 3 Mean recovery efficiencies of Phi6 in seeded wastewater samples using PEG precipitation method with and without overnight incubation

Wastewater Type	Phi6 mean recovery ± SD (%)	
	PEG with overnight (16-h) incubation	Rapid PEG without overnight incubation
Sanitary Sewer (n = 15)	32.07 ± 23.23	18.80 ± 11.48
WWTP Influent (n = 4)	31.31 ± 11.73	34.99 ± 26.38
All Samples (n = 19)	31.92 ± 21.27[a]	21.89 ± 15.88[a]

[a]No significant difference (n = 19, p = 0.1048) in mean recovery efficiencies between methods

Evaluation of PEG Precipitation Using Phi6 and Coronavirus OC43 as Potential SARS-CoV-2 Surrogates

When recovery efficiencies of Phi6 and OC43 were compared using five replicate influent samples from two WWTPs, no significant difference between the two surrogates was observed (Paired t test P value = 0.6137). These results are in line with recovery efficiencies for OC43 as reported by Pecson et al. (2021). However, the Phi6 recovery efficiencies observed in this study were almost 2 logs higher than the Phi6 recoveries achieved by other laboratories using the PEG method but with solids removal in the previous interlaboratory method assessment study (Pecson et al., 2021).

Discussion

This study demonstrates that *Pseudomonas* phage Phi6 seeded in different wastewater matrices can be concentrated and recovered by ultrafiltration-based method and PEG precipitation. In general, PEG method provided better virus recovery than the ultrafiltration-based methods as measured using RT-ddPCR. PEG precipitation is usually used as a secondary step for virus concentration in large volumes of water samples (De Keuckelaere et al., 2013; Polaczyk et al., 2008; Cuevas-Ferrando et al., 2020), but has also been used in concentrating enteric viruses directly from sewage as a primary concentration process prior to analysis by cell culture and molecular detection methods (Aw and Gin, 2010; Hovi et al., 2001; Myrmel et al., 2015; Shieh et al., 1995; Thongprachum et al., 2018). Enveloped viruses such as influenza A viruses and transmissible gastroenteritis virus (TGEV) have been detected in water samples using PEG precipitation combined with an initial filtration step (Blanco et al., 2019; Borchardt et al., 2017; Deboosere et al., 2011; Horm et al., 2012). However, only few studies have evaluated PEG

Table 4 Percent positive and mean concentrations of SARS-CoV-2 gene targets for PEG method with and without overnight incubation as measured using RT-ddPCR

Wastewater Type	% Positive (Mean GC per 100 ml ± SD)					
	With overnight (16-h) incubation			Without overnight incubation		
	N1	N2	E	N1	N2	E
Sanitary Sewer (n = 15)	33.3 ($4.36 \pm 9.21 \times 10^3$)	26.7 ($4.64 \pm 9.65 \times 10^3$)	26.7 ($3.39 \pm 6.70 \times 10^3$)	26.7 ($4.19 \pm 9.31 \times 10^3$)	26.7 ($3.95 \pm 8.80 \times 10^3$)	20 ($1.97 \pm 4.87 \times 10^3$)
WWTP Influent (n = 4)	75 ($8.57 \times 10^3 \pm 1.12 \times 10^4$)	50 ($2.0 \pm 2.15 \times 10^3$)	75 ($8.53 \pm 1.24 \times 10^4$)	75 ($3.97 \pm 3.71 \times 10^3$)	75 ($6.40 \pm 7.99 \times 10^3$)	25 ($7.92 \times 10^2 \pm 3.39 \times 10^1$)
All (n = 19)	42.1 ($5.25 \pm 9.49 \times 10^3$)	31.6 ($4.08 \pm 8.62 \times 10^3$)	36.8 ($4.47 \pm 8.06 \times 10^3$)	36.8 ($4.14 \pm 8.35 \times 10^3$)	36.8 ($4.46 \pm 8.48 \times 10^3$)	21.1 ($1.82 \pm 4.55 \times 10^3$)

precipitation as a primary concentration approach for viruses in sewage (e.g., Amdiouni et al., 2012; Hovi et al., 2001; Ye et al., 2016).

The exact mechanism of PEG precipitation of viruses from suspension is still not well understood. This could be due to that larger molecular aggregates such as viral particles are preferentially associated with inter-polymer spaces between PEG molecules and thus concentrated until their solubility is exceeded and precipitation occurs (Adams, 1973; Atha and Ingham, 1981). This may also precipitate out viruses attached to other particles in untreated wastewater which is particularly difficult to filter. Thus, this method may be better for those samples with a higher range of TSS and turbidities as evidenced by the wider range in SARS-CoV-2 N1 gene concentrations seen by Pecson et al. (2021) with PEG protocols which removed solids as compared to PEG protocols which retained solids.

