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Capsid integrity RT-qPCR for the selective detection of intact SARS-CoV-2 in wastewater

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HIGHLIGHTS

• Capsid integrity RT-qPCR was optimized to assess intact form of SARS-CoV-2 in wastewater.
• CDDP-RT-qPCR was effective for the selective detection of intact enveloped viruses.
• UF method was more effective than PEG method for the recovery of enveloped viruses.
• Intact SARS-CoV-2 was detected from wastewater.
• CDDP-RT-qPCR can provide a better estimate of the presence of SARS-CoV-2 in wastewater.

GRAPHICAL ABSTRACT

ABSTRACT

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) genomes have been detected in wastewater worldwide. However, the assessment of SARS-CoV-2 infectivity in wastewater has been limited due to the stringent requirements of biosafety level 3. The main objective of this study is to investigate the applicability of capsid integrity RT-qPCR for the selective detection of intact SARS-CoV-2 in wastewater. Three capsid integrity reagents, namely ethidium monoazide (EMA, 0.1–100 μM), propidium monoazide (PMA, 0.1–100 μM), and cis-dichlorodiammineplatinum (CDDP, 0.1–1000 μM), were tested for their effects on different forms (including free genomes, intact and heat-inactivated) of murine hepatitis virus (MHV), which was used as a surrogate for SARS-CoV-2. CDDP at a concentration of 100 μM was identified as the most efficient reagent for the selective detection of infectious MHV by RT-qPCR (CDDP-RT-qPCR). Next, two common virus concentration methods including ultrafiltration (UF) and polyethylene glycol (PEG) precipitation were investigated for their compatibility with capsid integrity RT-qPCR. The UF method was more suitable than the PEG method since it recovered intact MHV (mean ± SD, 38% ± 29%) in wastewater much better than the PEG method did (0.013% ± 0.015%). Finally, CDDP-RT-qPCR was compared with RT-qPCR alone for the detection of SARS-CoV-2 in 16 raw wastewater samples collected in the Greater Tokyo Area. Five samples were positive for SARS-CoV-2 when evaluated by RT-qPCR alone. However, intact SARS-CoV-2 was detected in only three positive samples when determined by CDDP-RT-qPCR. Although CDDP-RT-qPCR was unable to determine the infectivity of SARS-CoV-2 in wastewater, this method could improve the interpretation of positive results of SARS-CoV-2 obtained by RT-qPCR.

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1. Introduction

The ongoing coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a global concern that has spread throughout more than 200 countries and has caused approximately three million confirmed deaths worldwide (WHO, 2021). SARS-CoV-2 is mainly transmitted among people via respiratory droplets and contact routes. However, the presence of high concentrations (up to $10^8$ copies/g of feces) of SARS-CoV-2 RNA in the feces of infected people has also been commonly reported (Chen et al., 2020; Cheung et al., 2020; Lescure et al., 2020; Zhang et al., 2020b). Several studies have successfully isolated viable SARS-CoV-2 from the urine and feces of COVID-19 patients (Sun et al., 2020; Xiao et al., 2020), thus raising concerns about the possible transmission of SARS-CoV-2 through the fecal-oral route. Recently, it was found that 1.5 and 1.7 days were required for a 1.0 log reduction ($T_{90}$) of infectious SARS-CoV-2 at room temperature (20 °C) in wastewater and tap water, respectively (Bivins et al., 2020). At cool water temperatures, infectious SARS-CoV-2 was able to survive much longer. Indeed, Camilo et al. (2020) reported that the $T_{90}$ values of infectious SARS-CoV-2 were 7.7 and 5.5 days and the $T_{99}$ (time required for a 2 log reduction) values were 18.7 and 17.5 days in river water and wastewater at 4 °C, respectively. This evidence suggests that exposure to wastewater contaminated with SARS-CoV-2 might be associated with certain risks, particularly during the low-temperature season in places where sanitation infrastructure is not available or is insufficient (Bao and Canh, 2021; Pandey et al., 2021).

