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Science of the Total Environment 882 (2023) 163487

Contents lists available at ScienceDirect

Science of the Total Environment

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

Optimization and performance evaluation of an automated filtration method for the recovery of SARS-CoV-2 and other viruses in wastewater

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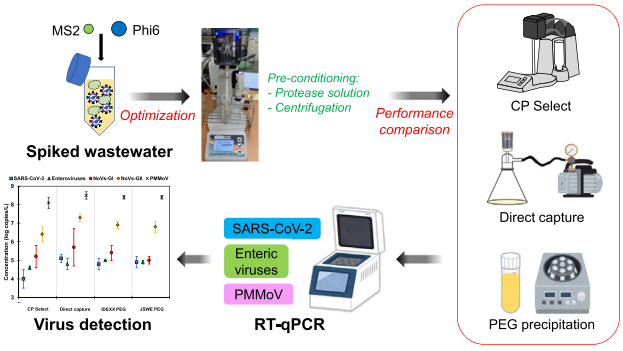
HIGHLIGHTS

- Testing CP Select automated filtration method for virus detection in wastewater
- The addition of protease solution increased SARS-CoV-2 recovery from wastewater.
- Higher performance was achieved using 0.05 μm PS hollow fiber filter tips.
- SARS-CoV-2 RNA was detected in 75 % samples using the optimized CP Select protocol.
- Comparable sensitivity of enteric virus detection was observed with other methods.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Damià Barceló

Keywords:
CP select
Enteric virus
SARS-CoV-2
Virus concentration
Wastewater-based epidemiology

ABSTRACT

A rapid virus concentration method is needed to get high throughput. Reliable results of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) detection in wastewater are necessary for applications in wastewater-based epidemiology. In this study, an automated filtration method using a concentrating pipette (CP Select; Innovaprep) was applied to detect SARS-CoV-2 in wastewater samples with several modifications to increase its sensitivity and throughput. The performance of the CP Select method was compared to other concentration methods (polyethylene glycol precipitation and direct capture using silica column) to evaluate its applicability to SARS-CoV-2 detection in wastewater. SARS-CoV-2 RNA was successfully detected in six of eight wastewater samples using the CP Select method, whereas other methods could detect SARS-CoV-2 RNA in all wastewater samples. Enteric viruses, such as noroviruses of genogroups I (NoVs-GI) and II (NoVs-GII) and enteroviruses, were tested, resulting in 100 % NoVs-GII detection using all concentration methods. As for NoVs-GI and enteroviruses, all methods gave comparable number of detected samples in wastewater samples. This study showed that the optimized CP Select method was less sensitive in SARS-CoV-2 detection in wastewater than other methods, whereas all methods were applicable to detect or recover other viruses in wastewater.

1. Introduction

The global pandemic of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) was first reported in Wuhan, China, in December 2019. Although SARS-CoV-2 spreads mainly through droplets via air, several studies have reported the presence of this virus in the stools of infected patients (Cevik et al., 2021; Chen et al., 2020; Cheung et al., 2020; Xiao et al., 2020). Based on these findings, the wastewater-based epidemiology (WBE) approach of SARS-CoV-2 detection in wastewater has been extensively used recently (Albastaki et al., 2021; Hemalatha et al., 2021; Prado et al., 2021; Saththasivam et al., 2021). WBE can be used as additional data to support

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http://dx.doi.org/10.1016/j.scitotenv.2023.163487
Received 5 February 2023; Received in revised form 6 April 2023; Accepted 9 April 2023
Available online 15 April 2023

clinical data to decide and establish policies; thus, preventive actions to reduce community infections can be made (Kitajima et al., 2020).

Low virus concentration is a shortcoming in analyzing or detecting viruses in water or wastewater. To tackle the problem, the virus concentration tends to be used before subjecting the whole sample to DNA/RNA extraction to increase detection sensitivity in the sample. Several virus concentration methods exist, but the efficiency of each method can be different depending on the virus type. For example, to concentrate nonenveloped enteric viruses, such as noroviruses (NoVs) and adenoviruses, in wastewater, virus adsorption and elution methods using electronegative membranes have been widely used (Haramoto et al., 2018). As for SARS-CoV-2 or its surrogate viruses, several studies have reported that polyethylene glycol (PEG) precipitation methods provide sensitive detection in influent wastewater samples (Barril et al., 2021; Kumar et al., 2020; Torii et al., 2021); however, they are time-consuming. Several other PEG precipitation conditions have been reported and successfully detected or gave promising recovery of SARS-CoV-2 in wastewater samples with shorter processing time (Dimitrakopoulos et al., 2022; Maksimovic Carvalho Ferreira et al., 2022; Torii et al., 2022): for example, the protocol that developed by IDEXX Laboratories (Westbrook, ME, USA) was found to have better recovery efficiency of φ6 and pepper mild mottle virus (PMMoV) than other PEG precipitation protocols (Torii et al., 2022).

Another approach to reducing the processing time, including the direct capture method, was also tried in several studies by extracting nucleic acids directly from wastewater samples (Mondal et al., 2021; Whitney et al., 2021). The direct capture method using the Promega (Madison, WI, USA) kit is a novel column-based technique that combines virus concentration and nucleic acid extraction and purification into a one-step method. With protease addition as pretreatment, contaminated or unwanted proteins and nucleases will break down in the sample (Eychner et al., 2015; Farkas et al., 2020). However, specific conditions of protease treatment should be considered as protease addition affected viral capsid (Langlet et al., 2018).

