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Assessment of two volumetrically different concentration approaches to improve sensitivities for SARS-CoV-2 detection during wastewater monitoring

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ABSTRACT

Wastewater monitoring for severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2), the virus responsible for the global coronavirus disease 2019 (COVID-19) pandemic, has highlighted the need for methodologies capable of assessing viral prevalence during periods of low population infection. To address this need, two volumetrically different, methodologically similar concentration approaches were compared for their abilities to detect viral nucleic acid and infectious SARS-CoV-2 signal from primary influent samples. For Method 1, 2 L of SARS-CoV-2 seeded wastewater was evaluated using a dead-end hollow fiber ultrafilter (D-HFUF) for primary concentration, followed by the CP Select™ for secondary concentration. For Method 2, 100 mL of SARS-CoV-2 seeded wastewater was evaluated using the CP Select™ procedure. Following D-HFUF concentration (Method 1), significantly lower levels of infectious SARS-CoV-2 were lost (P value range: 0.0398–0.0027) compared to viral gene copy (GC) levels detected by the US Centers for Disease Control (CDC) N1 and N2 reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) assays. Subsamples at different steps in the concentration process were also taken to better characterize the losses of SARS-CoV-2 during the concentration process. During the centrifugation step (prior to CP Select™ concentration), significantly higher losses (P value range: 0.0003 to <0.0001) occurred for SARS-CoV-2 GC levels compared to infectious virus for Method 1, while between the methods, significantly higher infectious viral losses were observed for Method 2 (P = 0.0002). When analyzing overall recovery of endogenous SARS-CoV-2 in wastewater samples, application of Method 1 improved assay sensitivities (P = <0.0001) compared with Method 2; this was especially evident during periods of lower COVID-19 case rates within the sewershed. This study describes a method which can successfully concentrate infectious SARS-CoV-2 and viral RNA from wastewater. Moreover, we demonstrated that large volume wastewater concentration provides additional sensitivity needed to improve SARS-CoV-2 detection, especially during low levels of community disease prevalence.

1. Introduction

SARS-CoV-2, the viral agent responsible for the current COVID-19 global pandemic, has spread to every country of the world and is responsible for 6.5 million deaths worldwide, one million in the United States alone as of August of 2022 (Oxford, 2021). While significant attention has been given to establishing human infection rates of SARS-CoV-2 through individual testing of mostly symptomatic patients, only more recently has a focus been placed on population-level monitoring via wastewater. Monitoring of wastewater, recently described as wastewater-based epidemiology (WBE) as a possible population screening tool has gained attention (Ahmed et al., 2020a; Ai et al., 2021). The advantage of wastewater screening is that both symptomatic and asymptomatic individuals (shedding virus particles through their stool) are represented within sanitary waste collection systems (Farkas et al., 2020). Wastewater analysis could act as a non-invasive way to assess community level infection rates to provide more timely and accurate assessments of viral presence and spread.

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Estimating levels of circulating SARS-CoV-2 through wastewater analysis has inherent limitations with current virus detection methodologies, particularly with regards to analytical volumes processed (Ahmed et al., 2021b; Ai et al., 2021; Cuevas-Ferrando et al., 2021; Gonzalez et al., 2020). Current methodologies used to concentrate and detect SARS-CoV-2 have, by necessity, been developed quickly to analyze wastewater samples ranging from 50 to 250 mL, while avoiding the time constraints and difficulties associated with large volume wastewater concentration approaches (Ahmed et al., 2021b; Ai et al., 2021; Randazzo et al., 2020). While small volume wastewater samples can be informative during high community infection rates, these volumes can limit the ability to use wastewater to estimate circulating levels of virus, especially during early stages of disease outbreaks. In addition, pathogenic viruses are often found in wastewater at low densities necessitating the concentration of larger volumes of wastewater (1–100 L) to achieve detectable virus levels (Korajkic et al., 2022). As this pandemic evolves or future pandemics occur, the ability to detect low levels of circulating viruses and estimate infection rates within communities, especially during early stages of an outbreak, is important and improvements in method sensitivity will be critical to detect early onset of disease outbreaks for more effective implementation of appropriate remediation to minimize and thwart disease spread.

An easy to use, quick, field-deployable concentration method that has the flexibility to effectively monitor emerging pathogens and/or pandemic related microorganisms (viruses, bacteria, protozoa, etc.) is needed. Ideally, the method will be capable of large volume processing allowing increased sensitivity, while being amenable to both molecular and culture-based analyses to better monitor or address future pandemic related microorganisms. Technologies such as hollow-fiber ultrafilters (HFUF), repurposed medical dialysis filters, have been utilized for this application and have shown effectiveness in recovery of diverse microorganisms of interest from a variety of liquid matrices (Cuevas-Ferrando et al., 2021; Liu et al., 2012; McMinn et al., 2017; Morales-Morales et al., 2003). Upon sample concentration, washing of captured microorganisms from the ultrafilter surfaces is performed using a mild surfactant solution which allows for recovery of both viable and non-viable microorganisms present (Hill et al., 2005; Korajkic et al., 2021). Methods capable of relaying infectious virus status are critical to understanding the risk to public health, establishing the fate and transport of viral pathogens like SARS-CoV-2 (and viral variants) through wastewater, and determining viral persistence. Once developed, these methodologies could play a major role in efforts to better inform public health officials in the United States and abroad for addressing the COVID-19 pandemic, while also providing necessary tools to address future pandemics (Kitajima et al., 2020).

