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Development of a large volume concentration method for recovery of coronavirus from wastewater

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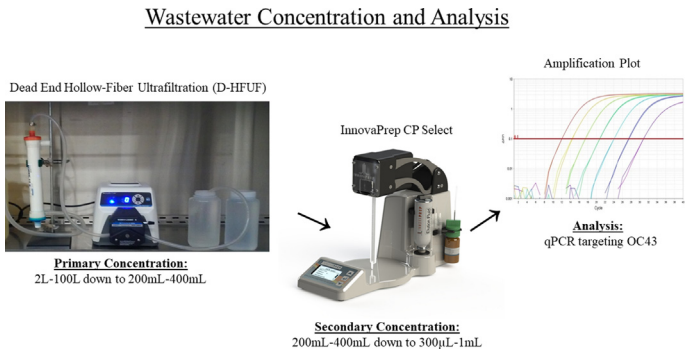
HIGHLIGHTS

- Current SARS-CoV-2 methods lack the ability to process large volumes of wastewater.
- A twostep concentration procedure was developed to process large wastewater volumes.
- D-HFUF was used for primary concentration and the CP Select™ was used for secondary.
- D-HFUF recovered 69 ± 18% of OC43 spike, the CP Select™ recovered 48 ± 2%.
- This method is capable of concentrating 8× the volumes of similar techniques.

GRAPHICAL ABSTRACT



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ABSTRACT

Levels of severe acute respiratory coronavirus type 2 (SARS CoV 2) RNA in wastewater could act as an effective means to monitor coronavirus disease 2019 (COVID-19) within communities. However, current methods used to detect SARS CoV 2 RNA in wastewater are limited in their ability to process sufficient volumes of source material, inhibiting our ability to assess viral load. Typically, viruses are concentrated from large liquid volumes using two stage concentration, primary and secondary. Here, we evaluated a dead-end hollow fiber ultrafilter (D-HFUF) for primary concentration, followed by the CP Select™ for secondary concentration from 2 L volumes of primary treated wastewater. Various amendments to each concentration procedure were investigated to optimally recover seeded OC43 (betacoronavirus) from wastewater. During primary concentration, the D-HFUF recovered 69 ± 18% ($n = 29$) of spiked OC43 from 2 L of wastewater. For secondary concentration, the CP Select™ system using the Wastewater Application settings was capable of processing 100 mL volumes of primary filter eluates in <25 min. A hand-driven syringe elution proved to be significantly superior ($p = 0.0299$) to the CP Select™ elution for recovering OC43 from filter eluates, 48 ± 2% compared to 31 ± 3%, respectively. For the complete method (primary and secondary concentration combined), the D-HFUF and CP select/syringe elution achieved overall 22 ± 4% recovery of spiked OC43 through ($n = 8$) replicate filters. Given the lack of available standardized methodology confounded by the inherent limitations of relying on viral RNA for wastewater surveillance of SARS CoV 2, it is important to acknowledge these challenges when interpreting this data to estimate community infection rates. However, the development of methods that can substantially increase sample

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volumes will likely allow for reporting of quantifiable viral data for wastewater surveillance, equipping public health officials with information necessary to better estimate community infection rates.

1. Introduction

Severe acute respiratory coronavirus type 2 (SARS CoV 2) is the etiological agent responsible for the current pandemic of severe pneumonia sweeping the globe (COVID-19). Symptoms of this disease include fever, cough, muscle ache, difficulties in breathing and diarrhea (Lin et al., 2020). Evidence suggests that SARS CoV 2 can infect various tissues, specifically those expressing the angiotensin-converting enzyme (ACE2) protein, which includes the gastrointestinal lining (Mohapatra et al., 2020). Analysis of stool from both symptomatic and asymptomatic patients shows high prevalence of viral shedding (30–90% of infected individuals) accompanied by high individual viral shed rates (up to 7.5 $\log_{10}$ per gram of feces) (Zhang et al., 2020; Wolfel et al., 2020). Evidence of high viral loads in stool substantiates the use of wastewater as a potential mechanism to monitor population infection rates. These data could then be applied to wastewater derived population assessments, possibly acting as an early warning tool to better assess, predict and contain viral outbreaks at the community level (Gertjan Medema et al., 2020).

Human pathogenic viruses in wastewater are typically found in low concentrations, necessitating the need to concentrate large volumes (>1 L) of wastewater to accurately quantify virus signal (Wyn-Jones et al., 2011; Jiang et al., 2001; Aslan et al., 2011; Sherchan et al., 2020; Cuevas et al., 2021). To date, limited existing studies have relied on concentrating wastewater volumes of <500 mL, generally resulting in samples at or below assay detection limits which precludes viral quantification; results were generally reported as presence/absence (Gertjan Medema et al., 2020; Nemudryi et al., 2020). In addition, new methodologies need to be developed to monitor SARS CoV 2 in wastewater, as existing virus concentration methodologies were designed for enteric, non-enveloped viruses whose structure is significantly different compared to that of enveloped respiratory viruses such as SARS CoV 2 (Haramoto et al., 2009; Ye et al., 2016). The lack of an appropriate methodology for SARS CoV 2 detection in wastewater has been identified as an existing major scientific research gap in this field (Kitajima et al., 2020; Ahmed et al., 2020). The inability to accurately quantify the virus in wastewater and other matrices inherently hinders the ability to track SARS CoV 2, evaluate its concentrations, and determine the efficacy of wastewater treatment removal processes.