Automated filtration using concentrating pipettes (CP Select) from InnovaPrep (Drexel, MO, USA) is a promising method for rapid and simple virus concentration with high detection sensitivity (Gonzalez et al., 2020; Juel et al., 2021; Kevill et al., 2022). However, the initial costs and cost per sample processed are high, as it requires quite an advanced and automated filtration system. Filtering the whole wastewater sample directly through this system will be impractical since it will increase the chance of clogging in the filter tip. Thus, centrifugation as pre-treatment is necessary to obtain the supernatant before subjecting to filtration. However, using only the supernatant might decrease the detection sensitivity since SARS-CoV-2 is mostly present in the solid portion of wastewater (Kitamura et al., 2021; Li et al., 2021). To minimize the virus loss in the resulting pellet after centrifugation, protease solution was added to help viral genomic releases from the suspended solid. In addition, an incubation time of 30 min after protease addition was needed to release viral particles attached to the solid portions of wastewater, indicating that all viral particles are expected to be present in the supernatant when it is filtered (Mondal et al., 2021). In the end, many factors besides recovery performance also need to be considered, such as cost, processing time, access to reagents, and scalability (Barril et al., 2021; Lu et al., 2020).

As a newly applied method, this study aimed to optimize the CP Select protocol, evaluate its performance, and compare it to other methods to assess the applicability of various concentration methods not only for SARS-CoV-2 but also for other viruses, such as enteric viruses, in wastewater.

2. Materials and methods

2.1. Wastewater samples

Seven influent wastewater samples were collected between December 6, 2021 and February 9, 2022: three grab influent wastewater samples from three treatment lines (KO, M2, and M3) of two different wastewater treatment plants (WWTPs) and four composite influent wastewater samples from four different WWTPs (FU, KY, KM, and KT) were collected. In several WWTPs, influent samples were collected more than once between December 2021 and February 2022. The samples used for each experiment are shown in Table 1. All WWTPs were located in Japan, where COVID-19 incidence was very low at the end of 2021. In addition, a grab influent sample was collected on October 26, 2021 from a large-scale septic tank of a COVID-19 quarantine facility in Japan (Iwamoto et al., 2022). The samples were collected into autoclaved polyethylene bottles, transported to the laboratory on ice, and kept at 4 °C until further analysis.

2.2. Enumeration of Escherichia coli

E. coli in wastewater samples was enumerated by a culture-based method using a CHROMagar ECC (Kanto Chemical, Tokyo, Japan), according to the manufacturer's protocol. The blue colonies were counted after 24 h incubation at 37 °C.

2.3. Viral stock preparation

Wastewater samples were seeded by *Pseudomonas* phage φ6 [NBRC 105899; National Institute of Technology and Evaluation (NITE), Tokyo, Japan], a surrogate of enveloped viruses (Aquino De Carvalho et al., 2017), along with coliphage MS2 (15597-B1, American Type Culture Collection, Manassas, VA, USA), a surrogate of nonenveloped viruses (Ye et al., 2016). *Pseudomonas* phage φ6 was propagated using *Pseudomonas syringae* (NBRC14084; NITE) as a host strain, as described previously (Torii et al., 2021). *Salmonella typhimurium* WG49 was used as a host strain for MS2. The φ6 and MS2 stocks were diluted 20- and 200-fold, respectively, before seeding into the samples. Because of the low incidence cases of COVID-19 in the area where the WWTPs were located between November and December 2022, a SARS-CoV-2 stock (Zeptometrix, Buffalo, NY, USA) with an initial concentration of ~10^6 copies/mL was also seeded to wastewater samples (Table 1). The nonseeded samples were also processed as the COVID-19 cases were quite unpredictable and fluctuated. The samples were seeded with that stock or diluted stock at a ratio of 1:1000. To mimic the real condition of sewage, the seeded samples were mixed in slow motion at 30 rpm using a rotator (Nichiryo, Koshigaya, Japan) for 10 min at room temperature. The recovery (%) of seeded viruses was calculated from the copy number obtained from the seeded samples using the copy number of stock or diluted stock for SARS-CoV-2, φ6, or MS2 as the baseline for total recovery.

2.4. Virus concentration using the CP Select method

A 40 mL seeded sample was added to a 50 mL tube and centrifuged at 3000 ×*g* for 10 min. The supernatant and pellet were tested for virus

Table 1
Wastewater samples used in this study.

WWTP type	WWTP ID	Sample ID	Sampling date (dd/mm/yyyy)	*E. coli* concentration (CFU/mL)
Quarantine facility		A	26/10/2021	15,000
Municipal WWTP	KO	B[a]	06/12/2021	66,000
		C	19/01/2022	52,000
		D	25/01/2022	47,000
		E	07/02/2022	42,000
		F	09/02/2022	39,000
	M2	G[a]	06/12/2021	17,000
		H	17/01/2022	16,000
		I	24/01/2022	8,000
		J	07/02/2022	16,000
	M3	K	17/01/2022	69,000
		L	24/01/2022	40,000
		M	07/02/2022	33,000
	FU	N[a]	07/12/2021	40,000
		O	08/02/2022	17,000
	KY	P	08/02/2022	22,000
	KM	Q	08/02/2022	49,000
	KT	R	08/02/2022	32,000