In this study, we evaluated and compared two methods, 1) a large volume method (D-HFUF) capable of analyzing large volumes (2 L) of wastewater, and 2) a small volume (100 mL) method (CP SelectTM) also using HFUF technology. For the first series of experiments, laboratory cultured stocks of SARS-CoV-2 were seeded into autoclaved primary influenza to assess the ability of the D-HFUF and the CP SelectTM for recovery of both infectious SARS-CoV-2 and gene copies levels of SARS-CoV-2 RNA from wastewater. We documented viral losses through each concentration process to aid in identifying areas where these concentration methodologies could be improved. For the second series of experiments, we applied the methods (D-HFUF and CP SelectTM) to concentrate endogenous levels of SARS-CoV-2 in wastewater samples to determine if larger volume analysis results in improved assay sensitivity.

2. Materials and methods

2.1. SARS-CoV-2 stock preparation

Vero-6 cells (Cat. ATCC CRL 1586, American Type Culture Collection, Manassas, VA) were grown to confluency in 75 cm², filter-capped flasks (ThermoFisher Scientific, Waltham, MA) and incubated at 37 °C; 5% CO₂. Each 75 cm² flask contained 30 mL of cell maintenance media; Dulbecco’s Modified Eagles Medium (DMEM), with high glucose with L-glutamine (ThermoFisher Scientific); along with 10% fetal bovine serum (FBS) (ThermoFisher Scientific). Prior to infection, used maintenance media was washed and cell monolayers were washed with 10 mL of Dulbecco’s Phosphate Buffered Saline (DPBS) (ThermoFisher Scientific). To acquire high levels of infectious SARS-CoV-2 (Washington State strain, USA/WA1/2020), the virus was propagated as described below. Briefly, 1 mL of frozen SARS-CoV-2 (10² plaque forming units/100 mL (PFU/100 mL) were thawed and diluted in 20 mL of sterile maintenance media. Three milliliter volumes of SARS-CoV-2 viral dilutions were added to 4–75 cm² flasks of 100% confluent Vero-6 monolayers and allowed to incubate for 1 h at 37 °C; 5% CO₂. Thirty milliliter of fresh maintenance media were added, and flasks were incubated for an additional 72 h at 37 °C, 5% CO₂. Following the 72 h incubation, flasks were subjected to two freeze/thaw cycles at ~80 °C and at room temperature, respectively. Cell debris was pelleted by centrifugation at 4,500g for 10 min. The resulting supernatant was collected and syringe filtered through a 0.22 μm filter (ThermoFisher Scientific) prior to aliquoting into 1 mL volumes in cryovials. Viral titers were assessed from filtered supernatant using both RT-qPCR and plaque assay as described below. Viral titers of 1 × 10⁶ infectious particles/mL were aliquoted and used for seeding experiments in this study. Virus seeding stocks were stored at ~80 °C until use.

2.2. Infectious SARS-CoV-2 Detection

Levels of infectious SARS-CoV-2 were measured according to Mendoz et al. (2020). Briefly, 6-well plates (Corning, Corning, NY) were seeded with 4 × 10⁵ Vero-6 cells per well and incubated at 37 °C at 5% CO₂ for 3 days for 90% confluent cell monolayer formation. Once confluent, the maintenance media was removed, and cell monolayers were washed with 1 mL of DPBS. Following the wash step, the monolayers were inoculated with 100 μL SARS-CoV-2 seeding suspension or wastewater concentrate, or 1:10 dilutions of SARS-CoV-2 seeding suspensions, with all virus inoculums run in triplicate. Following addition of virus inoculum, plates were allowed to incubate for 1 h at 37 °C; 5% CO₂. During the 1-h incubation, plates were gently rocked by hand every 15 min to facilitate viral attachment/adsorption. Following the 1-h infection, wells were overlaid with a liquid overlay media (LOM), consisting of a 3 mL of a 1:1 mixture of 3% carboxymethylcellulose and overlay diluent (2 × Minimum Essential Media [MEM] (500 mL), 10% FBS, 10 mL of 200 mM L-glutamine, 7.5 mL of 100 × sodium bicarbonate, and 10 mL of 100 × nonessential amino acids (ThermoFisher Scientific) were added to each plate well and allowed to incubate for 72 h at 37 °C; 5% CO₂. Following the 72 h incubation, the liquid overlay was removed, and cell monolayers were washed with 1 mL of DPBS. Cell monolayers were then fixed by adding 1 mL of 4% paraformaldehyde (ThermoFisher Scientific) and incubated at 4 °C overnight. Cell monolayers were then stained with 0.5% crystal violet solution (1 g crystal violet dissolved in 20 mL absolute ethanol and 80 mL sterile DI water; ThermoFisher Scientific) and incubated for 15 min at room temperature. Crystal violet was then removed from cells through three washes with 1 mL of DI water and allowed to dry. The resulting viral plaques were enumerated and reported as PFU/100 mL.