Unfortunately, there are challenges to multi-tiered viral concentration approaches; 1) virus signal can be lost during each step of concentration and 2) some of these approaches can significantly increase the sample processing time (polyethylene glycol precipitation up to 24 to 48 h). Typically, large-scale virus concentration methods employ two stages of concentration; primary concentration (liters to milliliters) and secondary concentration (milliliters to microliters). Various primary concentration methods have been developed to concentrate enteric viruses (Norovirus, Adenovirus and Enterovirus) from wastewater based on either electrostatic interactions (electropositive and electronegative filtration) between viral capsids and filter surfaces or by size exclusion based separation technologies such as ultracentrifugation and ultrafiltration (UF) (McMinn et al., 2018; McMinn et al., 2017; Lodder and de Roda Husman, 2005; Thongprachum et al., 2018; Ikner et al., 2012). Secondary concentration methods, such as flocculation-precipitation, membrane filtration, or centrifugal ultrafiltration and ultrafiltration, typically rely on particle separation through precipitation or size exclusion (Ye et al., 2016; Ahmed et al., 2020; Wang et al., 2005; Randazzo et al., 2020). These methods rely on the manipulation of viral capsid isoelectric points (use of reagents or strongly buffered solutions), or on lengthy processing times that could result in inactivation of sensitive infectious virus present. Size exclusion technologies using dead end hollowfiber ultrafiltration (D-HFUF) are an easy to use, rapid means for concentrating viruses from challenging matrices such as wastewater, making them ideal for recovery of infectious viruses present (McMinn et al., 2017; Pallin et al., 1997; Cuevas-Ferrando et al., 2020).

Working directly with samples having the potential of containing virulent SARS CoV 2 can lead to additional laboratory safeguards beyond what are typically found in most laboratories. Namely, the Centers for Disease Control and Prevention (CDC) recommends work involving concentration and isolation of SARS CoV 2 in wastewater to be done in a Biosafety Level 2 (BSL-2) laboratory setting under BSL-3 precautions (CDC, 2020). To avoid the complications associated with adhering to these requirements, many researchers have instead pasteurized samples containing putative SARS CoV 2 prior to sample manipulation, and/or utilized surrogate viruses that closely resemble SARS CoV 2 (Kitajima et al., 2020; Ahmed et al., 2020). For this study, betacoronavirus strain OC43 was chosen based on its close lineage (Boni et al., 2020) to SARS-CoV, SARS CoV 2 and middle east respiratory syndrome coronavirus (MERS-CoV), while requiring only BSL-2 precautions for its use in laboratory settings. Additionally, strain OC43 is recognized as an acceptable surrogate for SARS CoV 2 according to the American Society for Testing and Materials (ASTM) standards (ASTM, 2020).

In this study, we developed a large volume concentration method that can be applied for use to detect coronaviruses, including SARS CoV 2 in wastewater. To achieve this, we instituted an experimental approach that is divided into different research focuses: 1) determine stability of SARS CoV 2 betacoronavirus surrogate OC43 to a range of laboratory solutions to identify optimal handling and storage conditions, 2) evaluate various primary concentration conditions using D-HFUF technologies for improved coronavirus recovery and 3) identify optimal secondary concentration procedures employing size exclusion that allow for volume reductions suitable for molecular analysis.

2. Materials and methods

2.1. OC43 stock preparation

Human betacoronavirus OC43 (#VR-1558) was acquired from American Type Culture Collection (ATCC), (Gaithersburg, Maryland). Prior to experimentation, vials of OC43 were thawed and diluted in sterile 1XPBS (0.137 M NaCl, 0.0027 M KCl, and 0.0119 M phosphate, P3813, Sigma Aldrich, St. Louis, MO) to a 1×10^5 genomic copies/mL final concentration and were stored at −80 °C until use. The same stock of OC43 was used for all experiments described below.

2.2. OC43 RNA stability testing

Enveloped viruses including coronaviruses have been identified as being sensitive to degradative forces such as temperature, ultraviolet light and specimen/solution chemical components (Duan et al., 2003; Sands et al., 1979). With these findings in mind, it was important to assess the stability of OC43 RNA in the presence of solutions typically used for virus manipulations in laboratory settings prior to beginning spiking and recovery experimentation. In addition to solution stability, the viral RNA was evaluated for its ability to withstand numerous freeze thaw cycles in each solution type. For this experiment, 100 mL volumes of seven

