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Comparison of Electronegative Filtration to Magnetic Bead-Based Concentration and V2G-qPCR to RT-qPCR for Quantifying Viral SARS-CoV-2 RNA from Wastewater

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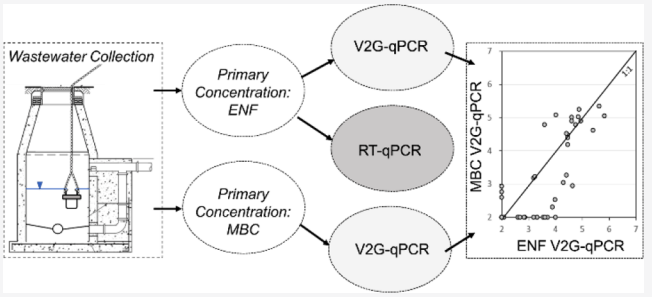
ABSTRACT: Methods of wastewater concentration (electronegative filtration (ENF) versus magnetic bead-based concentration (MBC)) were compared for the analysis of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), beta-2 microglobulin, and human coronavirus OC43. Using ENF as the concentration method, two quantitative polymerase chain reaction (qPCR) analytical methods were also compared: volcano second generation (V2G)-qPCR and reverse transcriptase (RT)-qPCR measuring three different targets of the virus responsible for the COVID-19 illness (N1, modified N3, and ORF1ab). Correlations between concentration methods were strong and statistically significant for SARS-CoV-2 ($r = 0.77$, $p < 0.001$) and B2M ($r = 0.77$, $p < 0.001$). Comparison of qPCR analytical methods indicate that, on average, each method provided equivalent results with average ratios of 0.96, 0.96, and 1.02 for N3 to N1, N3 to ORF1ab, and N1 to ORF1ab and were supported by significant ($p < 0.001$) correlation coefficients ($r = 0.67$ for V2G (N3) to RT (N1), $r = 0.74$ for V2G (N3) to RT (ORF1ab), $r = 0.81$ for RT (N1) to RT (ORF1ab)). Overall results suggest that the two concentration methods and qPCR methods provide equivalent results, although variability is observed for individual measurements. Given the equivalency of results, additional advantages and disadvantages, as described in the discussion, are to be considered when choosing an appropriate method.



KEYWORDS: *SARS-CoV-2, COVID-19, electronegative filtration, magnetic bead concentration, quantitative polymerase chain reaction (qPCR), V2G-qPCR, RT-qPCR*

1. INTRODUCTION

The COVID-19 pandemic, caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), has inspired novel research development and rapid detection-based testing approaches and applied pressure for establishing cost-effective monitoring programs to help better predict the outbreak in suburban and municipal areas.[1−10] Although primary transmission of the COVID-19 illness is from direct person-to-person contact and via close-contact inhalation (i.e., airborne routes), it is also excreted in the feces and urine of presymptomatic, symptomatic, and asymptomatic individuals in concentrations up to 10^5−10^7 genomic copies per liter (gc/L) raw wastewater.[5,11,12] Viral concentrations found in wastewater are determined to represent collective shedding of the community at any point in time, providing a temporal distribution of a community's contributions to a sewershed.[10] Due to this significance, wastewater-based epidemiology (WBE) monitoring programs have been established all over the globe as a response to provide early detection of viral presence within a community.[1,4,13−15] Moreover, the early detection of SARS-CoV-2 through molecular biology-based approaches coupled with WBE can inform policy decision makers before human health surveillance approaches (i.e., testing and tracing of infected individuals) are able to determine community-wide infection.[10,16−19] Thus, WBE is a cost-effective method of epidemiologic efforts in response to the COVID-19 pandemic for predicting a community's risk of infection quickly in contrast to human surveillance which requires intense, constant testing of large groups of individuals to provide community level health data.

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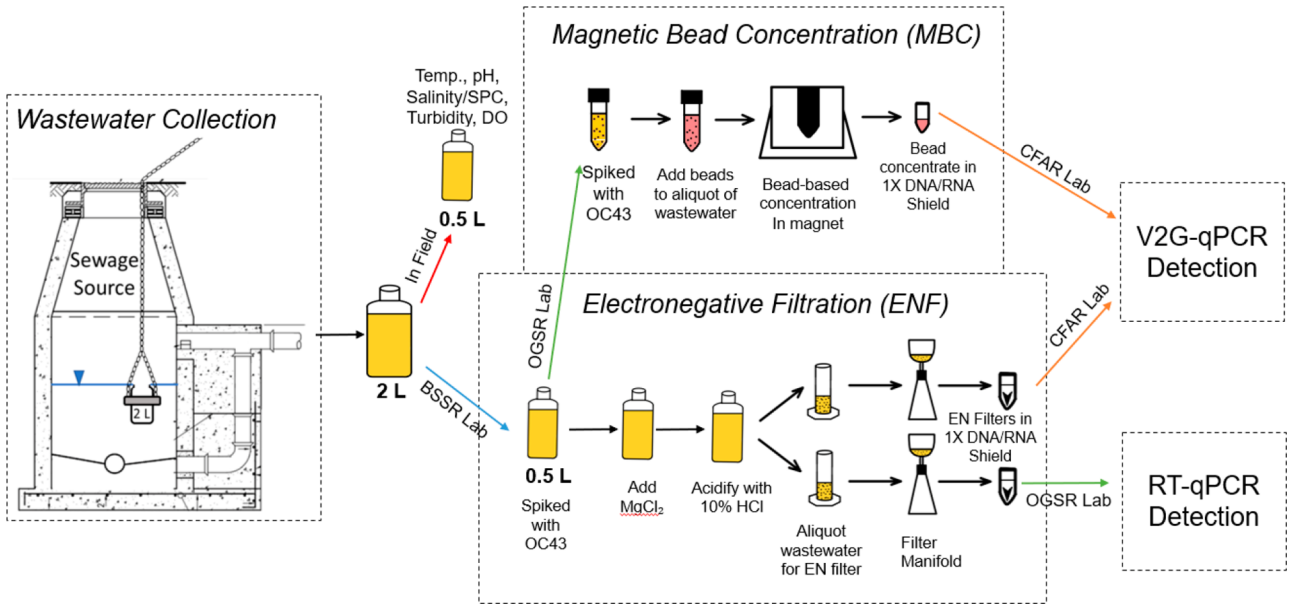