To date, in an attempt to monitor wastewater for tracking the spread of COVID-19 in human communities (wastewater surveillance), SARS-CoV-2 RNA has been detected in raw wastewater in many countries around the world, including in Japan, the United States, France, the Netherlands, Italy, Australia, Spain, India, Pakistan, and China, with concentrations ranging from $10^2$ to $10^6$ copies/L (Ahmed et al., 2020a; Haramoto et al., 2020; Kumar et al., 2020; Medema et al., 2020; Randazzo et al., 2020; Rimoldi et al., 2020; Sharif et al., 2020; Shcherch et al., 2020; Trottier et al., 2020; Wurtzer et al., 2020b; Zhang et al., 2020a). Furthermore, several studies have reported the presence of SARS-CoV-2 RNA in treated wastewater ($<10^{-5}$ to $10^{-5}$ copies/L) (Wurtzer et al., 2020a), river water ($10^2$ to $10^6$ copies/L) (Guerrero-Latorre et al., 2020), and sewage sludge ($10^{-4}$ to $10^{-8}$ copies/mL of primary sludge) (Peccia et al., 2020). However, the infectivity or even the structure of SARS-CoV-2 (e.g., intact or compromised) was not assessed since most previous studies utilized RT-qPCR for virus detection. Although cell culture assays are the gold standard for detecting viable viruses, they are laborious, time-consuming, and expensive. More importantly, biosafety level 3 laboratories are required to incubate infectious SARS-CoV-2 due to its high infection risk. Therefore, the assessment of SARS-CoV-2 infectivity in wastewater environments remains limited (Rimoldi et al., 2020). To better understand the infection risks via water/wastewater contaminated with SARS-CoV-2, a simple, rapid, and reliable method is necessary for detecting infectious SARS-CoV-2.

Capsid integrity RT-qPCR has been recently developed to overcome the limitations of conventional RT-qPCR and thus to discriminate infectious from damaged viruses. In this approach, samples are pretreated with capsid integrity reagents such as monoazide dyes (e.g., ethidium monoazide (EMA), propidium monoazide (PMA) and PMAxx (a new version of PMA)), or more recently metal compounds (e.g., cis-dichlorodiammineplatinum (CDDP) and platinum (IV) chloride (PtCl4)) prior to RT-qPCR. These reagents can bind to the genomes of enveloped viruses, particularly SARS-CoV-2, so a capsid integrity RT-qPCR can be used to detect the presence of infectious SARS-CoV-2 (Bao and Canh, 2021; Pandey et al., 2021; Kumar et al., 2020; Medema et al., 2020; Rimoldi et al., 2020; Sharif et al., 2020; Shcherch et al., 2020; Trottier et al., 2020; Wurtzer et al., 2020b; Zhang et al., 2020a). This current study aimed to investigate the applicability of capsid integrity RT-qPCR for the selective detection of intact SARS-CoV-2 in wastewater. Given the stringent requirements for handling SARS-CoV-2 (biosafety level 3), MHV was used as a model virus to optimize capsid integrity RT-qPCR since MHV also belongs to the genus Betacoronavirus and thus has a similar morphology to that of SARS-CoV-2 (Pandey et al., 2021). Additionally, MHV has previously been used as a useful surrogate for investigating the persistence of SARS-CoV-2 in water or evaluating the recovery efficiency of virus concentration methods for SARS-CoV-2 (Ahmed et al., 2020b; Bivins et al., 2020a). We tested three capsid integrity reagents (including EMA, PMA, and CDDP) to determine the most effective one for the selective detection of intact MHV by RT-qPCR. Next, the effects of virus concentration methods (including the UF and PEG precipitation methods) on viral structure were evaluated using spiked MHV in raw wastewater. Finally, the optimal concentration method and effective capsid integrity RT-qPCR were applied to assess the presence of SARS-CoV-2 in raw wastewater.

2. Materials and methods

2.1. Preparation of intact virus stock and infectivity assay

All the laboratory works were conducted at the Graduate School of Engineering, the University of Tokyo. MHV A59 strain (ATCC VR-764)
was propagated using DBT cells. The DBT cells were grown in Eagle's minimum essential medium (MEM, Wako, Japan) supplemented with 5% fetal bovine serum (FBS) in a 75-cm² flask. The semi-confluent DBT cells were inoculated with MHV and incubated in MEM with 1% FBS at 37 °C (5% CO₂) for 3 days. Then, the flask was frozen and thawed once to recover the MHV. The recovered MHV was purified by membrane filtration with a cellulose acetate filter (0.2 μm, DISMIC-25CS, Advantec, Tokyo, Japan) and gel filtration with an Illustra Microspin S-300 HR column (GE Healthcare, Tokyo, Japan) (Kitajima et al., 2010; Sangsanont et al., 2014). The purified MHV was regarded as intact MHV stock. The intact virus stock was aliquoted and maintained at −80 °C until use.

The infective MHV was enumerated by the most probable number assay (Meister et al., 2018; Torii et al., 2020) using DBT cells on 96-well plates, with four serial dilutions and five replicates. The samples to be quantified were serially diluted by 10-fold using maintenance medium, supplemented with 1% FBS. Each well was inoculated with 150 μL of the diluted samples. After 3 days of incubation at 37 °C with 5% CO₂, the presence of cytopathic effects was monitored by microscopy. The number of positive wells of each dilution was counted and analyzed by an R package (Ferguson and Itie, 2019).