2.3. Wastewater collection and treatment

Using a sterile 10 L carboy, primary wastewater influent samples (following primary settling and screening) were collected (from August through October) from an urban wastewater treatment (WWTP) facility located within the metropolitan sewerage district of Cincinnati, Ohio. This facility processes between 55 and 96 million gallons (MGD) of waste/day, serving a population of 237,000 residents (https://www.msdgc.org/). Wastewater samples were transported immediately (on ice) to the US Environmental Protection Agency (USEPA) laboratories for
located in Cincinnati, Ohio for processing. Upon arrival at the laboratory, wastewater samples were either autoclaved for 60 min at 121°C to inactivate any SARS-CoV-2 present (SARS-CoV-2 seeding and recovery experiment) or were left untreated and transferred to a Biosafety Level-3 (BSL-3) laboratory for assessing levels of endogenous SARS-CoV-2. In a previous study, sample pre-treatment (autoclaving) manipulation did not alter the recovery of enveloped viruses compared to unautoclaved wastewater (Ahmed et al., 2020b). All wastewater samples were stored overnight at 4°C prior to experimentation the following day. No SARS-CoV-2 gene copies or infectious particles) were detected in the autoclaved wastewater (Ahmed et al., 2020b). All wastewater samples did not alter the recovery of enveloped viruses compared to unautoclaved wastewater.

2.4. Concentration method 1

Autoclaved wastewater aliquots (2 L) were measured using a graduated cylinder and transferred into a sterile 2.5 L Erlenmeyer flask containing a stir bar. To each 2 L wastewater aliquot, 1 mL of SARS-CoV-2 seed (1 × 10^6 infectious particles/mL) was added and allowed to mix for 3 min prior to concentration. Dead-end hollow-fiber ultrafiltration (D-HFUF) concentration was used to concentrate 2 L volumes of wastewater using 15 S Asahi Kasei Reverse ultrafilters (Dial Medical Supply, Chester Springs, PA) and a peristaltic pump (Masterflex L/S Easy Load, Cole Parmer, Vernon Hills, IL) set at 300 rpm (1.5 L/min). Following sample filtration, filters were eluted following procedures detailed in McMinn et al. (2021).

Filter eluates were centrifuged (12,000 × g for 30 min) to remove particulates prior to secondary concentration. For secondary sample concentration, the Concentrating Pipette Select™ (CP Select™) (InnovaPrep, Drexel, MO) was used to reduce the approximate 100 mL volumes of primary filter elute or primary influent to volumes more applicable to molecular analyses (eluate volume ranged between 670 and 932 µL). Using InnovaPrep’s CP Select™ Wastewater Application settings, primary filter eluates were passed through the concentrating pipette tip (CPT) ultrafilter (InnovaPrep) and upon sample filtration ultrafilter tips were eluted (2 times) using a hand-driven syringe elution procedure previously described (McMinn et al., 2021).

2.5. Concentration method 2

For seeding and recovery experimentation, a 2 L sample of autoclaved primary influent was seeded with 1 mL of SARS-CoV-2 and was allowed to mix for 3 min at room temperature prior to aliquoting 100 mL volumes for further processing. For experiments targeting endogenous SARS-CoV-2, 100 mL volumes of primary influent were analyzed directly. Prior to CP Select™ concentration, all wastewater samples were centrifuged at 12,000 × g for 30 min to remove particulates. All samples were then processed as described above using the InnovaPrep CP Select™.

2.6. Determining SARS-CoV-2 loss during concentration procedures

Throughout both primary and secondary concentration, subsamples were collected (400 µL for culture and 200 µL for nucleic acid extraction) to determine SARS-CoV-2 losses at each step of the concentration procedure. For Method 1, subsamples were taken: 1) from the 2 L of autoclaved wastewater following the addition of SARS-CoV-2 seed and mixing for 3 min, 2) from the filter eluate (post D-HFUF concentration), and 3) following centrifugation of the eluate but prior to CP Select™ secondary concentration. For Method 2, subsamples were taken: 1) from the 100 mL autoclaved wastewater following the addition of SARS-CoV-2 seed and mixing for 3 min, and 2) following centrifugation of the 100 mL virus seeded wastewater. Both Method 1 and Method 2 were evaluated for their ability to concentrate endogenous SARS-CoV-2 from unamended primary influent samples collected as described above. Percent of virus losses were calculated using the following formula: SC/S × 100 = PR here: SC = SARS-CoV-2 concentrations in sample filtrate/eluate, S = SARS-CoV-2 concentrations in seeded material from prior concentration step (i.e., levels of SARS-CoV-2 in seeded sewage prior to D-HFUF concentration), PR = percent recovery. The percent loss (L) was calculated as follows: L = 100 - PR SARS-CoV-2 genomic copies or infectious virus.