Figure 1. Visual workflow of the sample splitting and processing for each concentration method performed. Two bottles (0.5 L) were split from the initial wastewater collection (2 L) in the field, and aliquots of wastewater collected per sampling site underwent both concentration approaches across the study period. Molecular laboratories received concentrate samples following the ENF and MBC processes in 1X DNA/RNA Shield and utilized RT-qPCR and V2G-qPCR for the detection of SARS-CoV-2 and other targets from treated wastewater.

Increased use of WBE monitoring programs as reliable strategies for noninvasive testing of communities for SARS-CoV-2 has led to the emergence of protocols and practices that are under current validation.[19−22] The University of Miami (UM), located in Miami-Dade County, Florida, USA, implemented a WBE program during the Fall 2020 semester dedicated to monitoring SARS-CoV-2 within the wastewater of UM's three campuses.[10] As an ongoing collaborative effort between three shared resources/laboratories of UM, the Biospecimen Shared Resource (BSSR), the Center for AIDS Research (CFAR), and the Oncogenomics Shared Resource (OGSR), a 5-week study during the summer of 2021 was conducted to compare two sample concentration methods of wastewater via electronegative filtration (ENF) and a manual magnetic bead-based concentration method (MBC), both methods adopting similar processes of currently validated workflows.[10,15,23−25] The comparison was made in the CFAR laboratory using three molecular targets (N3, B2M, and OC43). The molecular assays utilized to compare the concentration methods included a novel volcano second generation-quantitative polymerase chain reaction (V2G-qPCR), developed in-house, and a standard reverse transcriptase (RT)-qPCR nucleic acid detection approach. Each laboratory was given the opportunity to develop and optimize their methods given equipment and expertise available. At the CFAR laboratory, RNA extracted from wastewater samples prepared by both concentration methodologies was analyzed by V2G-qPCR. V2G efficiently amplifies both RNA and DNA templates so a separate cDNA synthesis step is unnecessary. This approach simplifies qPCR, reduces assay time, and is less costly than RT-qPCR kits utilized by other investigators.[10] Furthermore, all reagents were readily available to prepare in-house PCR mixes to quantify the SARS-CoV-2, beta-2 microglobulin (B2M), and human coronavirus OC43 (OC43) targets of interest. Primers and probes for each target were validated to be highly specific with sensitive detection limits of 1−2 copies per 4 μL of purified RNA. The avoidance of commercial kits and reagents was particularly fortuitous as reliable laboratory supplies—a large issue on a global scale—were difficult to acquire throughout the COVID-19 pandemic.[17,26,27] The standard RT-qPCR approach was analyzed in the OGSR, to provide a baseline comparison against the novel V2G-qPCR assay, on a separate set of ENF filter samples, processed with the same method and sample aliquots of wastewater.

B2M, used here as an indicator of human cellular contributions to the wastewater, can be found throughout the human body, within saliva, urine, feces, epithelial cells, and most other human cells; these bodily fluid inputs are common elements found within wastewater following molecular processes. Under circumstances of infection or inflammation, such as COVID-19 infection, B2M, a protein-coding gene, gets upregulated by the body and shed at a higher capacity into the sewershed. B2M was used in this study, versus the more mainstream fecal indicators typically associated with WBE studies, as an internal indicator of a target introduced into wastewater from a human source. Pepper Mild Mottle Virus (PMMoV) RNA is commonly used as a fecal indicator, but levels in wastewater can be influenced by nonhuman sources, such as kitchen sink disposal of peppers. Since PMMoV in wastewater can be derived from several sources, beyond a human dietary origin, measurements of PMMoV RNA may be an overestimate of the human-specific contributions of fecal matter to sewersheds. B2M RNA is found consistently and at significant levels within wastewater and thus was used as a separate molecular target to compare the ENF and MBC wastewater concentration methods.

The goal of this project was to compare the two distinct concentration methodologies to further illustrate the effectiveness of each method for capturing SARS-CoV-2 viral particles from wastewater as well as the downstream molecular assessment. Therefore, we describe key differences between the workflow and sample concentration process, as well as discrepancies between the molecular differences of processed

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municipal wastewater samples. The aim of this study was to determine if ENF and MBC provide comparable results for the detection of SARS-CoV-2 from municipal wastewater via qPCR analysis, and if V2G-qPCR is more effective at detecting the COVID-19 virus than the more mainstream RT-qPCR. The most effective methods of detecting the true viral presence of SARS-CoV-2 within local communities, as a response to the COVID-19 pandemic, are still being investigated, and this comparison adds to that research by confirming the validity of each concentration and molecular quantification method.