[a] SARS-CoV-2 stock was spiked to the wastewater samples.

recovery, along with the raw whole sample, to assess whether centrifugation as pretreatment is needed. To assess the effect of protease that was expected to increase detection sensitivity, a 500 μL protease solution (Promega) was added before centrifugation. The sample and protease mixture were incubated for two different timeframes (10 and 30 min) to optimize the protocol. The supernatant was recovered into a new 50 mL tube and filtered by the CP Select using various sizes of filter tips. Based on the manufacturer's manual, the expected final concentrated volume per elution ranged from 150 to 1000 μL using polysulfone (PS) hollow fiber filter tips. Thus, the particles attached to the filter tip were eluted three to five times with Elution Fluid-Tris (InnovaPrep) to obtain a final concentrated sample of 400 to 600 μL.

2.5. Virus concentration using the PEG precipitation method

Two different PEG precipitation methods were conducted in this study. The first method (later called "JSWE PEG") was conducted by following the Manual for Detection of SARS-CoV-2 RNA in Wastewater (Japan Society on Water Environment COVID-19 Taskforce, 2022) and previous studies (Hata et al., 2021; Torii et al., 2021), with slight modifications. In brief, 4.0 g PEG 8000 (Sigma-Aldrich, St. Louis, MO, USA) and 2.35 g NaCl (Kanto Chemical) were added into the 40 mL seeded sample without centrifugation at a final concentration of 10 % (w/v) and 1.0 mol/L, respectively. The mixture was incubated overnight at 4 °C, with continuous mixing using a magnetic stirrer. Subsequently, the mixture was centrifuged at 10,000 ×*g* for 30 min at 4 °C. The resulting supernatant was discarded, and the pellet was resuspended with 800 μL polymerase chain reaction (PCR)-grade water to obtain a concentrated sample of 900 to 1200 μL.

The second method (later called "IDEXX PEG") was conducted by following a protocol by IDEXX Laboratories, as described previously (Malla et al., 2022). Briefly, 4.0 g PEG 8000 and 0.94 g NaCl were added into the 40 mL seeded sample without initial centrifugation to obtain a final concentration of 10 % (w/v) and 0.4 mol/L, respectively. The mixture was centrifuged at 12,000 ×*g* for 99 min at 4 °C. The resulting supernatant was discarded, and the pellet was resuspended with 800 μL PCR-grade water to obtain a concentrated sample of 850 to 1000 μL.

2.6. Virus concentrations using the direct capture method

The direct capture method was conducted using the Maxwell Enviro Wastewater TNA kit (Promega), according to the manufacturer's protocol. A 40 mL seeded sample was added into a 50 mL tube, followed by 500 μL protease solution. The mixture was incubated for 30 min and centrifuged at 3000 ×*g* for 10 min at room temperature. The supernatant was transferred into two 50 mL tubes to obtain a total volume of ~20 mL for each. Then, 5.5 mL Binding Buffer 1 and 0.5 mL Binding Buffer 2 were added to those two tubes containing the samples to obtain a total volume of ~26 mL each. The tubes were shaken gently, and 24 mL isopropanol (Kanto Chemical) was added to those tubes to obtain a total volume of 50 mL each. The mixtures were shaken gently before passing through the PureYield Midi Binding Column using a vacuum manifold. The PureYield Midi Binding Column was then washed using 5 mL Column Wash 1, followed by 30 mL Column Wash 2. The nucleic acid in the PureYield Midi Binding Column was recovered by 0.5 mL nuclease-free water preheated at 60 °C to obtain a concentrated sample with a final volume of 300 to 400 μL.

2.7. RNA extraction

RNA extraction was conducted using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Then, 140 μL concentrated samples were processed to obtain 60 μL viral RNA extract.

2.8. Reverse transcription-quantitative PCR (RT-qPCR)

For SARS-CoV-2, φ6, and PMMoV assays, the viral RNA extract was directly applied for one-step RT-qPCR using a SARS-CoV-2 Detection RT-qPCR Kit for Wastewater (Takara Bio, Kusatsu, Japan). SARS-CoV-2 RNA was detected using the CDC N1N2 assay (Centers for Disease Control and Prevention, 2020), where probes of CDC N1 and N2 assays were labeled with a Cy5 reporter dye. In addition, φ6 (Gendron et al., 2010) and PMMoV (Haramoto et al., 2013; Zhang et al., 2006), an indigenous and the most abundant virus in wastewater (Kitajima et al., 2018), were detected in a duplex one-step RT-qPCR, where probes were labeled with HEX and FAM, respectively. Each 25 μL of an RT-qPCR mixture contained 5.0 μL RNA, 2.5 μL of a mixture of primers and probe, 12.5 μL One-Step RT-qPCR Mix, and 5.0 μL RNase-free water. The thermal conditions of RT-qPCR for all assays were performed as follows: initial incubation at 25 °C for 10 min, RT reaction at 52 °C for 5 min and 95 °C for 10 s, initial denaturation at 95 °C for 30 s, followed by 45 cycles of denaturation at 95 °C for 5 s and annealing and extension at 60 °C for 30 s.