2.7. Endogenous SARS-CoV-2 detection

Primary influent wastewater samples were collected and were immediately transferred to a BSL-3 facility (without autoclaving) and processed using the methods described above (Method 1 and Method 2). Endogenous levels of SARS-CoV-2 resulting from the two methods were quantified and then compared to COVID-19 case numbers (corresponding to the sample collection dates) for the sewershed, retrieved from Ohio Department of Health (ODH) COVID-19 Dashboard which displayed confirmed case counts by symptom onset date (Ohio).

2.8. Nucleic acid extractions and RT-qPCR

Viral RNA was extracted from 200 µL portions of each seed, sub-sample, and sample concentrate using a Qiagen All Prep PowerViral Kit (Qiagen, Valencia, CA) according to manufacturer’s instructions, resulting in 100 µL extract volumes. All sample extracts were analyzed in triplicate unless otherwise noted. One extraction blank (EB), including all reagents but no nucleic acid RNA template, was included with each extraction batch. Purified RNA extracts were stored at −80 °C for more than 30 days. SARS-CoV-2 RNA was quantified using a one-step quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) using the U.S. Center for Disease Control and Prevention N1 and N2 primers sets (Control, (2021)). N1 forward primer (2019-nCoV N1-F) GAC CCC AAA ATA AGC GAA AT, TaqMan probe (2019-nCoV N1-P) FAM-/ACC CCG CAT TAC TGT GTG TGG ACC-BHQ1, and reverse primer (primer-2019-nCoV N1-R) TCT GGT TAC TGC CAG TTG AAT CTG, and N2 forward primer (2019-nCoV N2-F) TTA CAA ACA TTG GCC GCA AA, TaqMan probe (2019-nCoV N2-P) FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1, and reverse primer (2019-nCoV N2-R) GGG GCA CAT TCC GAA GAA, were used to detect SARS-CoV-2-RNA. Viral RNA present was amplified using the RNA UltraSense™ One-Step Quantitative RT-PCR System (Applied Biosystems, Foster City, CA) following manufacturer’s instructions. Each reaction consisted of; 1.25 µL enzyme mix, 5 µL of 5 × reaction mix, 2.5 µL BSA fraction V (Sigma Aldrich), 0.05 µL ROX, 3.0 µL RT-PCR master mix (1 µM primer and 80 nM probe), 2 µL template, and 11.2 µL nuclease-free water, for a total reaction volume of 25 µL. One step RT-qPCR reaction conditions were as follows: 50.0 °C for 15 min, 95.0 °C for 2 min, 3 cycles at 95.0 °C for 15 s followed by 60.0 °C for 30 s. All sample RT-qPCR TaqMan assays were performed in triplicate using a QuantStudio3 real-time qPCR system with threshold manually set to 0.03 (Applied Biosystems). Potential inhibition was assessed by comparing cycle thresholds (Ct) of undiluted and samples diluted 10-fold in sterile AE buffer (10 mM Tris-Cl; 0.5 mM EDTA; pH 9.0), which is expected to delay the detection of the target by 3.3 Ct, as 2^(-ΔΔCt) = 10, assuming 100% assay amplification efficiency. Data acceptance thresholds of ± 1.0 Ct were selected based on the expectation that a Ct difference between an uninhibited sample and a 10-fold diluted sample be no more than one Ct if assuming 0.5 cycle natural variability (pipetting errors, nucleic acid losses through adhering to equipment surfaces, low level sample inhibition, etc.) between qPCR sample replicates (Cao et al., 2012). Six no template controls (NTCs) were included with each instrument run.

2.9. Standard curve preparation

Armored RNA Quant® SARS-CoV-2 (Asuragen, Austin, TX) (1 × 10^11 copies/mL) was extracted as described above and used as a reference RNA standard. The resulting RNA extract was quantified by RT-qPCR using the most probable number (MPN) approach (McMinn, 2016).
Briefly, utilizing a ten-fold decimal dilution series starting with the undiluted extract (five replicates each) GC present were quantified for each dilution series with dilutions spanning 10–10⁰ copies per 2 µL. All dilutions were prepared using sterile AE buffer for use as calibration standards. A master standard curve was created based on five individual standard curves for each assay (N1 and N2 RT-qPCR assays), resulting slope and y-intercepts were averaged between the five replicate standard curves and this data was then used for quantifying GC of SARS-CoV-2 present in samples during the study. Average slope, R², y-intercept, and amplification values were – 3.32 ± 0.05, 0.99 ± 0.00, 36.39 ± 0.72 and 1.03 ± 0.00 for N1 and – 3.23 ± 0.25, 0.98 ± 0.01, 35.19 ± 1.09 and 1.03 ± 0.00 for N2, respectively. N1 and N2 assay lower limit of quantification (LLOQ) was identified as an average of all 10 copy standards and equaled 34.279 and 33.519, Ct respectively, during this analysis. A total of 48 individual negative control reactions (combination of EBs and NTCs) were below the LLOQ, indicating absence of extraneous RNA contamination.

2.10. Data statistical analyses

Percent recovery data were log_{10} arcsine square root transformed prior to statistical analyses. Paired t-tests and one-way analysis of variance (ANOVA) with Tukey’s multiple comparison tests (GraphPad Prism 8.3.1, GraphPad Software, La Jolla, CA, USA) were used to determine effectiveness of each step of the concentration procedure. Levels of infectious SARS-CoV-2 virus were compared to the N1 and N2 RT-qPCR signals. Performance of Method 1 was then compared to Method 2 (α = 0.05 for all tests).