solutions were prepared as follows (powdered forms were re-suspended in sterile deionized water and filter sterilized through a 0.2 μm filter); 1) 1XPBS, 2) elution solution (0.01% Tween 80, 0.01% sodium hexametaphosphate, 0.001% Antifoam Y-20) (Sigma Aldrich) 3) Tryptic Soy Broth (TSB) (BD 211823, Fisher Scientific, Waltham, MA), 4) Dulbeco's Minimal Essential Media (DMEM) (Gibco 11,965,092, Fisher Scientific), 5) 25 mM Tris (T1378, Sigma Aldrich), 6) 5% Bovine Serum Albumin (BSA) DNase and protease-free powder (BP9706100, Fisher Scientific) and 7) 2.5 mM $MgCl_2$ (M8266, Sigma Aldrich). Each solution was aliquoted into three replicate volumes of 10 mL into sterile 15 mL polypropylene tubes (12-565-268, Fisher Scientific). Equal measured amounts (4.58 ± 0.01 log_{10} genomic copies/mL) of a diluted OC43 stock (1:10 dilution in 1XPBS) were added to each tube. Tubes were vortexed for 30 s and incubated at room temperature for 1 h prior to taking the first, time zero aliquot (300 μL) to be analyzed by RT-qPCR for OC43 concentrations. Tubes were then transferred to a −80 °C freezer for one week. Following storage at −80 °C for one week (sample Week 1) each virus seeded solution replicate ($n = 3$) was thawed at room temperature, subsampled, and placed back into a −80 °C freezer. This process was repeated each week for 2 additional weeks (samples Week 2 and Week 3).

2.3. Wastewater collection and treatment

Using a sterile 20 L carboy, a total of 10 primary treated wastewater samples, following primary wastewater settling and screening (which are free of large debris that could impede sample collection and processing), were collected weekly (from July through October of 2020). This wastewater treatment plant (WWTP) is located within the urban metropolitan sewerage district of Cincinnati, Ohio and processes between 55 and 96 million gallons (MGD) of waste per day. Wastewater samples were transported immediately (at room temperature) to Environmental Protection Agency (EPA) laboratories located in Cincinnati, Ohio for processing. Upon arrival at the laboratory, wastewater was autoclaved for 60 min at 121 °C to disinfect any SARS CoV 2 present, allowing subsequent testing to occur within the Biosafety Level 2 (BSL-2) equipped laboratories, conforming to CDC guidelines (CDC, 2020). Autoclaving wastewater was previously shown not to affect concentration procedures used for enveloped viruses compared to un-autoclaved wastewater (Ahmed et al., 2020). Wastewater was then allowed to cool overnight at 4 °C prior to experimentation the following day.

2.4. Primary concentration

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 aliquot, 1 mL of a 1:10 dilution of the OC43 stock preparation (described in Materials and Methods section 2.1) was added and allowed to mix for 3 min at room temperature. For dead-end hollow-fiber ultrafiltration (D-HFUF) concentration, 2 L volumes of wastewater were filtered through 15S Asahi Kasei Rexeed ultrafilters (Dial Medical Supply, Chester Springs, PA) using a peristaltic pump (Masterflex L/S Easy Load, Cole Parmer, Vernon Hills, IL) set at 300 rpm. For the standard protocol, filters were eluted by passing 200 mL of elution solution (0.01% Tween 80, 0.01% sodium hexametaphosphate, 0.001% Antifoam Y-20) in a clockwise, then counter-clockwise, and finally clockwise direction for 1 min each. The resulting filter eluate was either subsampled (300 μL to determine OC43 recoveries through primary concentration) or processed through secondary concentration. During optimization experimentation, the D-HFUF elution procedures were modified in separate attempts to improve recoveries of spiked OC43, as follows: 1) D-HFUF elution pump speeds were increased from 300 rpm to 500 rpm, 2) HFUF elution cycle times were increased from 1 min to 2 min for each direction, 3) Tween 80 concentrations were adjusted from 0.01% to 0.02%, 4) Sodium hexametaphosphate concentrations were adjusted from 0.01% to 0.02%.

For each wastewater concentration experiment, a blank (no virus spike) 2 L aliquot of autoclaved wastewater were run alongside spiked 2 L wastewater samples to determine if any background endogenous OC43 were detectable. There were no instances of background levels of OC43 in any wastewater blanks during testing.

2.5. Secondary concentration

Following sample filtration and primary filter elution, filter eluate volumes were recorded (eluate volume range: 155–207 mL) and eluates were evenly split, half of the eluate was centrifuged (12,000 ×g for 30 min) to remove particulates prior to secondary concentration. Five different Methods (Table 1) were investigated for using the Concentrating Pipette Select™ (CP Select™) (InnovaPrep, Drexel, MO) to concentrate 100 mL volumes of primary filter eluate (typically taking under 30 min), reducing volumes to those more applicable to molecular analyses (approx. 1 mL). Using the CP Select™ Wastewater Application settings (Table 1) provided by InnovaPrep, primary filter eluates were passed through the concentrating pipette tip (CPT) ultrafilter (Cat. #CC08003) and upon sample filtration, ultrafilter tips were eluted (between 1 and 3 times) using either of two CP elution fluid cans, (Method 1A) Tris buffer (Cat. #HC08001) containing 0.075% Tween 20 and 25 mM Tris or (Method 1B) Dulbecco's Modified Eagle Medium (DMEM)/N20 (custom order) (Table 1). CP Select™ eluates were collected in sterile 15 mL polypropylene tubes and their volumes measured and recorded. Amendments to the CP Select™ Wastewater Application were attempted to enhance recovery of OC43 through adjustments to valve open timing (Method 1C), from 800 ms to 999 ms, foam factor (Method 1D) from 10 to 50 and pulse settings (Method 1E) from 1 to 4. In addition, a hand-driven syringe elution procedure was explored to potentially improve OC43 recovery. For the syringe driven CP pipette elution, a 10 mL syringe with tip cap #305219 (BD Medical Systems, Franklin Lakes, NJ) was connected by a 3-in. section of Clearflex 70–1 PVC tubing (Canandigua, NY), to the elution port on the CPT ultrafilter following sample filtration. The syringe was loaded with 1 mL of elution solution (0.01% Tween 80, 0.01% sodium hexametaphosphate, 0.001% Antifoam Y-20), and this solution was forcibly pushed through the CPT ultrafilter. In some instances, the eluate was recirculated by re-passaging through the syringe filter (2–3 times) and/or allowing the elution solution to soak (2 min) the filter (Methods 2B-2E) (Table 1). An improved UF scouring-like effect was noted for the syringe filter driven elution as compared to the CP Select™ procedure, whereas sample turbidity was much greater using the syringe filter elution in comparison to the CP Select™ procedure.