Two-step RT-qPCR was conducted for MS2 (Friedman et al., 2011), enteroviruses (Katayama et al., 2002; Shieh et al., 1995), and NoVs of genogroups I (NoVs-GI) and II (NoVs-GII; Kageyama et al., 2003). A 30 μL viral RNA extract was further subjected to RT to obtain 60 μL cDNA using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's protocol. A 2.5 μL cDNA was mixed with 22.5 μL qPCR mixture containing 0.1 μL each of forward and reverse primers (100 pmol/μL), 0.05 μL probe (100 pmol/μL), 12.5 μL Probe qPCR Mix with UNG (Takara Bio), and 9.75 μL PCR-grade water. The thermal conditions of qPCR were performed as follows: 25 °C for 10 min and 95 °C for 30 s, followed by 45 cycles of 95 °C for 5 s and 60 °C for 30 s for NoVs-GI and NoVs-GII, 60 °C for 60 s for enteroviruses, and 56 °C for 60 s for MS2.

To obtain a standard curve, Positive Control DNA included in the SARS-CoV-2 Detection RT-qPCR Kit for Wastewater was used for SARS-CoV-2, φ6, and PMMoV assays. In contrast, gBlocks (Integrated DNA Technologies, Coralville, IA, USA) were used for MS2, enteroviruses, NoVs-GI, and NoVs-GII. These standards were serially diluted 10-fold using EASY Dilution (for Real-time PCR; Takara Bio) to obtain concentrations from 10^0 to 10^5 copies/μL. Negative control was also included in every qPCR run to confirm no contamination in the reagents. All samples, including standards and negative control, were performed in duplicate (qPCR technical replicates). Threshold cycle (Ct) values >40 were counted as negative.

2.9. Statistical analysis

Paired *t*-test was used to determine the most optimum modifications and compare the mean concentration and recovery of viruses in wastewater samples. Pearson correlation was used to observe the relationship between *E. coli* and SARS-CoV-2 concentrations in wastewater samples. Statistical analysis was performed using Microsoft Excel 2019 (Microsoft Corporation, Redmond, WA, USA). *P* values <0.05 were considered significant.

3. Results and discussion

3.1. Optimization of the CP Select protocol

Because the filtration-elution method using the CP Select is a relatively new virus concentration method, optimizing the manufacturer's protocol is needed to improve the detection sensitivity in wastewater samples. Several modifications were conducted in this study to increase the chance of virus detection, especially for SARS-CoV-2 in wastewater.

3.1.1. Sample preconditioning

Wastewater samples (Samples A and B; n = 2 each) were processed with two conditions (i.e., with and without centrifugation) to evaluate which condition is better to be applied in the protocol. In Table 2, using 0.45 μm PS hollow fiber filter tips, the supernatant from the initial centrifugation as pretreatment gave comparable results to the directly filtered sample for φ6 and MS2 (n = 4; paired *t*-test, $P > 0.05$). Using the supernatant is more practical because there is a chance of clogging in the filter tip if the whole raw sample is used (the turbidity of the wastewater sample usually

Table 2
Recovery of SARS-CoV-2 and surrogate viruses using centrifugation as pretreatment.

Target virus (no. of tested samples)	Sample fraction	No. seeded viruses (mean ± SD; copies)	No. recovered viruses (mean ± SD; copies)	% Recovery (mean ± SD)
SARS-CoV-2 (n = 2)	Whole	8.1×10^4	$8.9 \times 10^3 \pm 1.4 \times 10^3$	11.0 ± 1.8
	Supernatant		$2.5 \times 10^4 \pm 1.3 \times 10^4$	30.4 ± 15.5
	Pellet		$2.2 \times 10^3 \pm 9.8 \times 10^2$	2.7 ± 1.2
φ6 (n = 4)	Whole	$6.7 \times 10^6 \pm 6.7 \times 10^4$	$4.6 \times 10^5 \pm 1.2 \times 10^5$	6.9 ± 1.8
	Supernatant		$6.6 \times 10^5 \pm 9.6 \times 10^4$	9.9 ± 1.4
	Pellet		$4.1 \times 10^5 \pm 3.8 \times 10^5$	6.1 ± 5.7
MS2 (n = 4)	Whole	$5.0 \times 10^7 \pm 1.3 \times 10^7$	$1.4 \times 10^6 \pm 8.0 \times 10^5$	2.8 ± 1.6
	Supernatant		$1.4 \times 10^6 \pm 4.9 \times 10^5$	2.6 ± 1.1
	Pellet		$1.7 \times 10^5 \pm 1.1 \times 10^5$	0.4 ± 0.3

SD, standard deviation.

fluctuates). The recovery of SARS-CoV-2, φ6, and MS2 from the supernatant was 30.4 % ± 15.5 % (n = 2), 9.9 % ± 1.4 % (n = 4), and 2.6 % ± 1.1 % (n = 4), respectively. Low recovery of SARS-CoV-2 in the pellet portions was unexpected, as many studies reported that SARS-CoV-2 mostly attached to solid fractions in wastewater (D'Aoust et al., 2021; Kitamura et al., 2021; Torii et al., 2022). Nonindigenous SARS-CoV-2 (by seeding) in the samples might be a reason for low recovery in the pellet portion, as it takes a long retention time for the viruses to attach to the solid fraction present in wastewater samples. However, another study also found that 77 % of detectable indigenous SARS-CoV-2 fraction was found in the supernatant, and the rest was detected in the pellet (Forés et al., 2021), suggesting the applicability of using the supernatant only while conducting the CP Select method.