3. Results

3.1. Sample inhibition analysis

To assess the presence of inhibitory substances, all subsamples and sample concentrates were screened for inhibition of the RT-qPCR reactions. Deviations between undiluted and 10-fold diluted samples ranged from – 0.52–0.65 Ct for the N1 qPCR primer set and from – 0.11–0.57 Ct for the N2 RT-qPCR primer set. No notable evidence of sample inhibition (difference of ± 1.0 Ct between undiluted and diluted sample) was observed for any subsample or sample concentrate analyzed using the N1 and N2 RT-qPCR assays.

3.2. Infectious viral particles vs. viral RT-qPCR GC

To compare SARS-CoV-2 infectious particles with the GC signal (N1 and N2 qPCR), paired samples of SARS-CoV-2 seeding mixture, subsamples, and sample concentrates were analyzed. Irrespective of seeding mixture, subsample, or sample concentrate measured between culture and RT-qPCR, the N1 and N2 gene copies detected by RT-qPCR were nearly 4-log_{10} or higher in concentration (average viral PFU/mL vs N1 and N2 gene copies/mL). Overall average N1 GC signals throughout the study were 3.72 ± 0.38 log_{10} greater than SARS-CoV-2 infectious signals, while the N2 RT-qPCR signal averaged 3.69 ± 0.35 log_{10} greater. The largest differences in signal concentrations occurred during the final elution step (following CP Select™ concentration) where RT-qPCR assay signal concentrations averaged 4.15 ± 0.64 log_{10} and 4.16 ± 0.47 log_{10} higher than the infectious signal for the US CDC N1 and N2 assays, respectively. Overall, concentrations for both N1 and N2 assays (regardless of concentration step analyzed) were found to be significantly higher than infectious SARS-CoV-2 concentrations (P < 0.0001) assessed through experimentation, while there were no statistically significant differences between the two RT-qPCR assays.

3.3. SARS-CoV-2 loss at the primary concentration step of method 1

For Method 1, 1 mL of SARS-CoV-2 seed was inoculated and mixed into 2 L of autoclaved primary treated wastewater and concentrated using D-HFUF. SARS-CoV-2 losses were assessed following addition of the virus seed to the treated wastewater prior to D-HFUF concentration. Initial losses of the SARS-CoV-2 virus seeds prior to D-HFUF filtration were 81.1 ± 6.4% for culture and 73.4 ± 18.5% and 65.6 ± 13.6% for the N1 and N2 RT-qPCR assays, respectively. Losses of SARS-CoV-2 virus seeds following D-HFUF concentration averaged 82.2 ± 10.0%, 50.7 ± 9.6%, and 47.7 ± 7.5% for culturable SARS-CoV-2, N1, and N2 RT-qPCR assays, respectively. Overall, significantly higher losses were observed for culture-based SARS-CoV-2 (P value range: 0.0398–0.0027), as opposed to either N1 or N2 RT-qPCR assays through D-HFUF concentration.

3.4. SARS-CoV-2 loss during centrifugation

Following D-HFUF concentration for Method 1 and prior to direct analysis of seeded wastewater for Method 2, filter eluates and seeded wastewater samples were centrifuged to remove particulate matter prior to the InnovaPrep CP Select™ concentration step (InnovaPrep, 2021). Assessment of SARS-CoV-2 losses resulting from centrifugation of samples were measured from subsamples collected prior to and post centrifugation (as described in the Materials and Method section). Infectious SARS-CoV-2 losses during centrifugation for filter eluates (Method 1), ranged from 0% to 13% (Fig. 1). The observed losses from the same filter elutes analyzed using the N1 and N2 RT-qPCR assays ranged from 30% to 35% and 32–47%, respectively which were significantly higher compared with infectious virus losses (P value range: 0.0003 to <0.0001). For seeded wastewater samples (100 mL) that were processed using Method 2, infectious SARS-CoV-2 losses during centrifugation ranged from 16% to 94%, while observed losses using the N1 or N2 RT-qPCR assays ranged from 23% to 63% and 18–67%, respectively. There were no significant differences in centrifugation losses of infectious virus compared to GC levels of virus signal observed during Method 2. Infectious SARS-CoV-2 was lost at significantly higher levels (P = 0.0002) during centrifugation in Method 2 compared to Method 1 (Fig. 1). No other significant differences in losses were observed between the two methods and analytical techniques used during centrifugation.

3.5. SARS-CoV-2 loss during CP select™ concentration

SARS-CoV-2 viral losses were assessed during CP Select™ concentration under two different conditions (Fig. 1). Losses were either assessed from Method 1 filter eluates (post centrifugation) or were determined from seeded wastewater samples for Method 2 (post centrifugation). For Method 1 filter eluates, losses through CP Select™ concentration for infectious SARS-CoV-2 averaged 8.8 ± 0.8%, while N1 and N2 RT-qPCR signal loss averaged 30.8 ± 0.7% and 37.8 ± 6.8%,

Fig. 1. SARS-CoV-2 Infectious and GC Signal Losses During Method 1 and Method 2 Concentration.
respectively. There were no statistically significant differences in losses occurring between infectious SARS-CoV-2 and SARS-CoV-2 GC signal. For Method 2, infectious SARS-CoV-2 virus signal was lost at an average of 67.6 ± 13.4%, while losses for the N1 and N2 assays averaged 42.3 ± 8% and 46.0 ± 6.1%, respectively. There were no statistically significant differences in losses occurring between infectious SARS-CoV-2 and for either molecular marker during this stage of concentration.