Murine norovirus (MNV, S7-PP3 strain) was propagated using RAW.264.7 cells as a host, as described elsewhere (Kitajima et al., 2008). The propagated stocks were stored at −80 °C prior to the experiment.

2.2. Preparation of free viral RNA

Free viral RNA was prepared from purified virus stocks after RNA extraction (as described below). Free viral RNA stock was aliquoted and maintained at −80 °C until use.

2.3. Capsid integrity treatments

EMA powder (phenanthridinium, 3-amino-8-azido-5-ethyl-6-phenyl, bromide; Invitrogen, Carlsbad, USA) was dissolved in Milli-Q water to obtain a stock solution (10 mM). PMA solution (20 mM in H₂O) was obtained from Biotium, Inc., Fremont, CA, USA. CDDP (powder form, Sigma-Aldrich) was dissolved in DMSO to obtain a concentration of 100 mM. After preparation, the EMA, PMA, and CDDP stock solutions were stored at −20 °C in the dark until use.

EMA, PMA, or CDDP treatment was conducted according to our previous study (Canh et al., 2019). Briefly, EMA, PMA, or CDDP stock solution (14 μL) was added to the water samples (125 μL) to obtain the desired concentration. EMA or PMA spiked samples were incubated in the dark at 4 °C for 30 min and then exposed to a halogen light (650 W, Seleco Pacific, Auckland, New Zealand) for 3 min at a distance of 15 cm from the light source. During the light exposure, the samples were placed on ice water to minimize heating effects. It was confirmed that the light exposure had no damage effects on viral capsid (data not shown). For the CDDP treatment, CDDP spiked samples were incubated at room temperature (20 °C) for 30 min. Light exposure was not required for the CDDP treatment. Then, the samples treated with EMA, PMA, or CDDP were subjected to nucleic acid extraction (as described below).

2.4. Nucleic acid extraction, reverse transcription, and qPCR detection

Viral RNA was extracted from 140-μL water samples using the QIAamp viral RNA MiniKit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. The extracted viral RNA was subjected to reverse transcription (RT) to synthesize cDNA using the high-capacity cDNA RT kit (Applied Biosystems, Tokyo, Japan).

Real-time PCR (qPCR) was conducted using 20 μL of a reaction mixture that contained cDNA (5 μL), TaqMan Gene Expression Master Mix (10 μL) (Applied Biosystems), forward primer and reverse primer (final concentration, 0.5 μM for MNV and SARS-CoV-2 or 0.3 μM for MHV), TaqMan probe (final concentration, 0.125 μM for MNV and SARS-CoV-2 or 0.4 μM for MHV), and nuclease-free water. Quantification of MHV and MNV was performed using a primer and probe set according to previous studies (Besselsen et al., 2002; Kitajima et al., 2008). SARS-CoV-2 quantification was performing using a primer and probe set (CDC_N1) according to the Centers for Disease Control and Prevention (CDC) in the USA (CDC, 2020). The number of viral genome copies per qPCR reaction was determined from a standard curve using gBlocks or plasmid DNA (Integrated DNA Technologies, Coralville, IA, US) containing the target sequence for each amplification. Standard curves were generated by 10-fold serial dilutions (5 × 10⁻⁴–5 × 10⁻⁹ copies/reaction).

2.5. Heat treatment

Samples (200 μL each, approximately 10⁵ plaque-forming units (PFU)/mL) in 1.5-mL microtubes were incubated at 50, 60, 70, and 80 °C for 2 min in a dry block heater (Nissin, Tokyo, Japan), and immediately placed on ice water at the end of the experiment. One sample was maintained at 4 °C as a control.

2.6. Virus concentration

2.6.1. Ultrafiltration (UF)

The wastewater samples (100 mL) were first centrifuged at 3,500 × g for 15 min to remove particles. The supernatant was further concentrated using a UF device (Centricon Plus-70; Millipore) with a molecular weight cut-off of 30 kDa according to the manufacturer’s protocol to obtain a final volume of 0.4–1.0 mL.

2.6.2. PEG precipitation

PEG precipitation was conducted as described in a previous study (Torii et al., 2021). Briefly, sample centrifugation (100 mL) was performed at 3,500 × g for 15 min to remove particles. The supernatant (80 mL) was supplemented with 8 g of PEG8000 and 4.7 g of NaCl to reach final concentrations of 10% (w/v) and 1.0 M, respectively. The mixture was incubated at 4 °C overnight in a shaker. Then, the mixture was centrifuged at 10,000 × g for 30 min. After carefully discarding the supernatant, the precipitate was resuspended with 10 mM phosphate buffer. The concentrate had a final volume of 1.0 mL.