Table 1
Summary of secondary concentration procedures.

Method	Elution solution	Elution procedure	Pulse	Foam	Valve (ms)	Filter elution	Filter soak (2 min)
1A[a]	Tris[b]	CP	1	10	800	N/A	
1B[a]	DMEM[c]	Select™	1	10	800		
1C	Tris		4	10	800		
1D	Tris		1	10	999		
1E	Tris		1	50	800		
2A	Elution solution[d]	Syringe	N/A			Single	No
2B						Double	No
2C						Double	Yes
2D						Triple	Yes
2E						Triple	No

[a] Baseline settings for Pulse, Foam and Valve (Flow Start: 3 s., Flow End: 10 s., Flow Min Start: 40 s., Ext Delay: 3 s., Pump: 25% and Ext Pump Delay: 1 s.) as part of CP Select™ Wastewater Application settings.
[b] 0.075% tween 20, 25 mM Tris
[c] DMEM/N20 custom order foam elution can.
[d] Elution solution (0.01% Tween 80, 0.01% sodium hexametaphosphate, 0.001% Antifoam Y-20).

2.6. Nucleic acid extractions and RT-qPCR

Viral RNA was extracted from 200 μL portions of each spike and sample concentrate using a Qiagen All Prep PowerViral Kit (Qiagen, Valencia, CA), according to manufacturer's instructions. One extraction blank (EB), including all reagents but no sample template, was included with each extraction batch. Purified RNA extracts were analyzed within 24 h. The N-terminus region of human coronavirus strain OC43 gene targets were selected based on previous research (Yu et al., 2012). Forward primer (OC43-F) GCTCAGGAAGGTCTGCTCC, TaqMan probe (OC43-P) FAM-TTCCAGATCTACTTCGCGCACATCC-TAMRA, and reverse primer (OC43-R) TCCTGCACTAGAGGCTCTGC, were used to detect OC43 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 with each reaction mix consisting of; 1.25 μL enzyme mix, 5 μL of 5× reaction mix, 2.5 μL BSA, 0.05 μL ROX, 3.0 μL RT-PCR master mix (1 μM probe and 80 nM primer), 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: Holding Stage 1: 50.0 °C for 15 min, Holding Stage 2: 95.0 °C for 2 min, Cycling Stage: 95.0 °C for 15 s. then hold at 60.0 °C for 30 s. The RT-qPCR TaqMan assays were performed using a QuantStudio 3 real-time qPCR system and threshold was manually set to 0.03 (Applied Biosystems). Potential inhibition was assessed by performing 10-fold dilutions of each sample in sterile AE buffer (10 mM Tris-Cl; 0.5 mM EDTA; pH 9.0). Six no template controls (NTCs) were included with each instrument run.

2.7. Standard curve preparation

Reference DNA sources for OC43 consisted of a gBLOCK preparation (integrated DNA technologies, Coralville, IA). The gBLOCK construct was quantified using a Qubit fluorometer 3.0 (fisher scientific) diluted in sterile AE buffer to generate 10, 10^2, 10^3, 10^4, 10^5 and 10^6 copies/2 μL for calibration standards. All reference DNA material preparations were aliquoted into single use volumes sufficient to generate a standard curve in GeneMate Slick low-adhesion microcentrifuge tubes (ISC BioExpress, Kaysville, UT) and stored at −20 °C.

2.8. Data statistical analyses

Concentration data (i.e. solution stability trials) were $\log_{10}$ transformed while percent recovery data (all other experiments) were arcsine square root transformed, prior to data 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 modifications to primary concentration procedures, to evaluate the effect of varying solutions on stability of OC43 and to compare percent recoveries of different secondary concentration procedures ($\alpha = 0.05$ for all tests).

3. Results

3.1. RT-qPCR performance metrics

A standard master curve based on six individual standard curves was set up and used for quantification of all samples in the study. No amplification inhibition, based on 10-fold dilutions prepared for each individual sample, was detected (data not shown). Average slope, R^2, y-intercept and amplification values were -3.35 ± 0.03, 0.98 ± 0.002, 35.54 ± 0.17 and 0.99 ± 0.01, respectively. Assay lower limit of quantification (LLOQ) was identified as an average of all 10 copy standards and equaled 32.3 Cq, and all samples analyzed were above the defined LLOQ. A total of 330 individual negative control reactions (combination of EBs and NTCs) were below the LLOQ, indicating absence of extraneous RNA contamination (data not shown).