3.6. Overall recovery of SARS-CoV-2 through method 1 and method 2 concentration

Final cumulative methodological recoveries of infectious virus, GC N1 and N2 signals for SARS-CoV-2 seeded primary influent during Method 1 averaged 15%, 23%, and 21%, respectively. For Method 2, overall final methodological recoveries of infectious SARS-CoV-2 averaged 18%, while GC levels were recovered at an average of 27% for N1 and 32% for N2. Additionally, the overall concentration factor (beginning sample volume through final RNA extract volume) for Method 1 was 10000:1, while for Method 2 it was 1000:1.

3.7. Endogenous SARS-CoV-2 detection

To compare the performance of Method 1 (D-HFUF and CP Select®) to that of Method 2 (CP Select®) in a real-world wastewater surveillance effort (targeting endogenous levels of SARS-CoV-2), unamended wastewater samples were concentrated using each method and viral concentrations recovered were then compared. Wastewater sampling occurred during two different timepoints to evaluate each concentration method for their ability to detect fluctuating levels of endogenous SARS-CoV-2. For the first sampling timepoint, where community infection rates were lower (12.1 confirmed COVID-19 cases within sewershed), Method 1 detected 2.94 ± 0.08 and 2.64 ± 0.14 log_{10} GC/100 mL for N1 and N2 RT-qPCR signals, respectively. By contrast, either no N1 and N2 RT-qPCR signals were detected or the concentrations fell below assay LLOQ when Method 2 was used. Infectious endogenous SARS-CoV-2 viruses were also not detected (analytical volumes equal to 500 mL for Method 1 and 50 mL for Method 2) in any of the unamended wastewater samples tested.

4. Discussion

Wastewater surveillance has shown to be a useful means to track SARS-CoV-2 RNA prevalence within a given community. However, as reported in a recent review article, methodological improvements are needed to minimize false-positive and false-negative errors to improve detection of low-level SARS-CoV-2 signal in wastewater (Ahmed et al., 2022). Specifically, the establishment of targeted volume criteria for wastewater monitoring to provide uniform data collection requirements is needed to ease information amalgamation between laboratories (Ahmed et al., 2020a; Gonzalez et al., 2020). To date, many existing studies monitoring wastewater for SARS-CoV-2 have used small grab samples to link wastewater virus concentrations to community level incidences of disease (Ahmed et al., 2020a; Ahmed et al., 2020d; Ahmed et al., 2021b; Randazzo et al., 2020; Venugopal et al., 2020). In some cases, virus concentrations were drawn from data at or nearing the detection limit of analytical techniques (i.e., RT-qPCR, droplet digital PCR [ddPCR]), limiting the predictive capabilities of the approach when community infection rates are low during early phases of the outbreak (Bivins et al., 2021; Ciesielski et al., 2021). To date, improved methodologies capable of providing increased sensitivities for SARS-CoV-2 detection, like analyzing larger volumes of wastewater, are limited, and still needed. Increased sample volume could improve assay sensitivities to more accurately estimate active infection rates (both symptomatic and asymptomatic), especially during early phases of disease outbreaks within a community (Gerrity et al., 2021; Kitajima et al., 2020). This is especially true for wastewater monitoring efforts in cases

Table 1

| Sample Time Point | Infection Rate\(^a\) | Method          | Target | Replicate | Concentration\(^c\) | Average ± Standard Deviation |
|-------------------|-------------------|----------------|--------|-----------|------------------|-----------------------------|
| 1                 | 18.9              | 1              | N1     | 1         | 3.33             | 3.29 ± 0.08                 |
|                   |                   |                | N2     | 1         | 2.93             | 2.90 ± 0.04                 |
|                   |                   |                |        | 2         | 2.86             |                            |
|                   |                   |                |        | 3         | 2.92             |                            |
| 2                 | 12.1              | 1              | N1     | 1         | 2.24             | 2.29 ± 0.08                 |
|                   |                   |                | N2     | 1         | 2.38             |                            |
|                   |                   |                |        | 2         | 2.26             |                            |
|                   |                   |                |        | 3         | 2.07             |                            |
|                   |                   |                |        | 3         | 2.25             |                            |
|                   |                   |                |        | 3         | 2.25             |                            |
|                   |                   |                |        | 3         | 2.25             |                            |
|                   |                   |                | N2     | 1         | 2.65             | 2.94 ± 0.08                 |
|                   |                   |                |        | 2         | 3.01             |                            |
|                   |                   |                |        | 3         | 2.95             |                            |
|                   |                   |                | N2     | 1         | 2.74             | 2.64 ± 0.14                 |
|                   |                   |                |        | 2         | 2.71             |                            |
|                   |                   |                |        | 3         | 2.48             |                            |
|                   |                   |                | N2     | 1         | Below LLOQ\(^d\) | ND\(^d\)                    |
|                   |                   |                | N2     | 2         | Below LLOQ       | ND\(^d\)                    |
|                   |                   |                | N2     | 3         | Below LLOQ       | ND\(^d\)                    |