2.7. Screening of capsid integrity reagents

First, three different capsid integrity reagents (including EMA, PMA, and CDDP) were tested to identify the most effective concentration for each capsid integrity reagent. For this purpose, intact MHV and its free RNA were treated with EMA (0.1, 1, 10, and 100 μM), PMA (0.1, 1, 10, and 100 μM), and CDDP (0.1, 1, 10, 100, 500, and 1,000 μM) and then subjected to RT-qPCR. The control was conducted without EMA/PMA/ CDDP treatment. The highest concentration of EMA (100 μM), PMA (100 μM) and CDDP (1000 μM) was confirmed to have no inhibitory effects on RT-qPCR amplification (data not shown). All samples were tested in triplicate.

The most suitable concentration of each capsid integrity reagent was further tested with heat-inactivated MHV to determine the most effective capsid integrity reagent. The MHV sample (10⁵ PFU/mL) was heated at 80 °C for 2 min. At this heat condition, all MHVs were inactivated according to the infectivity assay (data not shown). Then, heat-inactivated MHV was treated with EMA, PMA, or CDDP at its most effective concentration and subjected to RT-qPCR. All samples were tested in triplicate.

Since sodium deoxycholate (SD) pretreatment has been found to improve the performance of capsid integrity treatments (EMA, PMA, and CDPP) to remove inactivated viruses (e.g., AIV) (Canh et al., 2019), the current study also investigated the effect of SD pretreatment on the performance of optimal capsid integrity treatment on inactivated MHV. For this purpose, heat-inactivated MHV samples (at 80 °C for
2.8. Screening of virus concentration methods

The UF and PEG precipitation methods were evaluated using raw wastewater to determine the suitable virus concentration method for the application of capsid integrity RT-qPCR. Raw wastewater samples (approximately 500 mL) were collected in June 2020 from wastewater treatment plants (WWTPs) located in the Greater Tokyo Area in Japan. The raw wastewater had the following water quality parameters: temperature, 22.9 °C; pH, 7.66; turbidity, 58.2 NTU; electrical conductivity, 556 μS/cm; and UV254 absorbance, 0.304 cm⁻¹. The samples were maintained at −20 °C until use.

Raw wastewater samples (100 mL) spiked with MHV (approximately 10⁸ copies/mL) were concentrated using UF or PEG precipitation (as described above) to obtain the final concentrated samples (0.4–1.0 mL). Then, the final concentrated samples were subjected to RT-qPCR and the most effective capsid integrity RT-qPCR (as determined from the previous section). The experiment was performed in triplicate.

2.9. Detection of SARS-CoV-2 in wastewaters

2.9.1. Sample collection and concentration

Sixteen raw wastewater samples were collected from six different WWTPs (A, B, C, D, E, and F) in the Greater Tokyo Area in Japan from January 7th to February 25th, 2021. The water samples were frozen at −20 °C until use (1–2 months). The capacity and detailed treatment processes of these WWTPs are shown in Table S1. The general information of COVID-19 epidemic in the Greater Tokyo Area was in the range between 100 and 600 cases per day per 1 million people and more details are shown in the supplemental information.

The most suitable concentration method (determined from the previous section) was used to concentrate the raw wastewater (100 mL) to obtain the final concentrated samples (0.4–1.0 mL). The final concentrated samples were immediately analyzed for SARS-CoV-2 by conventional RT-qPCR and the most effective capsid integrity RT-qPCR (determined from the previous section).

2.9.2. Process controls

The efficiency of the virus concentration process, RNA extraction, and RT-qPCR for the detection of SARS-CoV-2 was evaluated using spiked MHV (whole process control). Briefly, MHV (20 μL, approximately 10⁸ copies/mL) was spiked into raw wastewater samples (100 mL) and Milli-Q water (100 mL) as a control. The MHV spiked samples were incubated at room temperature (20 °C) for 1.5 h before performing the most effective concentration method (as determined in the previous section). The concentrated samples (140 μL) were then subjected to RNA extraction and RT-qPCR detection. The MHV recovery efficiency (whole process control, ER_MHV) was calculated according the following equation: 

\[
ER_{\text{MHV}}(\%) = \left( \frac{C_{\text{f}}}{C_{\text{i}}} \right) \times \left( \frac{1}{F} \right) \times 100,
\]

where \(C_{\text{i}}\) and \(C_{\text{f}}\) are the concentrations of MHV in Milli-Q water sample (without conducting the virus concentration method) and the concentrated sample, respectively. \(F\) represents the concentration factor.

The effectiveness of the capsid integrity treatment to eliminate SARS-CoV-2 RNA in water samples was investigated using MNV RNA since MNV has a similar genome structure to SARS-CoV-2 (positive single-strand RNA). Briefly, free genomes of MNV (1.4 μL, approximately 10⁸ copies/mL) were spiked into the concentrated water samples and Milli-Q-water samples as the control and was subsequently subjected to the most effective capsid integrity RT-qPCR (as determined from the previous section). The effectiveness of the capsid integrity treatment was identified based on a comparison between the reduction of MNV RNA in the target water samples and the reduction of MNV RNA in Milli-Q-water (control).