2. MATERIALS AND METHODS

2.1. Municipal Wastewater Sample Collection and Experimental Design. Wastewater was collected from a diverse set of locations representing wastewater from individual buildings of different types (dormitories and a hospital), collections of buildings (clusters), and from a large community (Central District, Miami-Dade County). To elaborate, samples were collected from the three UM campuses: 1) the Coral Gables campus, 2) the Rosenstiel School of Marine and Atmospheric Science (RSMAS)—marine campus, and 3) the Miller School of Medicine (MSoM) campus. To note, the hospital, UM Health Tower (UMHT), has been consistently treating COVID-19 patients throughout the year of 2021. Wastewater was collected weekly over a 5-week period from manholes or lift stations from July 13, 2021, until August 10, 2021. This period corresponded to the academic summer period when population densities were low at the Gables and RSMAS campuses. At the Coral Gables campus, four dormitory buildings and two clusters were sampled consistently, at the RSMAS campus, one cluster was evaluated, two samples were collected from the UMHT hospital, and the community watershed scale was assessed via samples collected from the Miami-Dade Central District Wastewater Treatment Plant (CDWWTP) located on the Virginia Key, Miami, FL, USA. A total of 10 sampling sites were surveyed weekly. Two samples were collected from three of the sites, one site each representing the building, cluster, and community scale of wastewater sample collection. One sample was a grab, and the other site was a composite. Thus, a total of 13 samples were collected weekly for the study period.

All sites at which grab samples were collected used a "bottle on chain approach" where a new 2 L bottle (HDPE) was lowered into the sewer and filled. The composite samples at UM (ISCO 6712) and at CDWWTP (HACH AS950) were collected via an autosampler programmed to fill a designated amount of sewage incrementally over 24 h the day prior. The 2 L bottle containing the wastewater sample was then split in the field into two containers (Figure 1): (1) a 0.5 L bottle (filled with 0.5 mL sodium thiosulfate (100 g/L) to remove the chlorine residual) taken to the BSSR for subsequent processing aliquot removal and concentration for SARS-CoV-2 quantification and (2) a 0.5 L plastic beaker utilized for water quality measurements in the field (pH, temperature, turbidity, dissolved oxygen (DO), and specific conductivity (SPC)) (Xylem YSI ProDSS). For details regarding the water quality of wastewater samples collected in this study, see Table S1 in the Supporting Information. Standard practices for field safety were utilized, including use of secondary containers to capture spilled wastewater and tap water rinses and 99.5% isopropyl alcohol disinfection of equipment.

Upon arrival at the BSSR, each sample was treated and split for appropriate assessment with each concentration method, ENF and MBC (see Methods 2.3, Figure 1). At the CFAR, V2G-qPCR was performed on electronegative filter samples as well as magnetic bead samples to quantify a modified N3 target of SARS-CoV-2; at the OGSR, electronegative filter samples were assessed for SARS-CoV-2 targets N1 and ORF1ab with the standard RT-qPCR method and compared (see Methods 2.4, Figure 1).

2.2. Sample Pretreatment. Upon their arrival at the BSSR laboratory, 0.5 L wastewater samples (n = 13 per week, transported on frozen ice packs from the field) underwent a treatment process where they were spiked with a heat-inactivated (15 min @ 56 °C) viral recovery control, OC43, to a level of 10^6 gc/L. Fifteen mL of OC43-spiked sewage was removed from the initial sample bottle and kept at 4 °C until samples were transported to the OGSR (adjacent building) and immediately concentrated using MBC (see Methods 2.3.2). An aliquot of the OC43-spiked wastewater was removed from the 0.5 L bottle so that the same sampling site could be analyzed with both concentration methods. For ENF, $MgCl_2$ was added to the remaining 485 mL to a concentration of 50 mM to increase viral absorption to the filters.[28] During continuous stirring, an initial pH was taken with a precalibrated pH probe directly inserted into the 0.5 L bottle and recorded once stable. To impart a positive charge to viral particles, the pH was then adjusted by adding acid (10% HCl) to a range of 3.5—4.5. These samples were immediately concentrated using ENF (see Methods 2.3.1) at the BSSR (Figure 1). All sample handling for pretreatment occurred within a Biosafety Level 2 (BSL-2) laminar flow hood, and standard laboratory safety practices were upheld.

2.3. Wastewater Sample Processing: Concentration. *2.3.1. Electronegative Filtration Method.* Hydrophilic, mixed cellulose ester membranes (47 mm diameter EMD-Millipore: #HAWP04700) with a pore size of 0.45 μm were utilized in the ENF method to capture the suspended particulates and viral particles within the wastewater sample.[10,29−32] Coupled with the pretreatment described in Methods 2.2, this protocol for ENF was modified in-house and did not include a bead beating step as some methods recommend. Our approach used a vacuum manifold and pump to pull pretreated wastewater, until apparent clogging occurred, through the membrane (volumes ranging from 15 to 150 mL) ultimately trapping the suspended solids by straining and capturing the free positively charged SARS-CoV-2 particles by charge attraction.[28] The electronegative filter membranes, containing a top layer of wastewater suspended solids and adsorbed SARS-CoV-2 particles, were folded and then placed in 1X DNA/RNA Shield (Zymo) where they were lysed and preserved resulting in a filter concentrate. For each sample, two filter concentrates were prepared: one for molecular analysis by V2G-qPCR and another by RT-qPCR. The volume of water filtered per sample for ENF was variable. The volume of sample filtered was dependent on the water quality (i.e., turbidity, amount of suspended solids, etc.), wastewater samples that were more turbid required smaller volumes (~15−50 mL) to completely saturate the filter membrane, and clearer water with less suspended solids required larger volumes (~60−150 mL) to completely saturate the filter with surface solids. Wastewater volumes utilized per sample for the ENF process can be viewed within the Supporting Information Table S2. Sterile preautoclaved graduated cylinders, forceps, and magnetic filter funnels were used per wastewater sample to ensure the absence

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ACS EST Water XXXX, XXX, XXX−XXX

of nucleic acids and to avoid cross contamination of wastewater collected between sampling sites.

