The Efficient and Practical virus Identification System with ENhanced Sensitivity for Solids (EPISENS-S): A rapid and cost-effective SARS-CoV-2 RNA detection method for routine wastewater surveillance

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HIGHLIGHTS

- The EPISENS-S consists of pelleting, RNA extraction, RT-preamplification, and qPCR.
- The EPISENS-S method was two orders of magnitude more sensitive than the PEG-qPCR.
- The applicability of the EPISENS-S method to municipal wastewater was confirmed.
- SARS-CoV-2 RNA concentrations in wastewater correlated well with the reported cases.
- The EPISENS-S is highly sensitive and suitable for routine wastewater surveillance.

GRAPHICAL ABSTRACT
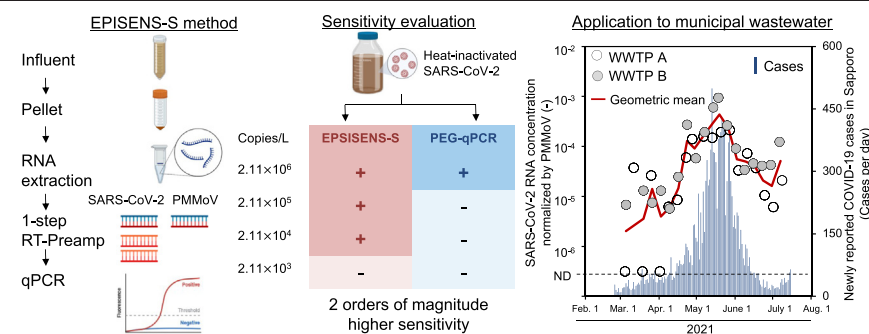
ARTICLE INFO

Editor: Warish Ahmed

ABSTRACT

Wastewater-based epidemiology has attracted attention as a COVID-19 surveillance tool. Here, we developed a practical method for detecting SARS-CoV-2 RNA in wastewater (the EPISENS-S method), which employs direct RNA extraction from wastewater pellets formed via low-speed centrifugation. The subsequent multiplex one-step RT-preamplification reaction with forward and reverse primers for SARS-CoV-2 and a reverse primer only for pepper mild mottle virus (PMMoV) allowed for qPCR quantification of the targets with different abundances in wastewater from the RT-preamplification product. The detection sensitivity of the method was evaluated using wastewater samples seeded with heat-inactivated SARS-CoV-2 in concentrations of 2.11×10^3 to 2.11×10^6 copies/L. The results demonstrated that the sensitivity of the EPISENS-S method was two orders of magnitude higher than that of the conventional method (PEG precipitation, followed by regular RT-qPCR; PEG-QVR-qPCR). A total of 37 untreated wastewater samples collected from two wastewater treatment plants in Sapporo, Japan when 1.6 to 18 new daily reported cases per 100,000 people were reported in the city (March 4 to July 8, 2021), were examined using the EPISENS-S method to confirm its applicability to municipal wastewater. SARS-CoV-2 RNA was quantified in 92 % (34/37) of the samples via the EPISENS-S method, whereas none of the samples (0/37) was quantifiable via the PEG-QVR-qPCR method. The PMMoV concentrations measured by the EPISENS-S method ranged from 2.60×10^6 to 1.90×10^8 copies/L, and the SARS-CoV-2 RNA concentrations normalized by PMMoV ranged from 5.71×10^{-6} to 9.51×10^{-4}. The long-term trend of normalized SARS-CoV-2 RNA concentration in wastewater was consistent with that of confirmed COVID-19 cases in the city. These results demonstrate that the EPISENS-S method is highly sensitive and suitable for routine COVID-19 wastewater surveillance.

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1. Introduction

Coronavirus disease 2019 (COVID-19), which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has become a global pandemic resulting in over 10 million deaths worldwide (World Health Organization, 2022). The symptoms of COVID-19 infection include fever, cough, and fatigue, but a considerable portion of infected individuals have been reported to be asymptomatic (Nishiura et al., 2020; Yanes-Lane et al., 2020). The cellular receptor of SARS-CoV-2 is angiotensin converting enzyme 2 (Yan et al., 2020), which is expressed in the epithelial cells of the human small intestine as well as the respiratory tract (Hamming et al., 2004; Xu et al., 2020), suggesting that SARS-CoV-2 replicates in the human gut and is then shed in the feces of infected individuals. The previous study reported that SARS-CoV-2 RNA has been detected in the feces of >50 % of infected individuals, including asymptomatic individuals, at the early stages of symptom onset (Tang et al., 2020; Wölfel et al., 2020; Xiao et al., 2020). These reports suggest that SARS-CoV-2 RNA can be detected from wastewater, and Kitajima et al., 2020 proposed wastewater-based epidemiology (WBE) as a promising tool to monitor COVID-19 prevalence in a given community.

Numerous studies have reported the detection of SARS-CoV-2 RNA in the wastewater of various countries since the early stages of the pandemic (e.g., Ahmed et al., 2020a; Fernández-de-Mera et al., 2021; Haramoto et al., 2020; la Rosa et al., 2020; Medema et al., 2020; Sherchan et al., 2020). In addition, some previous studies that succeeded in quantifying SARS-CoV-2 RNA in wastewater in high-prevalence area have reported that the concentration tended to increase prior to the increase in the number of newly reported cases based on clinical tests(Peccia et al., 2020).