3.1.2. Elution volume and frequency

Based on the manual protocol of the CP Select, a single elution step using a PS hollow fiber tip produced only ~150 μL. Thus, several elution times are needed to obtain enough concentrate volume for downstream analysis. Because the required volume needed for RNA extraction for this study was 140 μL concentrated sample, this study tried to obtain a final volume of the concentrated sample of ~500 μL or three to five times elution. Wastewater samples (Samples A and B; n = 2 each) were filtered using 0.45 μm PS hollow fiber filter tips after initial centrifugation to obtain the supernatant.

In Table 3, the recovery of φ6 in the first elution was significantly higher than in the second elution (n = 4; paired *t*-test, $P < 0.05$), showing that the first elution was enough to recover most of the viruses from the sample. Another study also found that the first elution step of the CP Select method could recover <30 % of human betacoronavirus OC43 in the samples, whereas the second elution increased the recovery yield by ~20 % (McMinn et al., 2021). A different result was obtained from MS2, as every elution gave comparable recovery (n = 4; paired *t*-test, $P > 0.05$). Thus, three to five times elution was considered practical for the optimized protocol to obtain a final concentrated volume of ~500 μL.

3.1.3. Protease treatment

Protease addition as pretreatment was expected to improve detection sensitivity, especially when using only the supernatant as preconditioning samples. Protease addition was assessed to evaluate the positive or negative impact on the overall virus recovery. Using the 0.45 μm PS hollow fiber filter tips, the addition of 500 μL protease solution with 30 min incubation before centrifugation significantly increased the recovery of SARS-CoV-2 (n = 4; paired *t*-test, $P < 0.05$; Table 4) in wastewater samples [Samples B, G, and N; n = 1 each, except for Sample B (n = 2)]. As mentioned before, it is expected that protease treatment helps destroy unwanted proteins and release genomic materials from the suspended solid in wastewater samples. The addition of protease solution in wastewater samples [Samples A, B, G,

Table 3
Recovery of SARS-CoV-2 and surrogate viruses from each elution.

Target virus (no. tested samples)	Elution times	No. seeded viruses (mean ± SD; copies)	No. recovered viruses (mean ± SD; copies)	% Recovery (mean ± SD)
SARS-CoV-2 (n = 2)	1st	8.1×10^4	$1.9 \times 10^4 \pm 1.0 \times 10^4$	22.9 ± 12.4
	2nd		$4.0 \times 10^3 \pm 1.7 \times 10^3$	4.9 ± 2.1
	3rd		$1.3 \times 10^3 \pm 6.8 \times 10^2$	1.6 ± 0.8
	4th		$7.5 \times 10^2 \pm 6.2 \times 10^1$	0.9 ± 0.1
	Total		$2.5 \times 10^4 \pm 1.3 \times 10^4$	30.4 ± 15.5
φ6 (n = 4)	1st	$6.7 \times 10^6 \pm 6.7 \times 10^4$	$4.7 \times 10^5 \pm 4.6 \times 10^4$	7.0 ± 0.7
	2nd		$1.1 \times 10^5 \pm 4.9 \times 10^4$	1.7 ± 0.7
	3rd		$5.0 \times 10^4 \pm 1.1 \times 10^4$	0.7 ± 0.2
	4th		$3.0 \times 10^4 \pm 1.3 \times 10^4$	0.4 ± 0.2
	Total		$6.6 \times 10^5 \pm 9.6 \times 10^4$	9.9 ± 1.4
MS2 (n = 4)	1st	$5.0 \times 10^7 \pm 1.3 \times 10^7$	$6.4 \times 10^5 \pm 3.8 \times 10^5$	1.3 ± 0.9
	2nd		$3.1 \times 10^5 \pm 1.3 \times 10^5$	0.6 ± 0.3
	3rd		$2.0 \times 10^5 \pm 1.2 \times 10^5$	0.4 ± 0.2
	4th		$2.1 \times 10^5 \pm 2.1 \times 10^5$	0.4 ± 0.4
	Total		$1.4 \times 10^6 \pm 4.9 \times 10^5$	2.6 ± 1.1

Table 4
Recovery of SARS-CoV-2 and surrogate viruses with protease treatment.

Target virus (no. tested samples)	Protease treatment	No. seeded viruses (mean ± SD; copies)	No. recovered viruses (mean ± SD; copies)	% Recovery (mean ± SD)
SARS-CoV-2 (n = 4)	No	$9.9 \times 10^4 \pm 3.4 \times 10^4$	$9.7 \times 10^3 \pm 6.7 \times 10^3$	9.5 ± 7.4
	Yes		$3.0 \times 10^4 \pm 1.8 \times 10^4$	26.6 ± 15.1
Φ6 (n = 5)	No	$1.6 \times 10^7 \pm 1.4 \times 10^7$	$1.3 \times 10^6 \pm 1.3 \times 10^6$	7.3 ± 4.0
	Yes		$1.2 \times 10^6 \pm 1.0 \times 10^6$	6.5 ± 3.8
MS2 (n = 5)	No	$6.4 \times 10^7 \pm 1.8 \times 10^7$	$8.4 \times 10^5 \pm 5.4 \times 10^5$	1.6 ± 1.4
	Yes		$1.1 \times 10^6 \pm 5.2 \times 10^5$	1.8 ± 1.0

Table 5
Detection of SARS-CoV-2 and recovery of surrogate viruses with different incubation times of the protease solution.