3.2. Solution stability testing

The RNA stability of OC43 was assessed in seven different solutions and under freeze-thaw cycling over the course of three weeks (Fig. 1). The elution solution was superior over six other solutions, resulting in significantly greater stability of OC43 RNA for the duration of the experiment (*p* value range: 0.0363 to <0.0001), indicating that the surfactants present in this solution had no adverse effects on viral stability. Furthermore, storage in 5% BSA resulted in significantly greater stability of OC43 RNA compared to 25 mM Tris and 2.5 mM $MgCl_2$ (*p* value range: 0.0230–0.0034), suggesting that proteinaceous substances could enhance viral stability under less than optimal storage conditions. Regardless of solution, the majority of viral RNA signal decrease occurred during the initial 1-h incubation at room temperature (time 0), suggesting that exposures to lengthy periods of ambient temperatures should be avoided. Based on these data, manipulations of OC43 (dilutions of stock) were performed in elution solution prior to experimentation.

3.3. Primary concentration

D-HFUF was evaluated for effectiveness in recovery of spiked OC43 from 2 L of autoclaved wastewater. Subsampling of centrifuged

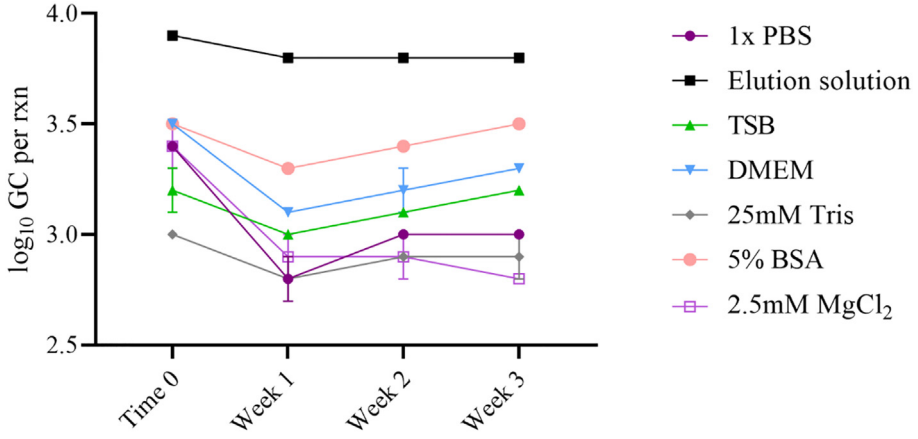

Fig. 1. Stability of OC43 RNA at −80 °C in seven different solutions commonly used for viral storage or as elution solutions (or additives to either solution type). Error bars represent standard deviation ($n = 3$). Time 0 sampling consisted of sampling the OC43 seeded solution following a 1-h incubation at room temperature.

D-HFUF eluates revealed that an average of 114 ± 18% of spiked OC43 was retained following this procedure, suggesting viruses were not attached to particulates following primary filter elution. These findings were also supported by our results from the direct extraction of pelleted material following centrifugation, which revealed low levels of 1.5 ± 1% of OC43 seed present. With this information, centrifugation was used to enhance the ability of the CP Select UF for timelier sample processing. During this study, different aspects of the filter elution procedure (post sample filtration) were modified in attempts to optimize the procedure for OC43. In each instance, the baseline protocol was compared to the modified elution protocols to determine if statistically different results were obtained. Primary filter elution protocol modifications included: increases in filter elution speed, increases in filter elution cycle time and changes to the elution solution ingredients.

Overall recovery of seeded OC43 from the baseline method averaged 69 ± 18% during experimentation. It was observed that adjusting the filter elution speed from 200 rpm to 500 rpm and increasing elution cycle times (from 1 min to 2 min) did not significantly outperform the baseline method (p value range: 0.5402–0.9943). Modifications to the elution solution using 2× the normal concentration of either hexametaphosphate or Tween 80 also did not significantly increase recoveries of OC43 over the baseline method (p value range: 0.3583–0.9640).

3.4. Secondary concentration

Modifications to the secondary concentration technique were evaluated in four trials. For all four trials, autoclaved wastewater was spiked with OC43 followed by primary concentration (as described above) and centrifugation to ensure that the matrix undergoing secondary concentration experiments realistically mimicked primary ultrafiltered concentrates. Methods 1A-1E (Table 1) focused on testing different modification of CP Select™ elution protocols, while Methods 2A–2E explored different syringe-driven elution protocols. Results indicated that a single elution step of the CP Select™ generally yielded <30% recovery, whereas a second elution of the same pipette tip increased the yield by an additional 20%. Based on these data, Methods 1A and 1E were conducted as double combined elutions, and various modifications of the elution protocols were then evaluated. During the same tests, CP Select™ waste was tested and only <1% of OC43 spike was detected, indicating that virus is being trapped effectively by the concentrating pipette tips.

In trial one, no statistically significant difference ($p = 0.2943$) was observed between methods 1A (25 ± 8%) and 1C (36 ± 10%), although both methods performed significantly better than methods 1B, 1D and 1E (p value range: 0.0011 – < 0.0001), each of which yielded less than 8% recovery (Fig. 2). Although method 1C generated slightly higher percent recoveries than method 1A, the final volume produced by method 1C was more than triple that of method 1A (i.e. 1770 μL ± 141 μL versus 334 μL ± 86 μL), precluding downstream analyses of large portions of the sample. Therefore, method 1A was selected as the CP Select™ baseline method and was utilized in the subsequent experiments.