2.3.2. Magnetic Bead-Based Method. The MBC method utilized Nanotrap Magnetic Virus Particles (Nano#44202; i.e., magnetic beads) from Ceres Nanoscience's Inc. to capture and concentrate the SARS-CoV-2 virus found in wastewater samples. Nanotrap particles are highly porous hydrogel particles designed to have high affinities for different classes of analytes including viruses. A two-part protocol, modified in-house, occurring during the MBC process performed 1) a minimum 10 min rest period allowing the suspended solids within the sample to settle and 2) a series of incubation and wash periods following the addition of the beads to the wastewater sample, allowing for the separation of SARS-CoV-2 viral particles from wastewater. To elaborate, 10 mL of the aliquoted 15 mL wastewater samples was extracted from each test tube and transferred into a prelabeled, sterile 50 mL centrifuge tube. Six hundred μL of preshaken magnetic beads was then added into each sample and left for 10 min to incubate to allow sufficient time for binding between the beads and ambient SARS-CoV-2 within the wastewater. Once the beads had incubated, the 50 mL tube was placed within the Ceres Nanoscience's magnet to remove them from suspension within the water column; tubes were left to incubate on the magnet for a minimum of 10 min where, following their attraction to the walls of the tube, the supernatant was poured out. Two wash steps, utilizing phosphate-buffered saline (PBS), occurred following similar methodology where a 1.5 mL tube (containing PBS and bead pellets) was left to incubate on a magnet for a minimum of 5 min, and the supernatant was removed. To the final pellet of bead particles, 300 μL of 1X DNA/RNA shield was added. The resulting concentrates were kept at −20 °C in storage, and an aliquot of 150 μL was set aside for later molecular analysis by V2G-qPCR.[33]

2.4. Molecular Assessment of Concentrates. At the CFAR laboratory, ENF and MBC concentrate samples were prepared for assessment by V2G-qPCR. To extract the RNA from the individual concentrates, a Zymo Quick RNA-Viral Kit was used consisting of a silica-based spin column protocol. For samples which underwent ENF, 250 μL of the concentrate within 1X DNA/RNA Shield was removed following a few flushes of the filter with repeat pipetting and applied to the column in combination with the kit's binding buffer. ENF concentrates were not vortexed, only flushed with pipetting, prior to RNA extraction to limit the number of large particulates that could be dislodged from the membrane ultimately capable of clogging the spin columns and reducing the efficiency of the extraction.

In contrast, the MBC samples underwent a brief vortex and 5 min separation period as they were applied to a magnet effectively pulling the beads from solution. The supernatant of the MBC samples was removed while still in the magnet, and 150 μL was applied to the spin column in combination with the kit's binding buffer. Concentrate samples were kept at 4 °C upon arrival at the CFAR laboratory and during the extraction process remained at room temperature. Extracted nucleic acid from ENF and MBC concentrates was immediately placed on ice and tested with V2G-qPCR analysis. A master mix was first prepared using in-house combinations of reagents and target-specific primers and probes (Table S3). Purified RNA from the EN filter and MBC concentrates was subjected to V2G-qPCR to measure SARS-CoV-2, B2M, and OC43 targets. Standards with known concentrations of 10^1−10^5 copies/μL were run to generate a standard curve from which the quantities of unknowns could be extrapolated. A minimum of 7 no-template controls (NTCs) were also included within each plate setup. Reagents for V2G-qPCR of SARS-CoV-2 RNA amplified the N3 target of the nucleocapsid gene near the 3′ end of the SARS-CoV-2 genome as modified from Lu et al., 2020.[14,33] Initial evaluations of the CDC primer/probe sets excluded N1 due to the strong secondary structure of the reverse primer. The N3 set performed better than the N2 set when using V2G although some reactions resulted in false positive results. For this reason, the N3 set was modified to improve specificity of V2G amplifications. Details about the modified N3 target reagents are provided in the Supporting Information (Figure S3). B2M has previously been developed as an internal gene expression (housekeeping) control, and it was adopted as a marker of human cells that are in wastewater. The B2M assay used in the current study amplifies the mature, spliced mRNA present in cells of human origin. The OC43 recovery control was chosen as it is an enveloped, positive-sense, single-stranded RNA coronavirus like that of SARS-CoV-2. This control was obtained from ATCC (#VR-1558) and produced, in-house, by cell culture over 5−7 days using Vero cells (ATCC) in RPMI media supplemented with 10% fetal bovine serum and penicillin/streptomycin. The culture supernatant was harvested and filtered through a 0.45-μm cartridge filter, and aliquots were used to measure the virion concentration. RNA was extracted from a 50-μL supernatant in quadruplicate and quantified in triplicate using V2G-qPCR to determine an average viral particle quantity per microliter.[10] A defined amount of OC43 particles was spiked into wastewater samples prior to processing (~10^6 gc/L). OC43 RNA in the final extracted RNA was measured by V2G-qPCR to determine percent (%) recovery; measuring the % recovery of OC43 RNA is a useful surrogate marker of % recovery of SARS-CoV-2 RNA by the ENF concentration method. Average percent recovery of the OC43 control was 20%.