Target virus	Protease incubation time (min)	No. positive samples/no. tested samples (%)	Concentration (mean ± SD; log copies/L)[a]	No. seeded viruses (copies)	No. recovered viruses (mean ± SD; copies)	% Recovery (mean ± SD)
SARS-CoV-2	10	5/6 (83)	4.6 ± 0.3	NA	NA	NA
	30	5/6 (83)	4.5 ± 0.3		NA	NA
Φ6	10	NA	NA	1.2×10^7	$4.2 \times 10^5 \pm 1.9 \times 10^5$	3.7 ± 1.7
	30	NA	NA		$5.1 \times 10^5 \pm 3.2 \times 10^5$	4.4 ± 2.8
MS2	10	NA	NA	3.3×10^7	$8.8 \times 10^5 \pm 4.2 \times 10^5$	2.6 ± 1.3
	30	NA	NA		$1.1 \times 10^6 \pm 6.3 \times 10^5$	3.4 ± 1.9

NA, not applicable.
[a] The mean concentration of each virus was calculated based on positive samples only.

and N; n = 1 each, except for Sample B (n = 2)] before initial centrifugation also did not seem to have any antagonistic effect on the recovery of other surrogate viruses (φ6 and MS2; n = 5 each; paired *t*-test, $P > 0.05$).

Optimization of incubation time was also assessed by comparing 10 and 30 min incubation times of protease to see if there is any difference in recovery between the two incubation times. Using 0.05 μm PS hollow fiber filter tips, incubation times of 10 and 30 min gave similar results of detection for SARS-CoV-2 (83 % positive) in wastewater samples (Samples C, D, H, I, K, and L; Table 5). In addition, there was no significant difference between the two timeframes for φ6 and MS2 recovery (n = 6; paired *t*-test $P > 0.05$), suggesting that protease incubation time of 10 min is enough as pretreatment before the sample is subjected to the CP Select method.

3.1.4. Filter tip types

Because there are many filter tips, SARS-CoV-2 detection in wastewater samples was assessed to find the best filter tip to be used in the protocol. In this experiment, 500 μL protease solution was added to the samples (Samples C, D, H, I, K, and L) and incubated for 10 min before being subjected to centrifugation (3000 ×*g*, 10 min, 25 °C) to get the supernatant.

As summarized in Table 6, the overall results showed that 0.05 μm PS hollow fiber filter tips gave a better performance for φ6 and MS2 recovery than 0.2 and 0.45 μm PS hollow fiber filter tips (n = 6; paired *t*-test, $P < 0.05$), except that 0.45 μm PS hollow fiber filter tips gave a comparable performance with 0.05 μm PS hollow fiber filter tips for φ6 recovery (n = 6; paired *t*-test, $P > 0.05$). In addition, 0.05 μm PS hollow fiber filter tips were more sensitive in SARS-CoV-2 detection (100 % positive) than the other two pipette types (0.2 and 0.45 μm) with 83 % positive detection each. It was expected that more solid particles would be attached to the tip of the filter with a smaller pore size because this method is based on size exclusion. This study also tried to assess another filter tip, the ultrafiltration PS hollow fiber filter tip, which is also widely used in the CP Select method (Forés et al., 2021; McMinn et al., 2021). However, in this experiment, this filter tip had difficulty even in filtering ultrapure water. Thus, this study only evaluated those three different filter tips. Several studies have detected SARS-CoV-2 and its surrogates by the CP Select method using a 0.05 μm PS hollow fiber filter tip (Ahmed et al., 2021; Gonzalez et al., 2020; Juel et al., 2021; Kevill et al., 2022), suggesting that this pore size is optimum enough to concentrate viruses in wastewater samples.

3.2. Performance comparison of virus concentration methods

Using the optimized protocol, the CP Select detected several viruses, including SARS-CoV-2, in wastewater samples (Samples E, F, J, M, and O–R). In Table 7, the CP Select method could detect SARS-CoV-2 in 6 of 8 (75 %) wastewater samples tested, whereas other methods could detect it in all samples. Because the optimized protocol used only the supernatant, there is a possibility of a high virus loss in the pellet or solid portion where SARS-CoV-2 was present the most (D'Aoust et al., 2021; Kitamura et al., 2021; Torii et al., 2022). Based on Fig. 1, the mean concentration of SARS-CoV-2 in detected samples using the CP Select method was 4.0 ± 0.5 log copies/L, which was significantly lower than other methods (n = 6; paired *t*-test, $P < 0.05$). Even though the CP Select method could detect NoVs-GII in all tested samples (Table 7), the performance of this method was lower than other method (n = 8; paired *t*-test, $P < 0.05$), with a mean concentration of 6.4 ± 0.4 log copies/L. A possibility of high inhibition in the negative samples has already been ruled out because comparable PMMoV concentrations as process control were observed among virus concentration methods (n = 8; paired *t*-test, $P > 0.05$), except for the CP Select method with a mean concentration of 8.1 ± 0.3 log copies/L. Lower mean concentration of SARS-CoV-2, NoVs-GII, and PMMoV by the CP Select method indicates another possibility that high protease concentration destroyed all viral particles; thus, nucleic acids present in the sample went through the filter tips as a discarded liquid portion. Another approach to detach viral particles in suspended solid is by adding 10 % Tween-20 (Polyoxyethylene (20) sorbitan monolaureate) with ratio 1:100 as manufacturer's recommendation; however, similar fraction of indigenous SARS-CoV-2 was recovered from supernatant and resulting pellet (Juel et al., 2021). In addition, no significant difference was observed between addition and without addition of Tween-20 (Forés et al., 2021), suggesting another approach beside addition of Tween-20 before centrifugation to improve the virus recovery especially in solid portion or pellet. The recovery of spiked φ6 and MS2 using the CP Select method were also lower than other methods (n = 8; paired *t*-test, $P < 0.05$) as shown in Fig. 2: 2.1 ± 1.1 % and 9.4 ± 7.3 % for φ6 and MS2, respectively.