\(^a\) Confirmed COVID-19 cases within sewershed  
\(^b\) Concentration reported as log_{10} GC per 100 mL  
\(^c\) Lower limit of quantification (LLOQ)  
\(^d\) Non-detect (ND) due to unquantifiable levels of RT-qPCR product
where viral variants and sub-variants (e.g., delta and omicron) are emerging, a critical timepoint where implementing mitigation efforts will be most effective. However, careful considerations such as choosing the appropriate method, and appropriate target volume assessments must be considered prior to sample analysis. Additionally, a concentration method must be validated to establish method performance since sensitivities to concentration processes can vary for each target pathogen.

In this study we evaluated two methods that have the potential to provide increased detection sensitivities that could be beneficial for monitoring disease incidence, especially during early phases of an outbreak. Prior to experimentation, virus stock titers revealed a nearly 4 log_{10} difference in concentration of infectious SARS-CoV-2 compared with that of the N1 or N2 RT-qPCR molecular signals, a trend that continued during all steps of the method comparison experimentation. Since RT-qPCR can detect both infectious and non-infectious virions, these results are not surprising and have been observed elsewhere (Berg et al., 2021; La Scola et al., 2020; Mak et al., 2020). One of the potential explanations for the 4-log_{10} difference in concentration between infectious SARS-CoV-2 and N1/N2 gene targets is the ability of the N1 and N2 primer sets to detect SARS-CoV-2 genomic and sub-genomic RNA (sgRNA) found in infectious and non-infectious particles (Lu et al., 2020). These RT-qPCR assays amplify two regions of the nucleocapsid gene, which are part of the viral genome that generates the most abundant sub-genomic RNA species during coronavirus replication and represents all the expressed sgRNAs by the virus (Kim et al., 2020), thus resulting in much higher sensitivities. Sender et al. also reported much higher molecular signals detected when RT-qPCR targets genomic and sub-genomic RNA as compared to theoretical total virus particles present in host cells (Sender et al., 2021).

Since wastewater is not typically used to track respiratory enveloped viruses and their interactions within and sensitivities to this matrix are not well established, we investigated virus losses upon addition of SARS-CoV-2 to autoclaved primary influent. We observed considerable SARS-CoV-2 seed losses (ranging from 66% to 81%) for both infectious and molecular signal. Our results support the notion that viral adsorption to wastewater particulates is likely occurring, as indicated by the initial reduction of culturable and N1/N2 RT-qPCR signals. This finding highlights important considerations when extrapolating SARS-CoV-2 wastewater-based monitoring data with active patient infection rates, where virus signal can be severely compromised within minutes of exposure to wastewater matrices (Berg et al., 2021; Noorimotlagh et al., 2021; Tiwari et al., 2021).

Size exclusion technologies are well established concentration methodologies that have the added advantage of more readily retaining microbial target infectious status as opposed to other similarly purposeful filtration methodologies (Ahmed et al., 2020c; McMinn et al., 2016; Nemudryi et al., 2020; Tiwari et al., 2021; Torii, Furumai, and Katayama, 2021). Additionally, size exclusion filtration, in a dead-end configuration, requires minimal expertise to setup and run, readily lending itself to field deployment where processing upwards of 100 L of environmental water at rates of 1 L/min can be achieved (Harmoto et al., 2018; McMinn et al., 2017; McMinn et al., 2021; McMinn et al., 2018). A critical step in establishing performance metrics of an effective concentration methodology is identifying sensitivities for a given viral target. This is certainly the case with SARS-CoV-2 (enveloped respiratory virus) since this virus is structurally different from enteric viral strains (non-enveloped) for which the majority of current environmental methods were developed (Franey et al., 2013). During Method 1, significant loss of SARS-CoV-2 infectious signal was observed compared to that of the N1 and N2 RT-qPCR signals during D-HUFU concentration. Infectious virus signal sensitivities during this processing step could be due to virions being irreversibly bound to particulates within the sample, damaged during filtration/elution processes, or sensitivity to the mild surfactant used to wash filter surfaces (Katz et al., 2018; Liu et al., 2012; Morales-Morales et al., 2003). Both the D-HUFU and CP select™ have been demonstrated to be effective concentration methods for SARS-CoV-2 and its surrogates from wastewater for detection using molecular methods (Ahmed et al., 2021a; McMinn et al., 2021). Unfortunately, as the proprietary foam-based elution options supplied by InnovaPrep are not designed for recovery of infectious virus; modifications to the elution procedure using a syringe-driven elution with the D-HUFU elution solution were developed (McMinn et al., 2021). This elution solution has been previously used to successfully recover both infectious and molecular viral targets (coliphage, beta-coronavirus OC43, enteric viruses, viral microbial source tracking (MST) markers), in a variety of matrices (Korajkic et al., 2022; Korajkic et al., 2021; McMinn et al., 2021), but its use to successfully isolate infectious SARS-CoV-2 (or other enveloped viruses) has not been demonstrated to date. In this study, infectious SARS-CoV-2 was successfully recovered using this modified elution procedure in conjunction with the D-HUFU elution solution, suggesting that this method can be used to isolate infectious viral particles, although further method refinement is needed.