In Japan, the number of COVID-19 cases per capita has been lower than in many other countries; as of June 12, 2022, Japan was ranked 141st in the world for cumulative cases per capita (7.12×10^3 cases per 100,000 people), which was lower than other developed countries (e.g., US: 2.55×10^4 cases per 100,000; UK: 3.29×10^4 cases per 100,000; and the Netherlands: 4.73×10^4 cases per 100,000) (World Health Organization, 2022). For this reason, it has been difficult to track temporal changes in the concentration of SARS-CoV-2 RNA in wastewater, because of its low concentration and the low sensitivity of conventional detection methods. The first detection of SARS-CoV-2 RNA in wastewater in Japan was reported by Haramoto et al., 2020, who employed their previously reported electronegative membrane-vortex method (Haramoto et al., 2012) to obtain a positive qPCR signal from only one secondary treated wastewater sample. A modified PEG + TRIzol method was used in another study in Japan, which detected SARS-CoV-2 RNA from four wastewater samples, but the samples were positive in either qPCR reaction in duplicate due to the method's lack of sensitivity (Torii et al., 2021).Kitamura et al., 2021 employed direct RNA extraction from a solid fraction of wastewater followed by RT-qPCR, which succeeded in detecting SARS-CoV-2 RNA from quite a few samples, but some samples collected during epidemic period tested negative. These previous reports from Japan collectively suggest that the sensitivity of the conventional methods is not high enough to quantify SARS-CoV-2 RNA in Japanese wastewater.

The lack of sensitivity in the detection method for SARS-CoV-2 RNA in wastewater has been a major obstacle of social implementation of WBE in Japan, and invention of a highly sensitive method for the detection of low concentrations of SARS-CoV-2 RNA in wastewater has been highly desired. Our group found that the majority of SARS-CoV-2 particles in wastewater were associated with solids (Iwamoto et al., submitted), which was also suggested by other studies reporting the detection of substantial amounts of SARS-CoV-2 RNA from primary sludge (Graham et al., 2021; Kim et al., 2022; Peccia et al., 2020) and wastewater solids (Kitamura et al., 2021). Therefore, it has been suggested that the analysis of solid phase wastewater could provide a higher SARS-CoV-2 RNA detection sensitivity compared to analysis of the water phase. Social implementation of WBE requires a method that is simple, not time consuming, and highly sensitive because acquisition of timely data is required for authorities to make political decisions to mitigate infections or promote socioeconomic activities.

Based on this background, we aimed to develop a highly sensitive and practical method for SARS-CoV-2 RNA detection in wastewater for routine wastewater monitoring for the social implementation of WBE. The method developed in the present study, Efficient and Practical virus Identification System with an ENhanced Sensitivity for Solids (EPISENS-S), employs a simple protocol consisting of low-speed centrifugation of wastewater, direct RNA extraction from the resultant pellet, RT preamplification, and qPCR using commercial kits. This method also includes the quantification of indigenous pepper mild mottle virus (PMMoV), an abundant RNA virus in wastewater (Kitajima et al., 2018), as an internal control and normalizer, to prevent misinterpreting the results of SARS-CoV-2 RNA concentrations in wastewater affected by temporal fecal strength and dilution led by precipitation (Graham et al., 2021; Kim et al., 2022).

2. Materials and methods

2.1. Wastewater sampling

A total of 37 influent wastewater samples (1 L each) were collected weekly from two wastewater treatment plants (WWTP A: $n = 19$, WWTP B: $n = 18$) in Sapporo city from March 4 to July 9, 2021, except for the sample collected on the week of March 12. All the samples were collected in sterile plastic bottles via grab sampling and immediately transported to the laboratory. The samples were processed with the concentration method described below on the day of sample collection.

2.2. Comparison of detection sensitivity

One liter of influent wastewater collected on November 4, 2021, from Plant A was seeded with heat-inactivated SARS-CoV-2 (ATCC VR-1986HK) to obtain the final concentration of 2.11×10^6 copies/L. The wastewater sample was used for the virus-seeding experiments, and the concentration of indigenous SARS-CoV-2 was considered to be negligible, when compared to the concentrations of seeded SARS-CoV-2. The seeded wastewater samples were diluted with the original influent wastewater to prepare samples with different concentrations of SARS-CoV-2. The diluted influent samples were then tested for SARS-CoV-2 RNA using the EPISENS-S and PEG-QVR-qPCR methods, as shown in Fig. 1.

2.3. EPISENS-S method

2.3.1. Pelleting and RNA extraction

Forty milliliters of influent wastewater were centrifuged at 10,000g for 10 min at room temperature to collect solids suspended in the wastewater as pellets. The supernatant was removed completely by decantation, and all the resultant pellets (approx. 0.25 g) were subjected to RNA extraction using the RNeasy PowerMicrobiome kit (Qiagen, Hilden, Germany) to obtain total RNA of 50 μL. The amount of pellets derived from 40-mL influent is generally within the capacity of the RNA extraction kit (the upper limit is roughly 0.25 g, according to the manufacturer's instructions).