3. Results and discussion

3.1. Effects of different capsid integrity reagents on intact MHV and its free RNA

The effects of different capsid integrity reagents (EMA, PMA, and CDDP) at different concentrations on intact MHV and its free RNA are presented in Fig. 1. As the EMA and PMA concentrations increased from 0.1 to 100 μM, the mean reductions of MHV RNA increased from 0.7 to 4.3 log₁₀ and from 2.4 to 5.1 log₁₀, respectively. CDDP also yielded a mean reduction from 0.5 to 4.2 log₁₀ as its concentration increased from 0.1 to 1,000 μM. These findings indicate that all assessed capsid integrity reagents could reduce the detection signal of MHV RNA by RT-qPCR and were more effective at higher concentrations.

Adverse effects on intact MHV virions were also evaluated. At concentrations of 0.1, 1.0, 10, and 100 μM, EMA showed mean reductions of intact MHV of 0.3, 1.4, 2.0, and 2.8 log₁₀ whereas PMA showed mean reductions of 0.4, 0.5, 0.8, and 1.0 log₁₀, respectively. CDDP had smaller effects on intact MHV since less than a 0.9 log₁₀ reduction was observed even at the highest concentration tested (1,000 μM). The reductions of intact MHV by PMA or CDDP were not significantly different from those of the control (in the absence of PMA or CDDP) \((p > 0.05, t\)-test), indicating that these capsid integrity reagents did not affect the detection of intact MHV by RT-qPCR. This result also implies that the intact MHV stock did not greatly contain free genomes of MHV. However, the reduction of MHV was obtained by EMA at concentrations higher than 0.1 μM \((p < 0.05)\). This result suggests that high concentrations of EMA can affect the detection of intact MHV by RT-qPCR.

Previous studies commonly used EMA and PMA at 100 μM and CDDP at 1,000 μM for performing capsid integrity RT-qPCR on non-enveloped enteric viruses without any effects on intact viral form (Canh et al., 2019; Fraisse et al., 2018). These concentrations also showed no inhibitory effects on RT-qPCR amplification. Therefore, it is possible that enveloped viruses such as MHV are more susceptible to capsid integrity reagents compared with non-enveloped enteric viruses. At high capsid integrity reagent concentrations (particularly EMA > 0.1 μM), the structure of MHV can be altered, which allows capsid integrity reagents to access its genomes so that subsequently these genomes are not detected by RT-qPCR. This result is consistent with a previous study indicating that PMA concentrations higher than 125 μM can cause a loss of infectivity for MS2 bacteriophages (Kim and Ko, 2012).

In the current study, the suitable concentration for each capsid integrity reagent was selected based on two criteria to rule out the effects of damage caused to the viral capsid: 1) the reduction of intact MHV did not differ significantly from that of the control and 2) the mean reduction was less than 0.5 log₁₀. Based on these criteria, EMA and PMA at a concentration of 0.1 μM and CDDP at a concentration of 100 μM were selected and considered to have a negligible effect on intact MHV. At these concentrations, CDDP (100 μM) was able to result in a 3.4 log₁₀ reduction of MHV RNA, which was higher than the 0.5 log₁₀ and 2.4 log₁₀ reductions obtained by EMA (0.1 μM) and PMA (0.1 μM), respectively.

In a previous study, platinum compounds (e.g., CDDP and PtCl₄) was also found to remove SARS-CoV-2 RNA in PBS buffer more effectively than monoxide dyes (e.g., EMA, PEMAX and PMAXx) (Cuevas-Serrano et al., 2021). Furthermore, PtCl₄ at 2.5 mM was recommended for the application of capsid integrity RT-qPCR because this concentration showed the highest reduction of gamma/heat-inactivated SARS-CoV-2 in fecal suspensions and nasopharyngeal swabs. However, the recommended concentration of PtCl₄ was much higher than the optimal CDDP concentration (100 μM) in the current study. It is possible that complex matrices in the tested samples can interact with PtCl₄ and...
reduce the effective concentration of PtCl₄. Therefore, the high concentration of PtCl₄ is needed to ensure the efficient performance of PtCl₄-

3.2. Determination of the optimal capsid integrity reagent

To determine the most effective capsid integrity reagent among EMA (0.1 µM), PMA (0.1 µM), and CDDP (100 µM), their performances were tested on heat-inactivated MHV (Fig. 2A). CDDP was able to remove heat-inactivated MHV (1.7 log₁₀) more effectively than EMA (0.6 log₁₀) and PMA (0.6 log₁₀). In the previous section, CDDP (100 µM) was also more effective than EMA (0.1 µM) and PMA (0.1 µM) at removing MHV RNA (Figs. 1 and 2A). Therefore, CDDP (100 µM) was considered the most effective among the tested capsid integrity reagents for discrimination of the damaged virus. This finding agrees with our previous study indicating that CDDP was more effective than EMA and PMA in discriminating between infectious and inactivated non-enveloped enteric viruses (e.g., AiV) (Canh et al., 2019).