At the OGSR, the EN filter concentrates were analyzed for SARS-CoV-2 alone with standard RT-qPCR; however, different targets within the viral genome were assessed (N1 and ORF1ab), differing from the single target assessment (nucleocapsid gene) used for V2G-qPCR. Specifically, the N1 nucleocapsid target assessed at the OGSR is located close to the 3′ end of the SARS-CoV-2 genome. The ORF1ab gene is located on the 5′ end of the SARS-CoV-2 genome. These two targets were chosen by the OGSR to better determine if, minimally, partial fragments of the single-stranded RNA of SARS-CoV-2, either on the 5′ or the 3′ end, were found within wastewater collected from the local community. The OGSR used a commercially available RT-qPCR kit, the MagMAX Viral/Pathogen II Nucleic Acid Isolation Kit IFU, and the manual method for a 200-μL sample input volume for extracting and purifying RNA from EN filter concentrates. The RT-qPCR process performed at the OGSR followed the Applied Biosystems TaqPath COVID-19 Combo Kit protocol (https://www.fda.gov/media/136112/download) using the PerkinElmer New Coronavirus Nucleic Acid Detection Kit IFU and corresponding protocol (https://www.fda.gov/media/136410/download) at a 20-μL reaction volume. All results from qPCR analyses are reported in gc/L of raw wastewater.

2.5. Data Analysis and Reporting Parameters. All processes were conducted quantitatively including using the

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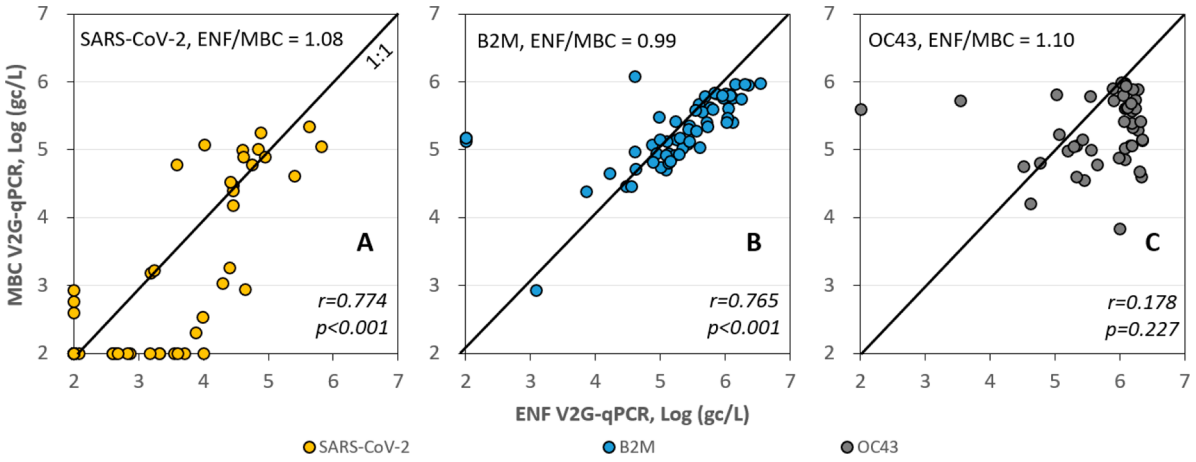

Figure 2. Correlations between ENF and MBC concentration methods for three molecular targets: A) SARS-CoV-2, B) B2M, and C) OC43 per sample per collection date for the 5-week study period. Spearman correlation coefficients and *p*-values calculated describe similar detection of SARS-CoV-2 and B2M from wastewater. Log transformed data with a detection limit of 10^2 gc/L wastewater.

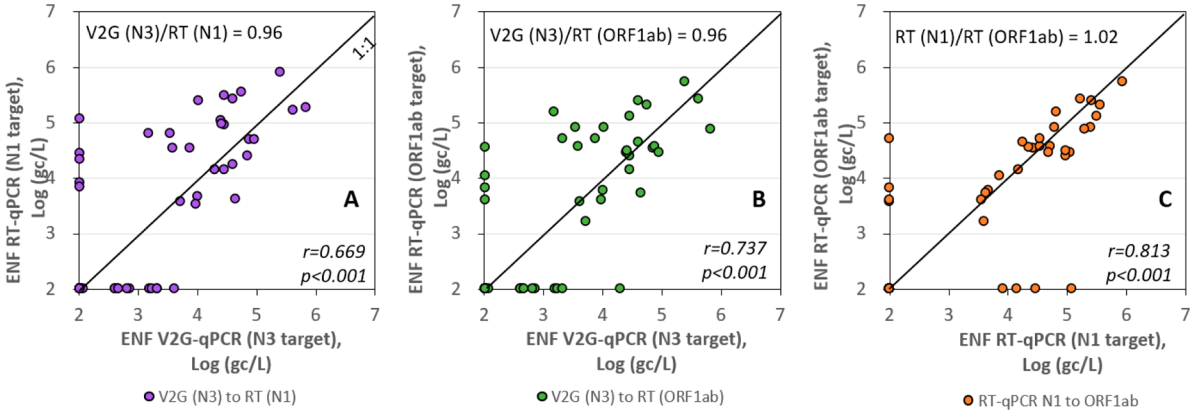

Figure 3. Correlation between SARS-CoV-2 measurements between a) V2G-qPCR (N3 target) and RT-qPCR (N1 target), b) V2G-qPCR (N3 target) and RT-qPCR (ORF1ab target), and c) RT-qPCR (N1 target) and RT-qPCR (ORF1ab target). All samples were processed using electronegative filtration. Log transformed data with a detection limit at 10^2 gc/L wastewater.