2.3.2. One-step RT-Preamplification

The RNA extract was subjected to one-step reverse transcription followed by pre-amplification (RT-Preamp) using the iScript™ Explore One-Step RT and PreAmp Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instruction with slight modifications. Briefly, 13.5 μL of the RNA extract was mixed with 16.5 μL of the RT-Preamp reaction mixture containing 15.0 μL of SsoAdvanced Preamp Supermix, 0.6 μL of iScript Explore Reaction Booster, 0.6 μL of iScript Advanced Reverse Transcriptase, and 0.3 μL of primer mix (Table S1A in the Supplementary Material) containing 3 pmol each of forward and reverse 2019-nCoV_N1 (CDC N1) and 3 pmol of PMMoV reverse (PMMV-RP1) primers (Table S1B in the Supplementary Material). The use of PMMoV reverse primer alone in this reaction terminates with reverse transcription for PMMoV RNA and prevents it from deviating from the quantitative range of qPCR for PMMoV, which is abundantly present in wastewater. The thermal cycling condition of RT-
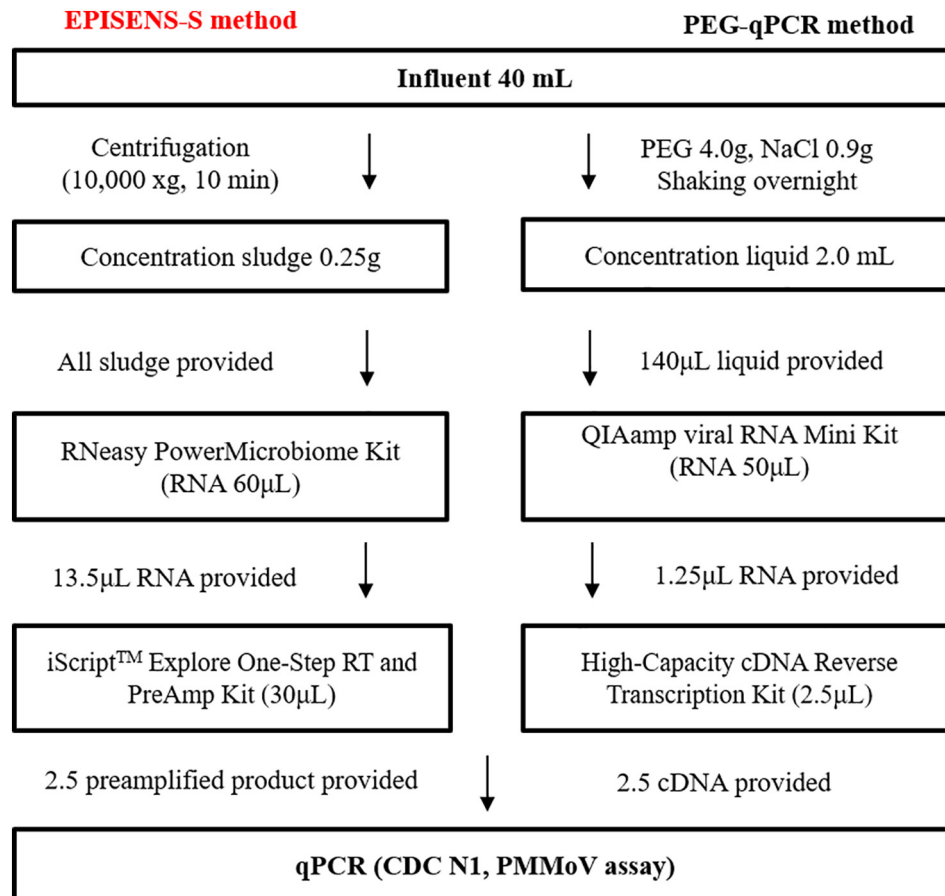
EPISENS-S method

PEG-qPCR method

Fig. 1. Detection flow of SARS-CoV-2 and PMMoV RNA in wastewater using the EPISENS-S and PEG-QVR-qPCR methods.

Preamp was 25 °C for 5 mins, 45 °C for 60 mins, and 95 °C for 3 min followed by 10 cycles of 95 °C for 15 s and 55 °C for 4 min. Tenfold serial dilution of plasmid containing the amplification region sequence of the qPCR assay (CDC N1, Integrated DNA Technologies) was included to generate a standard curve. Nuclease-free water was used as a negative control in the RT-Preamp reaction.

2.3.3. qPCR

qPCR for SARS-CoV-2 was performed in a total reaction volume of 25 μL, which consisted of 2.5 μL of the RT-Preamp product and 22.5 μL of the qPCR reaction mixture. The latter contained 12.5 μL of the QuantiTect Probe PCR Master Mix, 10 pmol each of forward (2019-nCoV_N1-F) and reverse (2019-nCoV_N1-R) primers, and 7.5 pmol of probe (2019-nCoV_N1-P) (Table S2 in the Supplementary Material). qPCR for PMMoV was performed in a total reaction volume of 25 μL, which consisted of 2.5 μL of the RT-Preamp product and 22.5 μL of the qPCR reaction mixture. The latter contained 12.5 μL of the QuantiTect Probe PCR Master Mix, 10 pmol each of forward (PMMV-FP1-rev) and reverse (PMMV-RP1) primers, and 7.5 pmol of probe (PMMV-Probe1) (Table 2). PCR amplification was performed with an ABI PRISM 7500 or 7500 Fast sequence detection system (Thermo Fisher Scientific) under the following conditions: 50 °C for 2 min and initial denaturation at 95 °C for 10 min to activate the DNA polymerase followed by 45 cycles of denaturation at 95 °C for 3 s and annealing and extension at 55 °C for 30 s. The thermal cycling conditions for SARS-CoV-2 and PMMoV assays were identical. Nuclease-free water was used as a negative control in the qPCR reactions. Amplification data were collected and analyzed with Sequence Detector software version 2.0 (Applied Biosystems 7500). The threshold value of relative fluorescence intensity (ΔRn) was adjusted to be 0.01 according to the manufacturer's (Qiagen) instructions for the qPCR master mix. Ten-fold serial dilutions of

standard plasmid DNA for SARS-CoV-2 and gBlocks for PMMoV (10^1 to 10^5 copies per reaction) suspended in nuclease-free water was used for the quantification of viral copy numbers in the PCR tubes. The slope (*S*) of the linear regression curve correlates with efficiency (*E*) of the PCR according to the formula $E = 10^{-1/S} - 1$.