In trial two, performance of method 1A was tested against two variants of syringe elution techniques (Fig. 2). The double-syringe elution (method 2B) significantly outperformed method 1A ($p = 0.0299$) with recoveries of 48 ± 2% and 31 ± 3%, respectively. Performance of method 2A (41 ± 2%) was lower, but not statistically different from method 2B ($p = 0.2115$).

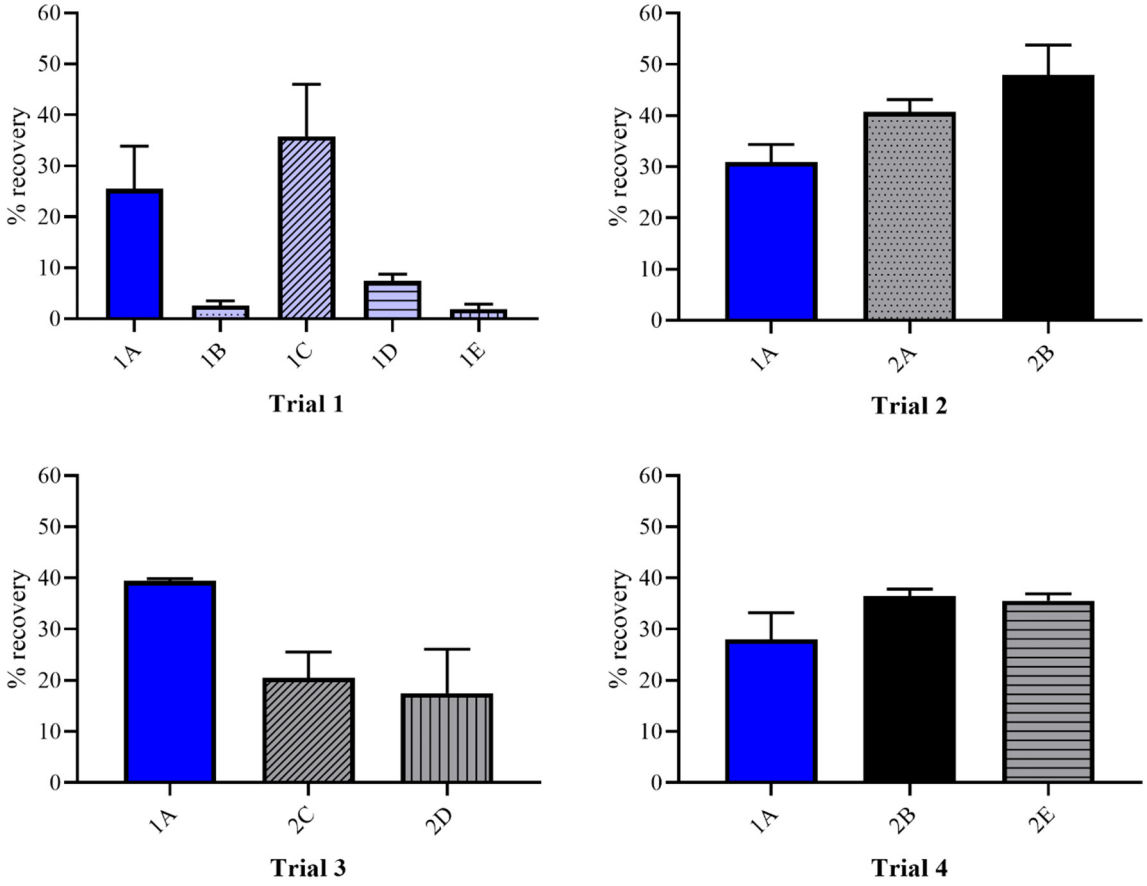

Fig. 2. Comparison of 10 different secondary concentration methods for their ability to recover OC43 RNA. Blue shaded bars (methods 1A-1E) represent different modification of CP Select™ elution protocols (1A-CP Select™ baseline, 1B- DMEM, 1C-Pulse, 1D- Valve, 1E-Foam), while black and gray bars represent different modifications of syringe elution protocols (2A-single syringe, 2B- double syringe, 2C-double syringe soak, 2D-triple syringe soak, 2E-triple syringe). Error bars represent standard deviation (n = 3). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In trial three, additional syringe elution protocols were compared with method 1A to determine whether additional modifications would yield results superior to method 1A (and alternatively method 2B). These modifications involved soaking the concentrating pipette tip for 2 min, followed by double (2C) and triple syringe (2D) elutions. Both methods were inferior to 1A (p value range: 0.0082–0.0173) generating percent recoveries of 20 ± 5% (2C) and 17 ± 9%, (2D), compared to 39 ± 0.4% for 1A, and were excluded from subsequent tests (Fig. 2).

In trial four, additional amendments to the syringe elution were investigated, which included a double syringe (2B) elution and a triple syringe (2E) elution (both lacking filter soaking) in comparison to method 1A (CP Select™ baseline). Recoveries of spiked OC43 using method 2B (36 ± 1%) were found to be significantly higher ($p = 0.0406$) than those using method 1A (28 ± 5%), while there was no statistically significant difference between recoveries of methods 2B and 2E in comparison ($p = 0.9301$).