recorded raw wastewater volume for the concentration step, final concentrate volumes, extraction volumes, and qPCR reaction volumes. These known volumes were then used to compute the concentration of each molecular target (SARS-CoV-2—N3, N1, ORF1ab, B2M, OC43) in units of gc/L of water following qPCR amplification. Shapiro-Wilk normality tests were run on each set of data, per concentration method and molecular target, spanning the 5-week study period. All qPCR data sets were nonparametric, with descriptive statistics available in Table S4. Spearman correlations (SPSS version 26) were computed to compare the log-transformed viral concentrations between concentration methods and between qPCR methods. Mann—Whitney U tests, also known as Mann—Whitney Wilcoxon tests, were used to evaluate whether the means of each data set were statistically equivalent to one another. Statistics were performed to compare ENF results to MBC results as analyzed by V2G-qPCR, for the three targets SARS-CoV-2, B2M, and OC43. Statistics also compared V2G-qPCR against RT-qPCR. SARS-CoV-2 targets for the qPCR comparison included the following: N3 vs N1, N3 vs ORF1ab, and N1 vs ORF1ab. All raw qPCR data generated were calculated to a gc/L basis, per concentration method, as each method utilized recorded volumes of raw wastewater, concentrates, extraction volumes, and qPCR reaction volumes per sample. Excel was used to plot the data to further illustrate the spread of data points across a 1 to 1 line (Figures 2 and 3), as well as to illustrate average abundance (gc/L) over the study period. See Figures S1 and S2 in the Supporting Information for time series plots comparing concentration and qPCR quantification methods.

3. RESULTS AND DISCUSSION

3.1. Comparison of Concentration Methods: ENF vs MBC. Within the complex medium that is wastewater, SARS-CoV-2 viral particles attach to the small particulates that are invisible to the human eye following their shedding from people into the sewage system. The primary concentration of wastewater, as described by Lu et al. 2020a,[34] is essential for accurate, sensitive, and efficient detection of SARS-COV-2 RNA downstream by qPCR; therefore, the effectiveness of the concentration method in capturing viral particles from water is imperative upstream of the molecular process. ENF is a process which pulls water, utilizing vacuum suction, through a membrane trapping most of the small particles, as well as any SARS-CoV-2 particles suspended within the sample. Comparatively, the MBC method which, following the addition of beads to a 10-mL aliquot of wastewater, was mixed and incubated effectively attracting the SARS-CoV-2

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particles to the beads allowing for straightforward concentration. Both methods studied here have been shown to isolate SARS-CoV-2 RNA from wastewater, as filtration (either ultra or ENF), bead-based concentration, and polyethylene glycol precipitation (PEG) have been the main standard methods utilized by laboratories for concentrating SARS-CoV-2 from wastewater since the start of the pandemic.[10,22,32−37] ENF and MBC are compared here as each workflow differs in treatment, setup, and overall handling of the samples.

V2G-qPCR data for ENF and MBC processes were statistically compared to determine if, based on the quantified presence, either method could be defined as more effective to detect SARS-CoV-2 from wastewater (Table 1). Comparison

Table 1. Summary Table of rho Coefficients and *p*-Values Resulting from Spearman Correlations Comparing Wastewater Samples over an Ordinal Scale[a]

variables compared	Spearman coefficient (r)	p-value
ENF vs MBC: SARS-CoV-2 (N3)	0.774*	**<0.001**
ENF vs MBC: B2M	0.765*	**<0.001**
ENF vs MBC: OC43	0.178	0.227
V2G-qPCR (N3) vs RT-qPCR (N1)	0.669*	**<0.001**
V2G-qPCR (N3) vs RT-qPCR (ORF1ab)	0.737*	**<0.001**
RT-qPCR (N1) vs RT-qPCR (ORF1ab)	0.813*	**<0.001**

[a]ENF and MBC methods compared for three molecular targets assessed with V2G-qPCR (SARS-CoV-2; N3, B2M, and OC43). V2G-qPCR and RT-qPCR methods compared for SARS-CoV-2 molecular targets of ENF samples (N3, N1, and ORF1ab). Mann−Whitney Wilcoxon tests were used to validate p-values. * denotes significant correlation between variables.