2.4. The PEG-QVR-qPCR method

2.4.1. PEG precipitation

PEG precipitation was performed with slight modifications to the previous report (Japan Society on Water Environment COVID-19 Taskforce, 2022) Briefly, 40 mL of influent wastewater were supplemented with 4 g of PEG 6000 (Wako, Osaka, Japan) and 0.9 g of NaCl (Wako) to obtain the final concentrations of 10 % (w/v) and 0.38 M, respectively. The wastewater was incubated at 4 °C overnight with agitation on a shaker at 200 rpm. Thereafter, the mixture was centrifuged at 12,000 *g* at 4 °C for 60 min, and the supernatant was discarded with decantation. The resultant pellet with approximately 1 mL of viscous residue was resuspended with 1 mL of TRIZol Reagent (Life Technology). The final volume of the concentrate was approximately 2 mL.

2.4.2. Viral RNA extraction and RT

Next, the aqueous phase (140 μL) of the concentrate was subjected to viral RNA extraction using the QIAamp Viral RNA Mini Kit (QVR) (Qiagen) according to the manufacturer's instructions to obtain a final RNA extract volume of 60 μL. The RT reaction to synthesize the cDNA was performed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Briefly, 10 μL of the extracted RNA was added to 10 μL of RT mixture containing 2 μL of 10× reverse transcription buffer, 0.8 μL of 25× deoxynucleoside triphosphates, 2 μL of 10× random hexamers,

50 units of MultiScribe™ reverse transcriptase, and 20 U of RNase inhibitor. The RT reaction mixture was incubated at 25 °C for 10 min, followed by 37 °C for 120 min, and finally 85 °C for 5 min to inactivate the enzyme.

2.4.3. qPCR

qPCR for SARS-CoV-2 and PMMoV were performed separately using a 25-μL reaction volume containing 2.5 μL of cDNA and 22.5 μL of the qPCR reaction mixture, as described above.

3. Results

3.1. Development of the EPISENS-S method

We developed a highly sensitive and simple method for the detection of SARS-CoV-2 RNA in wastewater, the EPISENS-S method, which includes quantification of PMMoV RNA as an internal control. (Fig. 1). The critical point of the EPISENS-S method is the combination of the RNA extraction kit suitable for solid fractions of wastewater and RT-Preamp. To identify the appropriate kit for RNA extraction from wastewater solids, which is a critical step in viral RNA detection from wastewater, five commercial kits were used for side-by-side comparisons of the detectability of SARS-CoV-2 RNA in a wastewater sludge collected from a septic tank at a COVID-19 quarantine facility in Japan. SARS-CoV-2 RNA was detected at a quantifiable amount (Ct = 38.02) by the PowerMicrobiome kit, whereas it was detected, but not quantifiable (DNQ, Ct > 40), or even not detected at all, by the four other kits (i.e., RNeasy PowerSoil Total RNA kit (Qiagen), NucleoBond RNA Soil Total RNA kit (Macherey-Nagel, Duren, Germany), ISoil (Nippon Gene, Tokyo, Japan), Quick-RNA Fecal/Soil Microbe Microprep kit (Zymo Research, Irvine, CA, USA)) (Table S3 in the Supplementary Material). We selected the RNeasy PowerMicrobiome kit, based on this result and its cost advantages ($10.54 per unit) and processing time (1 – 2 h), both of which are comparable to or lower than the other kits (Table S3 in the Supplementary Material).

To confirm that quantitative performance (i.e., accuracy of template quantity determination) was maintained after the preamplification process with 10 cycles of PCR, 10-fold serial dilution of plasmid DNA containing the target sequence (N gene of SARS-CoV-2) was used as a template in the one-step RT-Preamp followed by qPCR. The serial dilution of the plasmid DNA was also used as a template for regular qPCR (i.e., without the RT-Preamp process). The standard curves obtained from qPCR with and without the RT-Preamp process are shown in Fig. 2. The slopes of the standard curves of RT-Preamp followed by qPCR and regular qPCR were -3.24 and -3.23 corresponding to amplification efficiencies of 103 % and 104 %, respectively. The Y-intercept of the standard curves of RT-Preamp followed by qPCR and regular qPCR were 32.2 and 41.28, respectively. The Ct value decreased by 9.07 with the RT-Preamp process for a given concentration of DNA, which is consistent with the theoretical Ct value decrease of 8.84 for 10 cycles of PCR amplification following 2.22-fold dilution of DNA template with RT-Preamp mixture prior to qPCR. The results suggested that the Preamp reaction effectively increased the target gene copy numbers without any major effects on the qPCR quantitative performance.

We confirmed that RNA of PMMoV, which is abundantly present in wastewater, can be detected by qPCR following the one-step RT-Preamp reaction containing forward and reverse primers for SARS-CoV-2 and reverse primer only for PMMoV. The concentrations of PMMoV RNA detected from 40 mL-influent wastewater samples with one-step RT-Preamp using the specific reverse primer for RT followed by qPCR were significantly higher than those with the conventional RT-qPCR using random primers for RT ($P < 0.001$, Wilcoxon signed-rank test) (Table S4 in the Supplementary Material), which demonstrated that PMMoV RNA could be efficiently reverse-transcribed by the one-step RT-Preamp. It was also confirmed that the addition of the PMMoV reverse primer in the RT-Preamp reaction mixture did not interfere with the quantification of SARS-CoV-2 RNA with RT-Preamp and qPCR processes (Table S5 in the Supplementary Material).
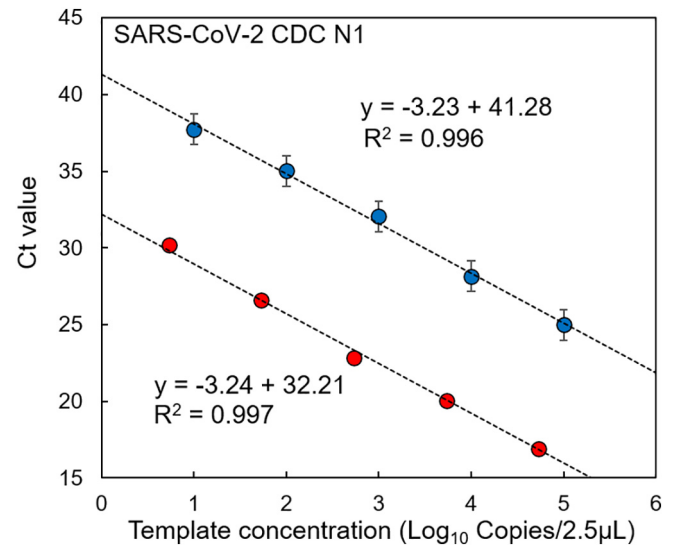