The average of OC43 recoveries for all 4 trials indicated that method 2B (42±7) significantly outperformed ($p = 0.0075$) method 1A (31 ± 7); therefore, method 2B was selected as the top secondary concentration method and was utilized to evaluate method performance.

3.5. Optimized method performance

The optimized primary concentration method was applied to 2 L volumes of OC43 seeded wastewater in combination with the CP Select™ secondary concentration procedure and syringe driven double filter elution (Method 2B), resulting in an overall average of 22 ± 4% recovery of OC43 through ($n = 8$) replicate filters.

4. Discussion

Accurate means of better assessing population infection rates in real time may be helpful in limiting the spread of SARS CoV 2. The current approach (person to person testing) using a variety of molecular and immunological analyses, has numerous limitations: availability of testing centers, test administered outside of individual's infection peak (Cheng et al., 2004; Hung et al., 2020; Oh et al., 2016), quality of specimen collection/handling (Ek et al., 2019), using an appropriate (costly) analytical test per sample type. These issues can be compounded by limited test capacity, so symptomatic patients, those with questionable travel histories, or those in contact with infected individuals are given testing priority (Babiker et al., 2020). Wastewater testing for SARS CoV 2 has recently been suggested as a more direct approach to assessing community infection rates avoiding many of the above complications (Peccia et al., 2020), although with its own set of technical and logistical challenges. A large proportion of early testing of SARS CoV 2 levels in wastewater have relied on methods that were rapidly developed and generally utilized smaller volumes (50-250 mL) that limit the detection sensitivity. More sensitive methods capable of processing larger volumes of wastewater especially in instances where SARS CoV 2 concentrations are expected to be low (e.g., during the early stages of a community outbreak) are needed for attaining quantifiable virus data.

Enveloped virus stability can be impacted by factors such as temperature, sample biological activity and aqueous media composition (Aquino de Carvalho et al., 2017). While these variables can be controlled in the laboratory to some degree (i.e. sample storage on ice), premature degredation of samples when storing and manipulating viral stocks for spiking experimentation remains a concern. In our study, stability of OC43 coronavirus was assessed in seven different solutions typically used to manipulate the virus or that are used during procedural aspects of numerous viral concentration methodologies. Of these solutions, the elution solution containing trace levels of surfactants was superior for maintaining the molecular signal through multiple freeze-thaw cycles and extended storage at −80 °C (typical virus stock storage temperature). During this experiment it was observed that most viral losses occurred during the initial 1-h incubation at room temperature and that little loss occurred thereafter through cold storage and multiple freeze-thaw cycles, regardless of the solution. Increased temperature has been shown to accelerate the denaturation of viral proteins and nucleic acids through the development of increased extracellular enzyme activity (Sinclair et al., 2012; Brown et al., 2009). Surfactants (similar to the elution solution used in this study) are typically used in virology to de-aggregate viral clumps and to enhance release of viral particles attached to substrates through mechanisms of viral protein coating (Lan et al., 2018; Pisharody and Mukherji, 2021). Tween 80 is a polysorbate surfactant that is commonly used to stabilize aqueous proteins through protein coating processes (Scopes, 1993; Davidson, 2012), which limit protein exposure to autooxidation processes, and reducing the production of hydroperoxides that could degrade exposed proteins (Shi et al., 2005; Lang et al., 2009). This stabilization quality could have played a unique role in delaying the degradation of the molecular signal of OC43 during the room temperature incubations and subsequent freeze-thaw cycling, although further testing will be needed to verify this hypothesis.

For large volumes (>1 L), primary virus concentration size exclusion filtration using HFUF has been used successfully for a multitude of different viruses in potable, reclaimed and environmental waters with recoveries ranging from 2% to 97% (Liu et al., 2012; Mull and Hill, 2012; Ikner et al., 2011; Kahler et al., 2015; Alavandi et al., 2015; Gerrity et al., 2021). To our knowledge, this is the first application of the D-HFUF system for concentration of enveloped viruses from large volumes of primary treated wastewater. Data on enveloped virus recovery from large volumes of wastewater is absent because they are not typically monitored in wastewater or wastewater impacted waterways (Kitajima et al., 2020). Following primary concentration of 2 L volumes of virus seeded wastewater, subsampling of filter eluates post centrifugation revealed that the vast majority of virus seed was suspended in solution. This finding contradicts those of others, where pelleted material was found to contain significant levels of virus (Ye et al., 2016). Our concentration approach, however, was different since primary treated wastewater was initially processed through primary concentration, then eluted using a surfactant solution. The surfactants present possibly released attached virus from particulates prior to centrifugation and pellet analysis. The D-HFUF system successfully recovered 69 ± 18% of spiked OC43 coronavirus from 2 L volumes of primary treated wastewater; these recovery values are comparable to those observed for enteric viruses in more purified environmental matrices. These findings indicate the efficacy of the D-HFUF system for targeting coronaviruses in large volumes of wastewater.