For NoVs-GI detection, the CP Select and direct capture methods provided slightly more detection in wastewater samples (88 % positive) than both of PEG precipitation methods, (75 % positive for the IDEXX PEG

Table 6
Detection of SARS-CoV-2 and recovery of surrogate viruses with different pipettes in 40 mL wastewater samples.

Target virus	Pore size (μm)	No. positive samples/no. tested samples (%)	Concentration (mean ± SD; log copies/L)[a]	No. seeded viruses (copies)	No. recovered viruses (mean ± SD; copies)	% Recovery (mean ± SD)
SARS-CoV-2	0.05	6/6 (100)	4.1 ± 0.4	NA	NA	NA
	0.2	5/6 (83)	4.5 ± 0.3		NA	NA
	0.45	5/6 (83)	4.4 ± 0.2		NA	NA
Φ6	0.05	NA	NA	1.1×10^7	$4.6 \times 10^5 \pm 2.8 \times 10^5$	4.1 ± 2.5
	0.2	NA	NA		$4.0 \times 10^5 \pm 2.5 \times 10^5$	3.5 ± 2.2
	0.45	NA	NA		$4.1 \times 10^5 \pm 1.5 \times 10^5$	3.6 ± 1.4
MS2	0.05	NA	NA	2.5×10^7	$1.7 \times 10^6 \pm 8.5 \times 10^5$	6.7 ± 3.4
	0.2	NA	NA		$5.9 \times 10^5 \pm 2.3 \times 10^5$	2.3 ± 0.9
	0.45	NA	NA		$3.3 \times 10^5 \pm 5.0 \times 10^4$	1.3 ± 0.2

[a] The mean concentration of each virus was calculated based on positive samples only.

Table 7
Detection of target viruses in wastewater samples by different virus concentration methods.

Concentration method	No. positive samples/no. tested samples (%)				
	SARS-CoV-2	Enteroviruses	NoVs-GI	NoVs-GII	PMMoV
CP Select	6/8 (75)	3/8 (38)	7/8 (88)	8/8 (100)	8/8 (100)
Direct capture	8/8 (100)	3/8 (38)	7/8 (88)	8/8 (100)	8/8 (100)
IDEXX PEG	8/8 (100)	3/8 (38)	6/8 (75)	8/8 (100)	8/8 (100)
JSWE PEG	8/8 (100)	3/8 (38)	4/8 (50)	8/8 (100)	8/8 (100)

precipitation method and 50 % positive for the JSWE PEG precipitation method). Comparable concentrations of NoVs-GI (n = 4; paired *t*-test, $P > 0.05$) were observed between virus concentration methods, except for the JSWE PEG precipitation method. As for enteroviruses, comparable detection was observed; however, the detected samples came from different samples; thus, it is hard to make a fair comparison between each method's performance.

Even though the number of tested samples was limited, this study showed the performance of the optimized CP Select method to detect indigenous viruses in wastewater samples compared with other methods. Several studies reported that precipitation-based method outperformed the CP Select method. For example, a comparative study using modified PEG precipitation method by initial addition of beef extract reported gave higher recovery in SARS-CoV-2 and other viruses than the CP Select method (Farkas et al., 2022). Another study also recommends to employ precipitation-based method using ammonium sulphate for concentrating viruses in wastewater samples that have high turbidity and surfactant load (Kevill et al., 2022). As different performances are shown by different concentration methods for different virus type, further research with larger sample size should be conducted to draw firmer conclusion of virus recoveries of each method.

E. coli as a fecal bacterial indicator was also tested to observe its potential relationship with the targeted virus concentration in wastewater samples. *E. coli* concentrations in the tested wastewater samples ranged from 1.6×10^4 to 4.9×10^4 colony-forming units (CFU)/mL (Table 1). Pearson correlation analysis results revealed no positive correlation between *E. coli* and SARS-CoV-2 concentrations in wastewater samples using all methods ($r < -0.30$), indicating the unsuitable use of *E. coli* as an indicator of SARS-CoV-2 in wastewater.