Fig. 2. Standard curves of CDC N1 assay using plasmid DNA containing the target region. The blue plots denote the standard curve of regular qPCR (without preamplification), and the red plots denote that of qPCR following preamplification. Error bars denote the standard deviation of Ct values in duplicate. The standard deviation of preamp-qPCR was so small that they were almost invisible in the figure.

3.2. Comparison of detection sensitivity between the EPISENS-S and PEG-QVR-qPCR methods

To evaluate the detection sensitivity of the EPISENS-S method relative to the PEG-QVR-qPCR method, wastewater samples inoculated with different amounts of heat-inactivated SARS-CoV-2 were examined using both methods (Table 1). PMMoV RNA was detected from all tested samples, with concentrations ranging from 2.19×10^7 to 1.98×10^7 copies/L and 3.30×10^7 to 8.83×10^7 copies/L copies/L by the EPISENS-S and PEG-QVR-qPCR methods, respectively (Table 1). With the PEG-QVR-qPCR method, SARS-CoV-2 RNA was detected in all samples containing the virus with concentrations of 2.11×10^6 copies/L, rarely detected from those with a concentration of 2.11×10^5 copies/L, and never detected from those with concentrations of 2.11×10^4 copies/L and 2.11×10^3 copies/L. SARS-CoV-2 RNA was detected from all the samples with a concentration of 2.11×10^4 copies/L when using the EPISENS-S method. This result demonstrates that the EPISENS-S method is two orders of magnitude more sensitive than the PEG-QVR-qPCR method, although the SARS-CoV-2 recovery efficiency calculated from the observed concentrations in wastewater containing 2.11×10^6 copies/L was higher for the PEG-QVR-qPCR method (5.9 ± 2.8 %) than the EPISENS-S method (1.1 ± 0.1 %).

To validate the applicability of the EPISENS-S method for the detection of indigenous SARS-CoV-2 RNA and PMMoV RNA in municipal wastewater, 37 influent wastewater samples collected from two WWTPs in Sapporo city were examined using both the EPISENS-S and PEG-QVR-qPCR methods (Table 2). PMMoV RNA was detected from all samples using both methods, with concentrations of 5.05×10^7 (range: 2.6×10^6–1.90×10^8 copies/L) and 2.56×10^7 (range: 1.67×10^6– 1.77×10^8 copies/L), respectively (Table 2, Table S6 in the Supplementary Material). There were no significant differences among PMMoV RNA concentrations measured with the PEG-QVR-qPCR method and the EPISENS-S method ($P = 0.25$, Wilcoxon signed-rank test). The results of PMMoV RNA concentration suggest that detection procedures were carried out without a significant difference in detection efficiency between samples measured with each method.

The quantified ratios of SARS-CoV-2 RNA with the EPISENS-S method were substantially higher than those of the PEG-QVR-qPCR method for both WWTP A (EPISENS-S: 95 %, 16/19; PEG-QVR-qPCR: 0 %, 0/18) and B (EPISENS-S: 100 %, 18/18; PEG-QVR-qPCR: 0 %, 0/18) (Table 2, Table S7 in the Supplementary Material). Most of the positive samples detected using the PEG-QVR-qPCR method were considered DNQ (Ct

Table 1

Detection of SARS-CoV-2 RNA and PMMoV RNA with the EPISENS-S and PEG-qPCR methods from wastewater samples artificially contaminated with various amount of heat-inactivated SARS-CoV-2.

Method	Target	Concentration of heat-inactivated SARS-CoV-2 RNA in wastewater (copies/L)[a]							
		2.11×10^6		2.11×10^5		2.11×10^4		2.11×10^3	
		Observed concentration (copies/L)	Positive ratio	Observed concentration (copies/L)	Positive ratio	Observed concentration (copies/L)	Positive ratio	Observed concentration (copies/L)	Positive ratio
EPISENS-S	SARS-CoV-2	$(4.08–4.64) \times 10^4$	3/3	$(2.66–3.95) \times 10^3$	3/3	$(1.75–1.02) \times 10^2$	3/3	N.D.	0/3
	PMMoV	$(2.19–2.93) \times 10^7$	3/3	$(2.53–19.8) \times 10^7$	3/3	$(2.30–2.73) \times 10^7$	3/3	$(2.41–4.45) \times 10^7$	3/3
PEG-qPCR	SARS-CoV-2	$(1.08–3.86) \times 10^5$	3/3	$(2.01) \times 10^5$	1/3	N.D.[b]	0/3	N.D.	0/3
	PMMoV	$(3.30–4.55) \times 10^7$	3/3	$(3.54–4.25) \times 10^7$	3/3	$(4.37–8.83) \times 10^7$	3/3	$(3.72–4.88) \times 10^7$	3/3

[a] Wastewater sample was seeded with various concentrations of heat-inactivated SARS-CoV-2 to obtain the respective final concentration.