Prior to CP Select™ concentration for both Method 1 and Method 2, particulate removal using centrifugation is recommended in InnovaPrep’s CP Select™ Wastewater Analysis Protocol to avoid filter fouling (InnovaPrep, 2021). However, viral losses determined through removal of particulates during centrifugation were observed and significantly impacted the SARS-CoV-2 RT-qPCR signals when compared to that of infectious signal, but only following D-HUFU concentration (Method 1). Reduced viral loss in filter eluates (Method 1) could be attributed to concentrates being suspended in an elution solution, which by design, limit virus attachment to particulates and filter surfaces through use of mild surfactants (Hill et al., 2005). Wastewater monitoring efforts have identified that significant levels of SARS-CoV-2 are recovered from particulates separated during sample processing, highlighting the importance of processing both liquid and solid fractions of wastewater to assure optimal SARS-CoV-2 signal detection (Casanova et al., 2009; Parkas et al., 2018; Graham et al., 2021; Mackowiak et al., 2018). There are numerous options available for processing viral nucleic acids from solids, but unfortunately, obtaining infectious virus signal from solids still proves difficult since processes used to release attached viruses can inadvertently result in virus inactivation (Parkas et al., 2017; Mackowiak et al., 2018). Nevertheless, the methods used in the current study were successful in recovery of seeded infectious SARS-CoV-2 virus from wastewater. Optimization of an effective SARS-CoV-2 wastewater monitoring method will need to address issues with processing particulates within samples, as they can be a significant source of the overall viral signal as well as effectively recovering infectious viruses from this matrix. Fortunately, a solids analysis (processing of retained solids post centrifugation) could be easily incorporated into each of the two concentration methods described herein.

To assess the performance of the two concentration methods under real-world wastewater surveillance scenarios, we processed unamended primary influent samples to quantify levels of endogenous SARS-CoV-2. During the first sampling event coinciding with the higher incidence of COVID-19 positive cases in the sewershed, Method 1 detected significantly higher concentrations of both N1 and N2 RT-qPCR signals, compared to Method 2. During the second sampling event with lower confirmed COVID-19 positive cases, N1 and N2 RT-qPCR signals were also detected using Method 1, but they were below the assay LLOQ for Method 2. Concentration patterns of SARS-CoV-2 detected in the tested wastewater samples paralleled the numbers of confirmed COVID-19 cases during each of the two timepoints sampled. However, extrapolation of these findings for public health relevance is difficult, as many wastewater surveillance efforts to date have attributed fluctuations in wastewater signal to patient shedding patterns, wastewater flow, or the absence of mild or asymptomatic carriers in reported public health data (Ahmed et al., 2021b; He et al., 2020; Li et al., 2021; Pan et al., 2020; Randazzo et al., 2020). While additional field studies are needed to generate similar comparative data, our results suggest sample volume could be a critical component for obtaining endogenous virus signal.
during wastewater surveillance and for enhancing our ability to assess low community levels of infection more accurately.

Similar to a few existing examples in the literature (Rimoldi et al., 2020; Westhaus et al., 2021), no endogenous infectious SARS-CoV-2 was detected in the wastewater samples collected for this study. These results indicate that SARS-CoV-2 is either quickly inactivated in wastewater, irreversibly adheres to wastewater particulates, or as recent studies have suggested, infected individuals do not excrete detectable infectious virus in their stool (I.S., 2001; Ahmed et al., 2020b; Albert et al., 2021; Carducci et al., 2020; Cerrada-Romo et al., 2022; Tiwari et al., 2021). Taken together, these findings suggest the need to further understand the feasibility and importance of monitoring infectious SARS-CoV-2 in environmental matrices (e.g., wastewater).

5. Conclusion

Useful insights on SARS-CoV-2 presence in populations have been collected from current wastewater monitoring efforts, but inherent limitations associated with small-volume sample analysis make it difficult to obtain data pertinent for assessing disease burden within a given community. Implementing a method capable of processing larger wastewater volumes has the potential to concentrate virus signal 20-fold over small volume methodologies. Large volume methods will be key in characterizing viral stability in environmental systems, assessing the risks of secondary exposure through contaminated waters, and ensuring current wastewater treatment processes are adequate for viral removal/inactivation. It is hoped that efforts such as those described herein will lead to the eventual identification and establishment of standardized wastewater methods allowing for more meaningful data comparisons generated from multiple geographically dispersed sewersheds. Large volume methods could enhance the generation of data during instances of low community infection (e.g., corresponding to early onset of the disease outbreak), aiding in better estimating viral prevalence, tracking viral diversity, and recognizing geographic hotspots for implementation of disease abatement efforts.

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Declaration of Competing Interest

The 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.

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