of ENF to MBC results shows that when data were taken as a whole, the ratio of ENF to MBC was 1.08 on average for SARS-CoV-2, 0.99 for B2M, and 1.10 for OC43 (Figure 2). This provides that each method, when used for concentrating wastewater, elicits a similar resulting detection of SARS-CoV-2 RNA found downstream with molecular processes. Spearman correlations resulted in correlation coefficients of r = 0.77 for SARS-CoV-2, r = 0.77 for B2M, and r = 0.18 for OC43 on samples analyzed by V2G-qPCR, processed with ENF, and compared here against MBC (Table 1). Average abundance for SARS-CoV-2 fluctuated most of the three molecular targets assessed with V2G-qPCR, in that it differed by almost 4-fold across the study period with concentrations ranging from 10^2 to 10^6; B2M was consistently detected around 10^5 to 10^6 gc/L, and OC43 generally measured between 10^5 and 10^6 gc/L across the 5-week interval (Figure 2). OC43 was added to the samples at a constant concentration therefore resulting in a limited range of detection. This small range of detection contributed toward the lower r values for this molecular target. Overall, these results describe that the ability of ENF and MBC, as sample processing workflows, is similar when the molecular target assessed is SARS-CoV-2 or abundant B2M found from wastewater. Results for OC43 were consistent with a ratio of near 1 for ENF versus MBC but suffered from the lack of range thereby providing low correlations. No significant difference was observed between ENF and MBC for SARS-CoV-2 (p = 0.46) and B2M (p = 0.39). Results suggest that each concentration method used provided statistically similar results, following Spearman correlations and Mann−Whitney Wilcoxon tests, in detecting SARS-CoV-2 over an ordinal scale within a community.

An important aspect of B2M is that it allowed us to determine that the wastewater being flushed into the sewershed and later collected by our team was in fact from a human source, as B2M is found in most cells and bodily fluids.[38] In addition to urine and feces, B2M can be detected from saliva and epithelial cells shed from various places in the human body and, under circumstances of infection or inflammation, is known to upregulate and shed in higher concentrations.[39−41] From these efforts, combined with previous ongoing experiments,[10,42] we have established B2M to be a useful target for evaluating SARS-CoV-2 detection from wastewater, as it can be used as a "human" indicator for future work and serves as a potential normalization parameter for the SARS-CoV-2 signal of WBE research. OC43, similarly, has been determined to be an effective recovery control resulting in ∼20% average viral recovery of SARS-CoV-2 RNA following qPCR detection. However, we may be overestimating the degradation of SARS-CoV-2 in this case if applied in a direct comparison, as RNA of OC43 is thought to degrade easier and faster than RNA of SARS-CoV-2.[43]

As rapid-detection approaches for measuring the abundance of SARS-CoV-2 within communities have only increased since the pandemic's onset in late 2020, the viability of methodology must also be assessed, which is attempted here. Mann−Whitney Wilcoxon tests confirmed there was also no significant difference found between the mean presence detected of SARS-CoV-2 from wastewater analyzed by V2G-qPCR across the 5 weeks of sampling between ENF and MBC methods (p = 0.46). Similarly, this was the case for B2M (p = 0.39). Given that both ENF and MBC were found here to provide similar results, other factors should be considered when choosing among methods (Table 2), such as the flexibility in adjusting processing volumes, procurement of supplies, and availability of automated processes. ENF's additional benefits include reliable, consistent sample processing results with little room for error of the resulting concentrate following the pretreatment of a wastewater sample. MBC is a straightforward approach which uses few reagents, little space, and a small, powerful magnet to process concentrates with similar viral loads to ENF. Limitations of ENF include a more complex pretreatment process ($MgCl_2$ addition and acidification) and the need to sterilize equipment between uses. In contrast, for MBC the largest drawback is time, in which samples undergo timed incubations and the need for a large number of magnets if many samples are to be analyzed in tandem. Furthermore, the resulting MBC concentrate's viability is dependent on the quality of the wash steps performed on the beads. A more comprehensive comparison of benefits and limitations for ENF to MBC is also provided within Table 2. A longer study period coupled with more samples collected and processed consistently with these described methods would provide a more robust comparison; however, across n = 60, we can minimally provide that ENF and MBC are both useful tools and effective concentration methods providing comparable results for WBE research.

3.2. Comparison of Downstream Molecular Detection: V2G-qPCR vs RT-qPCR. The standard RT-qPCR approach, a molecular biology nucleic acid detection assay that is used routinely for a widespread range of viral measurements, was also compared to the novel method that has been utilized by UM's WBE research program, V2G-qPCR.

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Table 2. Advantages and Disadvantages of Electronegative Filtration and Magnetic Bead-Based Concentration Plus V2G-qPCR and RT-qPCR

advantages	disadvantages
Electronegative Filtration	
•volumetric adjustment of wastewater sample allows for variable input volume per filter concentrate created	•requires individual sample volumetric adjustment and wait times
•multiple filters can be prepared from the same sample overcoming limitations in volume loss through sample splitting	•performed manually; no automation currently available
•relatively quick processing time	•requires pretreatment of samples via addition of $MgCl_2$ and acidification
•does not require sample elution as the filter is placed into the DNA-RNA shield	•much of the equipment used is nondisposable (e.g., filter funnels, graduated cylinders, forceps) requiring sterilization between use
•supplies easier to procure during the pandemic when specialized equipment was limited	•requires a vacuum source which may limit use outside laboratory settings
Magnetic Beads	
•both manual and automated formats available; automated approach can be used for primary concentration and for nucleic acid extraction	•same sample volume used regardless of water quality
	•requires a supply of beads
•manual formats do not require electricity allowing for use in field	•number of samples in batches are constrained by equipment available by the manufacturer
	•several timed steps which can slow down the process
V2G-qPCR	
•can use either RNA or DNA as input as reagents are tailored to being able to read both nucleic acid templates	•in-house approach, with limited widespread knowledge base of application
•noncommercial kits/reagents are used with supplies easier to procure during the pandemic	•novel assay designed for implementation of the COVID-19 public health response, not yet verified outside of WBE and HIV research
•assay can be performed on different qPCR platforms	•can only be run as singleplex (one molecular target) or duplex (two molecular targets); needs more work to validate multiplexing
•cost effective (about $1US per sample)	•requires optimization to minimize PCR inhibition
RT-qPCR	
•globally accepted approach and adopted by many laboratories worldwide	•requires the use of RNA as input, required production of cDNA for amplification
•utilizes commercially available kits, with corresponding protocols (easy to change an established method)	•prone to PCR inhibition, limited on the capacity and efficiency of the commercial kit utilized
•can be run as a singleplex, duplex, or multiplex (one, two, or multiple molecular targets)	•prone to dimer formation and nonspecific product amplification depending on commercial primers utilized
•most equipment that comes in contact with the sample is disposable limiting the need for sterilization between samples	
•relatively cost-effective with options for reducing costs	