[b] N.D., not determined.

Table 2

Comparison of the detection of indigenous SARS-CoV-2 and PMMoV RNA in untreated wastewater with the EPISENS-S and PEG-qPCR methods.

	Plant A				Plant B			
	SARS-CoV-2		PMMoV		SARS-CoV-2		PMMoV	
	PEG-qPCR	EPISENS-S	PEG-qPCR	EPISENS-S	PEG-qPCR	EPISENS-S	PEG-qPCR	EPISENS-S
Positive ratio	11 % (2/19)	95 % (18/19)	100 % (19/19)	100 % (19/19)	50 % (9/18)	100 % (18/18)	100 % (18/18)	100 % (18/18)
Quantified ratio	0 % (0/19)	84 % (16/19)	100 % (19/19)	100 % (19/19)	0 % (0/18)	100 % (18/18)	100 % (18/18)	100 % (18/18)
Mean concentration (range copies/L)		2.19×10^3 $(3.55 \times 10^2 - 1.32 \times 10^4)$	2.00×10^7 $(1.66 \times 10^6 - 1.74 \times 10^8)$	5.89×10^7 $(2.57 \times 10^7 1.55 \times 10^8)$		2.34×10^3 $(1.32 \times 10^2 3.89 \times 10^4)$	3.31×10^7 $(1.66 \times 10^6 1.78 \times 10^8)$	4.27×10^7 $(2.57. \times 10^6 1.91 \times 10^8)$

values > 40.0), according to the MIQE guidelines (Bustin et al., 2009). The concentrations of SARS-CoV-2 RNA observed by the EPISENS-S method ranged from 3.55×10^2 to 1.32×10^4 copies/L and 1.32×10^2 to 3.89×10^4 copies/L at WWTP A and WWTP B, respectively (Table 2). This result demonstrated that the EPISENS-S method is applicable for the detection of indigenous SARS-CoV-2 RNA in wastewater, and its detection sensitivity is higher than that of the PEG-QVR-qPCR method, which is consistent with the detection results of the seeded virus described above.

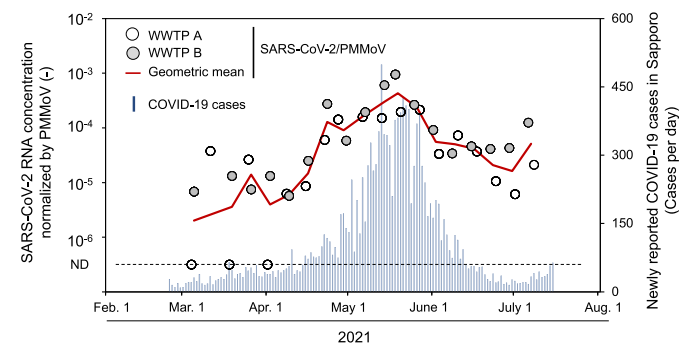*3.3. Quantification of SARS-CoV-2 RNA in wastewater using the EPISENS-S method*

In order to confirm the usefulness of the EPISENS-S method in tracking the dynamics of SARS-CoV-2 infections in a community, SARS-CoV-2 RNA concentrations in wastewater collected at two WWTPs in Sapporo city were determined by the EPISENS-S method and compared with the numbers of newly reported COVID-19 cases in the city. Total service population of the WWTPs covers approximately 20 % of the entire population of Sapporo city (approximately 1.96 million people). The concentrations of SARS-CoV-2 RNA in wastewater normalized by PMMoV were constantly low (up to 3.79×10^{-5}) until April 10, but increased rapidly from April 15 with a peak value of 2.12×10^{-4} on May 27, and 9.51×10^{-4} on May 18, at WWTPs A and B, respectively (Fig. 3). There were fewer than 65 clinically confirmed cases in the city prior to April 15, but this number increased rapidly to a maximum of 499 on May 13, corresponding to 25 per 100,000 people (Fig. 3, Fig. S1 in the Supplementary Material). The concentrations of SARS-CoV-2 RNA in wastewater normalized by PMMoV (log10-transformed) correlated well (Pearson's r = 0.83) with the 7-day moving average of newly reported COVID-19 cases in Sapporo city, even in such low-prevalence areas (Fig. 4). This result demonstrated that the concentrations of SARS-CoV-2 RNA in wastewater measured with the EPISENS-S method correlated well with the infection trends, even in such low-prevalence areas.

4. Discussion

To accomplish the social implementation of WBE, sensitive methods for the detection of SARS-CoV-2 RNA from wastewater have been developed

in previous studies, but they require special and expensive equipment (e.g., Precellys, ddPCR, InnovaPrep) (Ahmed et al., 2020b; Kim et al., 2022; McMinn et al., 2021). In addition, many of the previous studies focused on liquids in wastewater for the detection of SARS-CoV-2 RNA, rather than solids (Reynolds et al., 2022; Tiwari et al., 2022; Zdenkova et al., 2022). There has been an urgent need for a practical method for the detection of SARS-CoV-2 RNA in wastewater to monitor COVID-19 in low-prevalence communities (Kitajima et al., 2020). In the present study, we successfully developed a simple and highly sensitive and simple method for the detection of SARS-CoV-2 RNA in wastewater, which was named the EPISENS-S method. This new method employs two unique steps, total RNA extraction from the solid fraction of wastewater and the one-step RT-Preamp prior to qPCR.