Moreover, the effects of SD surfactant on the performance of CDDP treatment were also investigated to improve the discrimination of inactivated MHV. As shown in Fig. 2B, SD surfactant (0.1%) combined with CDDP (100 µM) showed a higher reduction of inactivated MHV...
(3.8 log10) than CDDP alone (1.7 log10). However, the combination of SD and CDDP was found to greatly reduce the detection of intact MHV (3.4 log10). It is possible that SD surfactant might damage the MHV capsid since surfactants have been found to dissolve the lipid bilayer membrane of enveloped viruses (e.g., influenza virus) (Kawahara et al., 2018). Therefore, surfactants (particularly SD) should not be used in combination with CDDP for capsid integrity RT-qPCR to assess the infectivity of enveloped viruses. This finding is inconsistent with previous studies indicating that surfactants (e.g., SD, and Triton X-100) can enhance the performance of capsid integrity RT-qPCR on non-enveloped enteric viruses with no impact on their capsid or infectivity (Canh et al., 2019). It is possible that non-enveloped viruses might not be susceptible to the effects of surfactant since they do not contain a lipid bilayer membrane in their structure.

Based on these results, capsid integrity RT-qPCR with 100 μM CDDP (CDDP-RT-qPCR) was selected as the most effective method and was used for further analyses in this study.

3.3. Performance of capsid integrity RT-qPCR to evaluate MHV inactivation by heat treatment

The performance of capsid integrity RT-qPCR using CDDP (CDDP-RT-qPCR) was compared to that of RT-qPCR alone and the infectivity assay to evaluate MHV inactivation after heat treatment at 50, 60, 70, or 80 °C (Fig. 3). Although RT-qPCR alone was unable to reduce MHV at any of the temperatures tested, loss of infectivity was observed at 1.6 log10 at 60 °C and more than 2.8 log10 (below the detection limit) at 70 and 80 °C. This result indicates that RT-qPCR alone was unable to discriminate between infectious and inactivated MHV. By contrast, CDDP-RT-qPCR showed a reduction of 1.0 log10, 1.0 log10, and 1.7 log10 at 60, 70, and 80 °C, respectively, indicating that CDDP-RT-qPCR was more effective in accessing the infectivity of MHV compared with RT-qPCR alone. This result also suggests that CDDP enters heat-inactivated MHV and subsequently blocks the viral genome from being detected by RT-qPCR. However, compared with the infectivity assay, CDDP-RT-qPCR still yielded a lower reduction of MHV, suggesting that CDDP-RT-qPCR did not fully differentiate between infectious and inactivated MHV. This is consistent with previous studies indicating that capsid integrity RT-qPCR overestimated the actual number of infectious viruses when examining non-enveloped enteric viruses after heat, chlorine, and UV treatments (Fuster et al., 2016; Leifels et al., 2015; Prevost et al., 2016; Randazzo et al., 2018). In fact, the loss of capsid integrity was not always correlated with the loss of viral infectivity (Hamza et al., 2011). Therefore, it is possible that viruses can lose their infectivity through alterations on viral capsid structures while still maintaining their capsid integrity to prevent the entry of capsid integrity reagents.

Enveloped viruses such as MHV have an additional lipid bilayer membrane compared with non-enveloped viruses. After inactivation treatment, enveloped viruses might interact with capsid integrity reagents at different efficiencies compared with non-enveloped viruses. In the current study, CDDP-RT-qPCR was able to reduce 3.4 log10 of MHV RNA (maximum capacity) but only 1.7 log10 of heat-inactivated MHV even at the highest temperature tested (80 °C for 2 min). Compared with our previous studies on enteric viruses (e.g., AIV), CDDP was capable of removing 5.7 log10 of AIV RNA and 4.8 log10 of heat-inactivated AIV (80 °C for 1 min) (Canh et al., 2019, 2020). In other words, CDDP yielded a 0.9 log10 difference in the reduction between inactivated AIV and its free RNA, which was smaller than the 1.7 log10 difference in the reduction between inactivated MHV and its free RNA. Most likely, the penetration of CDDP into the capsid of a non-enveloped virus (AIV) was more effective than that into an enveloped virus (MHV) at a similar inactivation condition. However, more studies are necessary to accurately compare between enveloped and non-enveloped viruses to investigate the performance of capsid integrity of RT-qPCR at a similar concentration of virus stock and capsid integrity reagents.