Results show that V2G-qPCR provides statistically equivalent results to that of RT-qPCR with Spearman correlations of V2G-qPCR (analyzing the N3 target) compared against RT-qPCR (analyzing the N1 target) resulting in $r = 0.67$, $p < 0.001$ and between V2G-qPCR (analyzing the N3 target) and RT-qPCR (analyzing the ORF1ab target) resulting in $r = 0.74$, $p < 0.001$ (Table 1). Mann−Whitney Wilcoxon tests further confirmed the lack of statistical differences between the N3 and N1 targets ($p = 0.44$) and between N3 and ORF1ab targets ($p = 0.60$). The two RT-qPCR SARS-CoV-2 targets, N1 and ORF1ab, were also compared against one another and resulted in a Spearman coefficient of $r = 0.81$ and $p < 0.001$ (Table 1). Moderately strong correlation coefficients allow us to describe that the different molecular targets assessed in CFAR and OGSR for SARS-CoV-2, N3, N1, and ORF1ab, have similar quantities given the differing qPCR approaches. The choice of any one of these molecular targets coupled with wastewater samples allows for relatively similar detection of COVID-19 within the community, following the methods described above. As explained here, V2G-qPCR was found to be statistically like the mainstream RT-qPCR, describing that V2G-qPCR is an effective assay which could replace RT-qPCR when analyzing wastewater for SARS-CoV-2 and other targets. This novel qPCR assay, coupled with ENF, can provide a rapid-detection result in as little as under 12 h, starting from sample collection in the field to the qPCR result. Furthermore, it utilizes combinations of readily available reagents and eliminates the requirement for prior cDNA synthesis of extracted viral RNA. All are benefits, given the nature of the global response to the COVID-19 pandemic, with supply chain issues, and the dire need for quick turnaround of results. V2G-qPCR ultimately allows for more assays to be run in the same amount of time as the standard RT-qPCR method following the RNA extraction of wastewater concentrate samples.

When the qPCR target comparisons were analyzed, the ratio for both V2G (targeting N3) to RT (targeting N1) and V2G (targeting N3) to RT (targeting ORF1ab) was 0.96 on average and resulted in a ratio of 1.02 between the two RT-qPCR targets N1 and ORF1ab (Figure 3). This demonstrates an equivalent ability of utilizing V2G-qPCR assessing for one target, N3, instead of RT-qPCR assessing for two targets, N1 and ORF1ab, for detecting SARS-CoV-2 from wastewater. V2G-qPCR as an assay, capable of being utilized for WBE monitoring, has been described here as an effective tool in determining the average viral presence of COVID-19 within the UM community. The strong correlation of N1 to ORF1ab validates the RT-qPCR approach used here, as the average log-transformed presence of SARS-CoV-2 for each assay was expected to be similar, and significant correlation was observed (Figure 3). Each of these genes are located on opposite sides of the SARS-CoV-2 viral genome, and this correlation would be expected if all fragments of the viral RNA are represented within the sample. See Table 2 for a listing of the advantages and disadvantages of each qPCR method.

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4. CONCLUSIONS

The overall aim of this study was to investigate the differences between the ENF and MBC concentration approaches for detecting SARS-CoV-2 RNA from wastewater followed by the aim to determine the validity of V2G-qPCR as a plausible replacement assay for the mainstream, commercial RT-qPCR approach. Results show that, overall, both concentration methods as well as each quantification method (V2G-qPCR versus RT-qPCR) provide equivalent results. The resulting similarity provided here between RT-qPCR and a novel V2G-qPCR, which takes less time and foregoes a cDNA synthesis prior to amplification, is valuable for pushing the forefronts of rapid-detection-based approaches and can complement other isothermal or sequence-based methods.[44,45] Electronegative filtration is considered one of the standard methods utilized for primary concentration of viral particles from wastewater in WBE research and provides dependable detection upon saturation of a filter of SARS-CoV-2 with downstream qPCR. Here, we compared this widely used filtration method with a newer technology, a magnetic bead-based viral concentration. The comparison between primary concentration provided that each is not only effective at detecting SARS-CoV-2 from wastewater but that the concentration step was recognized as a factor possibly aiding in that detection. As the use of WBE as a public health mechanism is growing in popularity, this study provides benefit to the validation of methods commonly used to perform the complex process.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestwater.2c00047.

Additional water quality summary, sample processing data, visualized comparisons, and explanation of modified N3 SARS-CoV-2 molecular target (PDF)

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Notes

The authors declare no competing financial interest.

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