First, the solid fraction of wastewater was simply collected by centrifugation, which was then directly subjected to total RNA extraction with the RNeasy PowerMicrobiome Kit (Qiagen). Previous studies have suggested

Fig. 3. Concentrations of SARS-CoV-2 RNA normalized with PMMoV RNA in wastewater in Sapporo city, as determined using the EPISENS-S method, and newly reported COVID-19 cases in the city. The circle plots denote the normalized concentration of SARS-CoV-2 RNA in wastewater collected at WWTP A or B. The red line denotes the geometric mean normalized concentrations of SARS-CoV-2 RNA in wastewater collected from WWTPs A and B in the same week. The dotted line denotes the theoretical lower limit of detection of the EPISENS-S method.
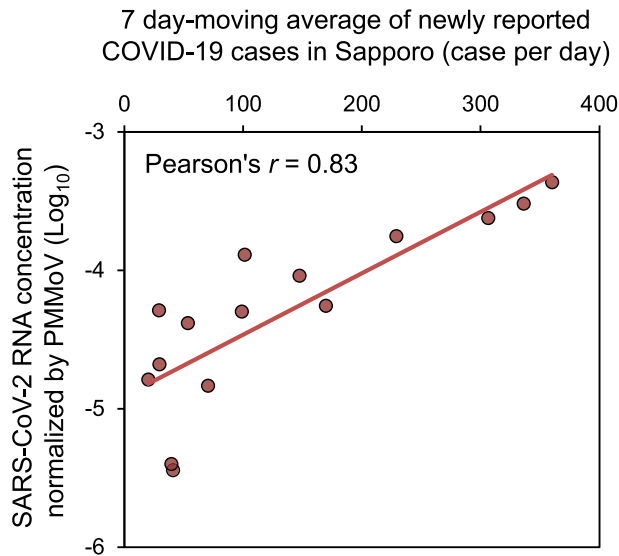
Fig. 4. Correlation of SARS-CoV-2 RNA concentration normalized by PMMoV RNA concentration, determined using the EPISENS-S method, and newly reported COVID-19 cases in Sapporo city ($n = 15$). The horizontal axis shows the 7-day (from 3 days before the day of sampling to 3 days after the sampling) moving average; the vertical axis represents the geometric normalized concentration of SARS-CoV-2 RNA in wastewater collected at two WWTPs in the same week. If SARS-CoV-2 RNA was not quantified from both samples collected at the WWTPs in the same week, the wastewater data were excluded from the statistical analysis.

that a majority of the enveloped virus particles exist in the solid fraction of wastewater, rather than the liquid fraction, due to the physicochemical properties of the envelope (i.e., the lipid membrane) (Kitamura et al., 2021; Ye et al., 2016), and that the measurement of SARS-CoV-2 RNA in primary sludge might be a more sensitive approach than that in influent wastewater (Graham et al., 2021; Kim et al., 2022). We used influent wastewater in the present study for detecting SARS-CoV-2 RNA because it is suitable to interpret the total amount of SARS-CoV-2 RNA entering wastewater treatment plant that is associated with the number of infected individuals in a corresponding area, but the EPISENS-S method is potentially applicable to the detection of viral RNAs in primary sludge as well. In fact, the RNeasy PowerMicrobiome kit was selected based on our side-by-side comparison of commercial kits using a wastewater sludge sample, which showed that this kit was the most efficient for extracting viral RNA from the sludge sample (Table S3 in the Supplementary Material). The concentration and RNA extraction processes in the EPISENS-S method are less time-consuming than those employed in previous studies. The concentration step of the EPISENS-S method takes only 10 min for pelleting via low-speed centrifugation, whereas PEG precipitation usually requires at least three hours, with agitation (Flood et al., 2021; La Rosa et al., 2020). In addition, the RNA extraction process with the RNeasy PowerMicrobiome Kit requires approximately 1.5 h per 12 samples, which is much less time consuming than the 3 h required for the RNeasy PowerSoil Total RNA Kit (Qiagen) (Peccia et al., 2020).

Second, this study multiplexed the one-step RT-Preamp process for SARS-CoV-2 and PMMoV, by selecting of appropriate combination of the primers added in the reaction mixture of RT-Preamp. Specifically, a forward and reverse primer set for SARS-CoV-2 and only reverse primer for PMMoV were used for RT-Preamp considering that the concentrations of PMMoV in wastewater is generally orders of magnitude higher than those of SARS-CoV-2. In this RT-Preamp process, single-stranded positive-sense PMMoV RNA was reverse-transcribed in the presence of the reverse primer but not preamplified due to the absence of a forward primer. This process was designed based on our observation that preamplification for PMMoV can cause the deviation from the upper quantitative range of qPCR because of its high abundance in wastewater. To verify the performance of the proposed system, the effect of the one-step RT-Preamp process on the qPCR

results of SARS-CoV-2 was analyzed by comparing the standard curves generated with and without RT-Preamp reaction (Fig. 2). We confirmed the effectiveness of the one-step RT-Preamp process in increasing specifically the amount of template cDNA of SARS-CoV-2 prior to qPCR (data not shown) and enhancing detection sensitivity without losing the qPCR quantitative performance, which is consistent with a previous study (Ishii et al., 2013). The successful quantification of PMMoV RNA in all the wastewater samples indicated the applicability of the multiplex RT-Preamp process for quantifying both SARS-CoV-2 and PMMoV RNA. Although PMMoV is an enveloped virus and considered to be abundantly present in the liquid phase, our results demonstrated that a substantial amount of PMMoV could be constantly detected from the solid phase as well, and therefore, could serve as a normalizer for SARS-CoV-2 RNA in the EPISENS-S method.