In a previous study comparing three methods to concentrate enveloped murine hepatitis virus (MHV) from wastewater samples, PEG and ultracentrifugation recovered 5% of the seeded MHV, whereas the ultrafiltration produced significantly higher recovery, 25% (Ye et al., 2016). A possible explanation to lower virus recovery efficiencies obtained with PEG precipitation could be a high salt (NaCl) concentration (0.5 M) added to the samples. High salt concentrations may inactivate enveloped viruses during PEG precipitation process. For example, Hamelin and Lussier, (1979) showed that infectivity of cytomegalovirus (an enveloped virus) declined from 24.7 to 6.6% as the NaCl concentration was progressively increased from none to 1.0 M NaCl. It has also been reported that infectivity of retro- and lentiviruses decreases significantly in a high salt elution buffer (1 M NaCl) (Zimmermann et al., 2011). In this study, 0.2 M NaCl was used in the PEG protocol.

The choice of PEG precipitation over other concentration methods was also based on the affordability of the procedure and the shortages of the filtration materials due to the increasing numbers of laboratories worldwide that monitor SARS-CoV-2 in wastewater. PEG precipitation is a simple and low-cost alternative (e.g., < $2 USD per sample for PEG method vs. > $34 USD for ultrafiltration-based method) for the concentration of viruses in wastewater without requiring any preconditioning of the sample. The PEG method used in this study has also been evaluated in a recent interlaboratory methods assessment for SARS-CoV-2 genetic signal in raw sewage using betacoronavirus OC43 as a matrix spike. By comparing 36 standard operating procedures used by 32 participating laboratories, PEG precipitation has shown a high degree of reproducibility across laboratories (Pecson et al., 2021).

Although PEG precipitation provided higher recovery efficiencies for Phi6 and SARS-CoV-2 in wastewater when compared with ultrafiltration, the protocol is slower particularly with an overnight incubation. However, in this study, the results of PEG precipitation with and without an overnight incubation for Phi6 and SARS-CoV-2 were not statistically significant. This is in agreement with other studies that reported a 2-h precipitation is sufficient for viruses (Deboosere et al., 2011; Polaczyk et al., 2008). Therefore, the PEG protocol could be shortened to increase throughput or accommodate existing analysis workflows for rapid results.

In addition to investigating recovery efficiencies of artificially seeded viruses using different concentration methods, this study compared the detection of SARS-CoV-2 genetic signals in wastewater using RT-qPCR and RT-ddPCR. Overall, RT-ddPCR showed higher sensitivity rate compared to RT-qPCR. While RT-qPCR shows equivalent detection rate of the SARS-CoV-2 N2 gene as RT-ddPCR, RT-ddPCR performed better for the E gene in wastewater. This may be due to RT-ddPCR allowing for greater PCR efficiency when lower concentrations of the target gene are present and its ability to cope with higher levels of inhibitory substances in wastewater. While a high number of samples in this study were found to be positive for one or more of the SARS-CoV-2 gene targets, a direct comparison of the virus concentrations between sanitary sewer and WWTP influent samples would be inaccurate due to the different sampling methods. For sanitary sewer, grab sampling was used to collect wastewater directly from manholes or lift station, whereas composite sampling technique was used for the WWTP. Different wastewater sampling techniques may influence the ability to detect and quantify viral genetic markers using PCR-based methods. For example, a grab sample taken during low flow periods may miss detecting the SARS-CoV-2 genetic markers in wastewater. A similar situation can occur for composite samples particularly for long sampling periods (e.g., 24 h) as the viral signals may be diluted. Therefore, determination of the optimal sampling strategy and timing will greatly enhance the ability to accurately detect SARS-CoV-2 in wastewater. Heaton et al. (1992) showed that over 60% of men and women defecated between 5 am and 12 pm each day. These patterns may have changed since the study, but sample collection time is still an important factor to consider when conducting a wastewater surveillance for SARS-CoV-2.

The concentration and detection procedures outlined in this study will facilitate rapid and high-throughput detection of SARS-CoV-2 in wastewater samples. The methods were used successfully in field studies for the detection of SARS-CoV-2 RNA in various wastewater samples.

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Data availability Full data are available upon request sent to corresponding author.

Declarations

Conflict of interest The authors declare no conflicts of interest nor competing interests.

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