Molecular assays are often used to monitor enteric pathogens due to difficulties in isolating and culturing them directly, but for large volume testing an additional concentration process is required to reduce the volume from the initial concentration process to volumes applicable to nucleic acid detection (≤1 mL). Numerous small-volume procedures exist for this purpose, including polyethylene glycol (PEG) precipitation, absorption-flocculation (charged membrane, Al $(OH)_3$ and $MgCl_2$), and centrifugal ultrafiltration columns. With each method listed, limitations such as lengthy incubation times (>24 h), filter fouling with turbid samples, and the use of strong acids or bases make many of these methods unsuitable for concentrating enveloped viruses in an efficient manner. Recently, the CP Select™ system was introduced to overcome some of these limitations. In this study, 31 ± 3% of spiked OC43 was recovered using the manufacturer's baseline protocol, and optimization using syringe driven elution resulted in recoveries as high as 48 ± 2%. These recovery efficiencies are similar or superior to those reported for enveloped viruses using PEG/$Al(OH)_3$, (range: 11–50%), centrifugal ultrafiltration (range: 11–56%), membrane filtration (range: 27–66%) and ultracentrifugation (range: 1–34%) (Ye et al., 2016; Kitajima et al., 2020; Ahmed et al., 2020; Wang et al., 2005; Randazzo et al., 2020; Heijnen and Medema, 2011; Torii et al., 2020; Jafferali et al., 2020; La Rosa et al., 2021) (Table 2). Although certain caveats exist for the CP Select™ system (e.g., it is expensive and could require multiple units to process large numbers of samples), the main advantage of the system is the speed of

Table 2
Previously reported percent recovery values of enveloped viruses from wastewater compared to this study.

Virus	Percent recovery[a]	Volume (mL)	Method	Reference
φ6	50	40	PEG + TRIzol	(Torii et al., 2020)
Bovine Coronavirus (BCoV)	23	50	Double ultrafiltration with Sartorius centrifugal ultrafilters	(Jafferali et al., 2020)
Bovine Respiratory Syncytial Virus (BRSV)	8	125	InnovaPrep CP Select™	(Gonzalez et al., 2020)
Human Coronavirus (HCoV)	2	250	Two phase PEG/Dextran separation	(La Rosa et al., 2021)
Mouse Hepatitis Virus (MHV)	66	50	Absorption/extraction with $MgCl_2$	(Ahmed et al., 2020)
MHV	15	250	Ultrafiltration (Centricon 10kDA)	(Ye et al., 2016)
φ6	18			
Porcine Epidemic Diarrhea Virus (PEDV)	11	200	Aluminum hydroxide adsorption-precipitation	(Randazzo et al., 2020)
SARS CoV	21	100	Particle adsorption-elution and PEG	(Wang et al., 2005)
BCoV	2	10,000	Dead end hollow fiber ultrafiltration/ Centricon 30 kDa or 100 kDa	(Gerrity et al., 2021)
Betacoronavirus (OC43)	22	2000	Dead end hollow fiber ultrafiltration/ InnovaPrep CP Select™	This study

[a] For comparison purposes with the present study only highest (i.e. optimal) reported percent recoveries are shown.

sample processing (approx. 24 min./100 mL), ease of use, and robustness when handling turbid samples. However, results suggest that CPT filter elution could be enhanced, as evidenced by increased recovery after a syringe mediated elution step leading to increased recoveries of OC43.

The current reported recovery rates for enveloped viruses from raw and/or primary treated wastewater range from 2% for Human Coronavirus 229E (HCoV) to 66% for Murine Hepatitis Virus (MHV) and are based on volumes ranging from 40 to 250 mL (Table 2). The percent recovery for the combined method based on 2 L volume of primary treated wastewater we report in this study (22 ± 4%) falls in the range of previously reported values. While this may not appear to be a meaningful improvement over existing protocols, the ability to increase the sample volume tested combined with an adequate percent recovery effectively elevates the viral signal quantity by an order of magnitude above other methodologies. Considering the effective volumes processed per RT-qPCR reaction, the highest percent recovery method (Ahmed et al., 2020) assayed 2.5 mL of original sample, whereas our method assayed 4.0 mL of original sample. A simple adjustment to increase the volume of sample template during RT-qPCR from 2 μL (our study) to 5 μL would increase assay volumes to 10 mL of original sample per reaction. The use of larger volumes (2 L) will not only increase the sensitivity of detection but also result in more robust detection levels and more accurate assessments of viral load. This increased sensitivity could make the difference between reporting based on presence/absence versus reporting quantifiable data during wastewater surveillance. Most importantly, it could allow public health authorities to identify the occurrence of SARS CoV 2 in wastewater earlier, providing more time for mitigation and potentially avoiding a large-scale outbreak.

Here we describe the first application of D-HFUF for concentrating an enveloped virus from large volumes of wastewater, coupled with a secondary concentration procedure using a rapid ultrafiltration technology (<25 min) to reduce the elution volume for improved molecular characterization of a viral target. Our results provide support for using large volume wastewater concentration to enhance SARS CoV 2 surveillance, thus providing improved data sets needed by epidemiologist and other health scientists to better diagnose and mitigate community infection rates and viral spread. However, it is important to note that application of virus concentration methodologies to raw or primary treated wastewater remains a challenge, in part, due to the complexity and variability of wastewater. This study was conducted using autoclaved wastewater collected from a single facility over a short period of time using an exogenous coronavirus strain. Additional research is warranted to confirm findings using unamended wastewater samples collected from a larger number of facilities targeting endogenous coronaviruses.

5. Disclaimer

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CRediT authorship contribution statement

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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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