3.4. Screening of virus concentration methods for the application of capsid integrity RT-qPCR

Two virus concentration methods (the UF and PEG methods) were investigated to recover MHV in raw wastewater (Fig. 4). The recovery yields were determined by RT-qPCR alone and CDDP-RT-qPCR (the most effective capsid integrity RT-qPCR determined from the previous section) (Fig. 4A). According to RT-qPCR alone, the UF method yielded a MHV recovery of 17.8% ± 7.4%, which was significantly higher than the recovery of 0.034% ± 0.024% obtained by the PEG method. This result agrees with findings from a previous study indicating a greater recovery of φ6 in wastewater samples by the UF method (6.4–35.8%) compared with the PEG method (1.4–3.0%) (Torii et al., 2021). When comparing the effectiveness of different concentration methods for quantifying SARS-CoV-2 RNA in wastewater, LaTurner et al. (2021) also found that the UF method yielded a higher concentration of SARS-CoV-2 RNA in wastewater concentrates than did the PEG method. However, the recovery of intact viruses or the effects on viral capsids by these concentration methods were not investigated since the previous studies applied only RT-qPCR or droplet digital RT-PCR to quantify viruses. In the current study, when determined by CDDP-RT-qPCR, the recovery yields of the UF method (38.0% ± 28.7%) were also greater than those obtained by the PEG method (0.013% ± 0.015%) (Fig. 4A). These results suggest that the UF method was able to recover intact MHV more effectively than the PEG method. In a previous study, the UF method was also found to recover infective MHV and Pseudomonas phage φ6 more effectively than the PEG method according to the infectivity assay (Ye et al., 2016). Therefore, it is better to apply the UF method for evaluating virus concentration when investigating the infectivity of enveloped viruses in wastewater.

Furthermore, for the UF method, the recovery efficiency of MHV determined by conventional RT-qPCR (17.8% ± 7.4%) did not differ significantly from the recovery efficiency of MHV determined by CDDP-RT-qPCR (38.0% ± 28.7%) (Fig. 4A). Additionally, the different levels obtained between RT-qPCR alone and CDDP-RT-qPCR in the samples concentrated by the UF method (0.4 log10) were comparable to those in the control (0.4 log10) (MilliQ-water samples without performing the virus concentration methods) (Fig. 4B). These results suggest that the UF method did not significantly affect the structure of MHV. However, in the samples concentrated by the PEG method, the difference (1.2 log10) between RT-qPCR alone and CDDP-RT-qPCR was significantly greater than that of the control (Fig. 4B). This may have occurred because the structure of MHV was partially destroyed by the PEG method.
method. This finding is consistent with a previous study indicating that the PEG method could affect the infectivity of enveloped viruses. Indeed, Ye et al. (2016) found that the infectivity of MHV was decreased by 2 log_{10} when MHV was incubated with PEG for 16 h. In this current study, the mixing process of wastewater samples and PEG was conducted for approximately 20 h. Therefore, it is possible that the PEG method in this current study can cause a certain loss of MHV infectivity due to the disruption of PEG on their lipid bilayers (Ye et al., 2016).

Since the UF method displayed a greater recovery of intact MHV and caused less damage to the MHV capsid than the PEG method, it was selected for concentrating SARS-CoV-2 in wastewater in the following steps of this study.

3.5. Application of capsid integrity RT-qPCR for the selective detection of SARS-COV-2 in wastewater samples

Sixteen raw wastewater samples collected from the Greater Tokyo Area were concentrated using the UF concentration method and were quantified for SARS-CoV-2 by RT-qPCR alone and CDDP-RT-qPCR (Table 1). The efficacy of the whole detection processes (including the UF concentration method, RNA extraction, and RT-qPCR amplification) was investigated for all wastewater samples using spiked MHV. The recovery efficiencies were mostly greater than 10%, indicating that the entire detection process was efficient for detecting viruses in the tested wastewater samples. Furthermore, the efficiency of CDDP treatment to eliminate free viral RNA present in wastewater was investigated using MNV RNA. The CDDP treatment was able to remove more than 5.7 log_{10} of MNV RNA in all concentrated samples, indicating that the CDDP treatment effectively removed free viral genomes present in the concentrated wastewater samples.