To widen the applications of WBE, a rapid, sensitive, and cost-effective method for concentrating viruses in wastewater samples is needed. Regarding processing time, the CP Select method was the fastest among the methods tested in this study. It takes only <30 min for the overall concentration process: ~25 min for the pretreatment (protease incubation and centrifugation) and < 5 min to filter the sample and eluate the attached solid portion to obtain the virus concentrate. In addition, a study reported that this method could simultaneously concentrate bacteria, but the test bacterial concentration was far lower than another method (Ahmed et al., 2021). Because the CP Select method usually requires initial centrifugation, most bacteria will be concentrated in the pellet. Further optimization needs to be performed to assess the applicability of the CP Select method to measuring bacterial concentrations in wastewater samples. The initial cost (for initial centrifugation) and the cost per sample processed are more expensive than other concentration methods, such as the PEG precipitation and membrane-based filtration methods. As for the direct capture method, it takes >50 min until the virus concentrate is obtained: ~40 min for sample pretreatment and another >10 min to mix and filter the mixture of the sample and reagents. Despite promising recovery performance among other methods in this study, the direct capture method requires more labor than other methods. In terms of cost, the overall cost of the direct capture method for sample processing is expensive, similar to the CP Select method. The PEG precipitation method is less expensive with comparable virus recovery performance compared to other methods. However, the PEG precipitation method takes a longer time in sample processing. For example, the IDEXX PEG method takes >2 h, whereas the JSWE PEG takes >9 h to conduct, suggesting that the IDEXX PEG protocol is a preferable option for the PEG precipitation method. In the end, the virus concentration method for better application of WBE would likely depend on each laboratory resource, as every method has advantages and disadvantages.

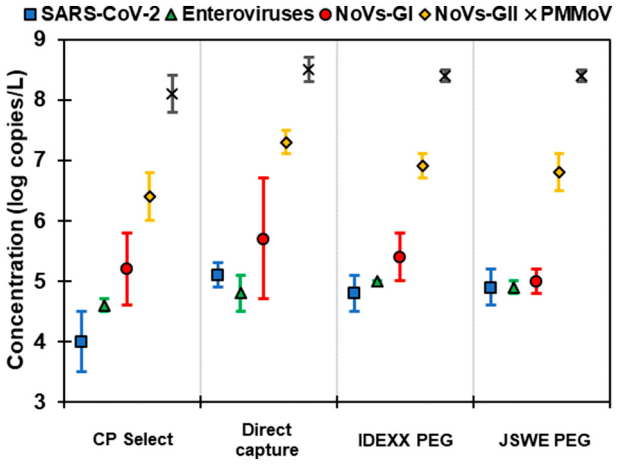

Fig. 1. Mean target virus concentration in wastewater samples by different virus concentration methods. The mean concentration of each virus was calculated based on positive samples only.

4. Conclusions

This study evaluated the optimization of the CP Select method and compared its performance to other virus concentration methods. The addition of the protease solution with 10 min incubation time as pretreatment of the CP Select method using 0.05 μm PS hollow fiber filter tips resulted in the more sensitive SARS-CoV-2 detection in wastewater samples. Based on the observed SARS-CoV-2 detection, the CP Select method could detect SARS-CoV-2 in six of eight wastewater samples tested, whereas other methods (column-based direct capture method and PEG precipitation method) could detect SARS-CoV-2 in all eight samples. As for enteric

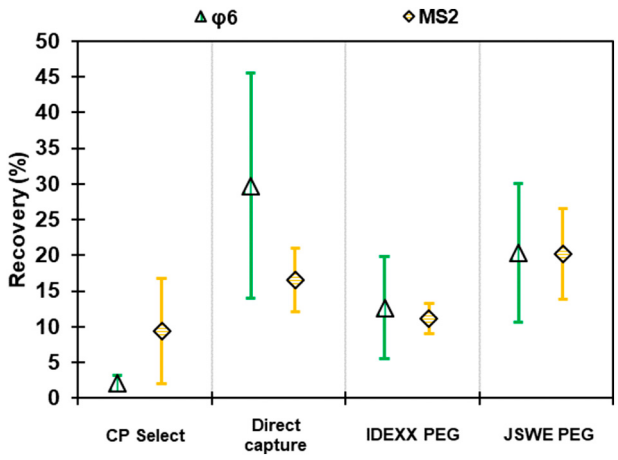

Fig. 2. Mean recovery (%) of spiked virus surrogate in wastewater samples by different virus concentration methods.

viruses in wastewater samples, the CP Select method gave a comparable performance for virus detection and a more rapid processing time compared to other methods, suggesting a potential application of this method in the future with further optimization. In addition, the performance of the CP Select method should be further assessed to observe the applicability of this method for the simultaneous concentration of not only viruses but also bacteria and protozoa.

CRediT authorship contribution statement

Made Sandhyana Angga: Formal analysis, Investigation, Methodology, Writing – original draft. **Bikash Malla:** Formal analysis, Investigation, Methodology, Writing – review & editing. **Sunayana Raya:** Investigation. **Masaaki Kitajima:** Resources, Writing – review & editing. **Eiji Haramoto:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Supervision, Writing – review & editing.

Data availability

Data will be made available on request.

Declaration of competing interest

Eiji Haramoto received research funding from the Takara Bio Inc. The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

Funding: This study was partially supported by Takara Bio Inc., the Japan Science and Technology Agency (JST) through J-RAPID program (grant number JPMJJR2001) and e-ASIA Joint Research Program (grant number JPMJSC20E2), the Minister of Health, Labor and Welfare of Japan (grant numbers JPMH20HA2007 and JPMH20HA2009), and the Japan Society for the Promotion of Science (JSPS) through Grant-in-Aid for Scientific Research (B) (grant number JP20H02284). The authors acknowledge the staff of the WWTPs for their support and permission on wastewater sampling and Central Scientific Commerce, Inc. for their kind support in using the CP Select.

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