In the present study, the development of a highly sensitive method was achieved by identifying an RNA extraction kit that efficiently removes PCR inhibitory substances via inhibitor removal technology (Qiagen) and increasing the amount of equivalent wastewater volume subjected to qPCR. The newly developed EPISENS-S method employs pelleting of solids from 40 mL of wastewater, from which RNA is extracted in a 50-μL solution. Thereafter, 13.5 μL of RNA extract (i.e., 27 % of 50 μL) is used for RT-Preamp reaction in a 30-μL mixture, and then 2.5 μL of the RT-Preamp product is subjected to qPCR. Theoretically, an equivalent wastewater volume of 10.8 mL (27 % of 40 mL) can be analyzed using the EPISENS-S method, whereas only 0.058 mL (0.14 % of 40 mL) is analyzed in the PEG-QVR-qPCR method. The newly developed EPISENS-S method can analyze two orders of magnitude higher equivalent wastewater volume per reaction (10.8 mL, or 27 % of 40 mL) than the conventional PEG-QVR-qPCR method (0.058 mL, or 0.14 % of 40 mL), which corresponds to 9.31×10^2 copies/L and 1.71×10^4 copies/L of the theoretical limit of detection, respectively, under the assumption that the recovery efficiency of viral RNA in the whole process is 100 %. The results of virus-seeding experiments, which showed two orders of magnitude higher sensitivity in the EPISENS-S method than the PEG-QVR-qPCR method, were consistent with the theoretical calculation of the equivalent wastewater volume subjected to qPCR analysis (27 % vs. 0.14 %). The results demonstrated that this high sensitivity was achieved by an increased wastewater equivalent volume, and that further improvements in the sensitivity of the EPISENS-S method could be expected by enhancing recovery efficiency, which was determined to be 1.1 % in the present study.

We have also demonstrated the applicability of the EPISENS-S method for the quantification of indigenous SARS-CoV-2 RNA in municipal wastewater in Japan, where the number of COVID-19 cases per capita has been relatively low. The concentration of SARS-CoV-2 RNA in untreated wastewater in Sapporo city measured by the EPISENS-S method increased prior to the increase in newly reported COVID-19 cases in the city during the sampling period. Our observation is consistent with some previous studies reporting that SARS-CoV-2 RNA concentrations in wastewater might foreshadow the numbers of clinically confirmed COVID-19 cases in a given area (Peccia et al., 2020). The EPISENS-S method allows for tracking the infection dynamics via WBE, even in a low-prevalence area, owing to its high sensitivity.

The social implementation of WBE requires a virus detection method from wastewater that is highly sensitive, simple, and time effective. The newly developed EPISENS-S method meets these requirements, and the required costs per sample of the EPISENS-S method for the analyzing SARS-CoV-2 RNA and PMMoV RNA are $10.5 for RNA extraction, $10.4 for RT-Preamp, and $2.10 for qPCR, demonstrating that EPISENS-S is a cost-effective method. Further research is recommended to demonstrate the expandability of the method. First, the applicability of this method to non-enveloped human pathogenic viruses should be examined, although our results suggested that the EPISENS-S method is effective for the recovery and detection of PMMoV, a non-enveloped plant virus. Secondly, settleability of wastewater solids by centrifugation (i.e., pelleting) is highly dependent on wastewater characteristics. For example, when wastewater is diluted with stormwater in a combined sewer system, the density of wastewater solids decreases, and pellets are rarely formed. It is also difficult to apply this method in secondary-treated wastewater or environmental water, which contains only a small amount of suspended solids. Although

the EPISENS-S method has been proven to be effective for untreated and undiluted wastewater samples, development of a more widely applicable method for virus detection from a variety of water samples is recommended.

5. Conclusions

We developed a highly sensitive and practically usable method, EPISENS-S, consisting of direct extraction of total RNA from pelleted wastewater solids and RT-Preamp prior to qPCR for quantification of SARS-CoV-2 RNA in untreated wastewater. The use of forward and reverse primers for SARS-CoV-2 and a reverse primer only for PMMoV in the RT-Preamp step allowed for multiplex RT-Preamp reaction and subsequent qPCR quantification of the targets with different abundances in wastewater.

The theoretical lower limit of detection of the EPISENS-S method for SARS-CoV-2 RNA detection from wastewater was 9.31×10^2 copies/L, which corresponds to two orders of magnitude greater sensitivity than the PEG-QVR-qPCR method. The greater sensitivity of the EPISENS-S method over the PEG-QVR-qPCR method was confirmed by the experiments using wastewater samples artificially contaminated with various amounts of heat-inactivated SARS-CoV-2.

The EPISENS-S method exhibited a higher detection ratio of indigenous SARS-CoV-2 RNA in wastewater than the PEG-QVR-qPCR method, which further confirmed the applicability and sensitivity of this method for detecting SARS-CoV-2 RNA in wastewater. The SARS-CoV-2 RNA concentration in wastewater measured by the EPISENS-S method correlated well with the newly reported COVID-19 cases in the corresponding area, demonstrating that the EPISENS-S method is useful for routine wastewater surveillance of COVID-19 in a given community with a relatively low prevalence.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.157101.

CRediT authorship contribution statement

Hiroki Ando: Formal analysis, Investigation, Methodology, Validation, Writing – original draft. **Ryo Iwamoto:** Investigation, Methodology, Writing – review & editing. **Hiroyuki Kobayashi:** Supervision, Funding acquisition. **Satoshi Okabe:** Supervision, Writing – review & editing. **Masaaki Kitajima:** Supervision, Conceptualization, Investigation, Methodology, Funding acquisition, Writing – review & editing.

Declaration of competing interest

Acknowledgements

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