Of the 16 raw wastewater samples, SARS-CoV-2 was detected in 5 samples (B1, C1, D1, F1, and F4) with concentrations ranging from 2.3 × 10^3 to 6.6 × 10^4 copies/L when determined by RT-qPCR alone (Table 1). This result is consistent with previous studies conducted in Japan indicating that the concentration of SARS-CoV-2 was <1.1–4.1 × 10^4 copies/L or higher than the detection limit (4.0 × 10^3 copies/L) in raw wastewater (Haramoto et al., 2020; Hata et al., 2021; Torii et al., 2021). A higher load of SARS-CoV-2 was also reported in raw wastewater collected in other countries, particularly loads of 1.3–3.2 × 10^5 copies/L in Spain (Randazzo et al., 2020), 10^4–10^6 copies/L in France (Wurtzer et al., 2020b), and 2.6 × 10^2–2.2 × 10^6 copies/L in France (Wurtzer et al., 2020b), and 2.6 × 10^2–2.2 × 10^6 copies/L in France (Wurtzer et al., 2020b).

![Fig. 4. Comparison between the UF and PEG methods for concentrating MHV in raw wastewater.](image)

**Table 1**

| Samples  | Process control | Reduction of MNV RNA^b | PCR^c (copies/L) | CDDP^c (copies/L) | LoD^c (copies/L) |
|----------|----------------|------------------------|------------------|-------------------|-----------------|
| Date     | Name | Plant | Recovery of spiked MHV^a (%) | Log_{10} | <LoD | <LoD | 5.0 × 10^3 | <LoD | <LoD | 5.9 × 10^3 | <LoD | <LoD | 6.9 × 10^3 | <LoD | <LoD | 3.4 × 10^4 | <LoD | <LoD | 4.9 × 10^4 | <LoD | <LoD | 7.4 × 10^3 | <LoD | <LoD | 7.9 × 10^3 | <LoD | <LoD | 1.0 × 10^4 | <LoD | <LoD | 6.8 × 10^3 | <LoD | <LoD | 3.3 × 10^4 | <LoD | <LoD | 3.9 × 10^4 | <LoD | <LoD | 5.1 × 10^4 | <LoD | <LoD | 5.8 × 10^4 | <LoD | <LoD | 3.3 × 10^4 | <LoD | <LoD | 5.3 × 10^3 | <LoD | <LoD | 5.3 × 10^3 |
| 2021/1/7 | A1   | A     | 17% >6.3 | <LoD | <LoD | 5.0 × 10^3 |
| 2021/1/13 | A2  | 31% >6.3 | <LoD | <LoD | 5.9 × 10^3 |
| 2021/1/20 | A3  | 42% >6.3 | <LoD | <LoD | 6.9 × 10^3 |
| 2021/1/7 | B1  | B     | 37% >6.3 | <LoD | <LoD | 4.9 × 10^4 |
| 2021/1/13 | B2  | 39% >6.3 | <LoD | <LoD | 7.4 × 10^3 |
| 2021/1/20 | B3  | 40% 5.8 | <LoD | <LoD | 7.9 × 10^3 |
| 2021/2/3 | C1  | C     | 41% >6.3 | <LoD | <LoD | 1.0 × 10^4 |
| 2021/2/10 | C2  | 44% 5.8 | <LoD | <LoD | 6.8 × 10^3 |
| 2021/1/20 | D1  | D     | 18% >6.3 | <LoD | <LoD | 3.3 × 10^4 |
| 2021/1/28 | D2  | 25% 5.7 | <LoD | <LoD | 3.9 × 10^4 |
| 2021/2/17 | D3  | E     | 10% >6.3 | <LoD | <LoD | 5.1 × 10^4 |
| 2021/2/25 | D4  | 7% >6.3 | <LoD | <LoD | 5.8 × 10^4 |
| 2021/2/3 | F1  | F     | 13% >6.3 | <LoD | <LoD | 4.1 × 10^4 |
| 2021/2/10 | F2  | 14% 5.9 | <LoD | <LoD | 5.3 × 10^3 |
| 2021/2/17 | F3  | 11% >6.3 | <LoD | <LoD | 5.3 × 10^3 |
| 2021/2/24 | F4  | 5% 6.0 | <LoD | <LoD | 5.3 × 10^3 |

^a MHV was used to evaluate the recovery of the whole process control using RT-qPCR.

^b MNV RNA was used to evaluate the ability of CDDP treatment to eliminate free viral genomes in water samples. The reduction limit was 6.3 log_{10}.

^c A primer and probe set (CDC_N1) was used to detect SARS-CoV-2.

^d SARS-CoV-2 was positive in one of two PCR reactions (Ct values: 36.5–39.1).

^e SARS-CoV-2 was positive in two of two PCR reactions (Ct values: 36.4–37.9).
SARS-CoV-2 and wastewater, this method could improve the interpretation of positive SARS-CoV-2 results obtained by RT-qPCR.

CRediT authorship contribution statement

Vu Duc Canh: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. Shotaro Torii: Investigation, Methodology, Writing – review & editing. Midori Yasui: Investigation, Writing – review & editing. Shigeru Kyuya: Resources, Writing – review & editing. Hiroyuki Katayama: Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare no conflicts of interest associated with this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.148342.

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