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Evaluation of three viral concentration methods for detection and quantification of SARS-CoV-2 in wastewater

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ABSTRACT

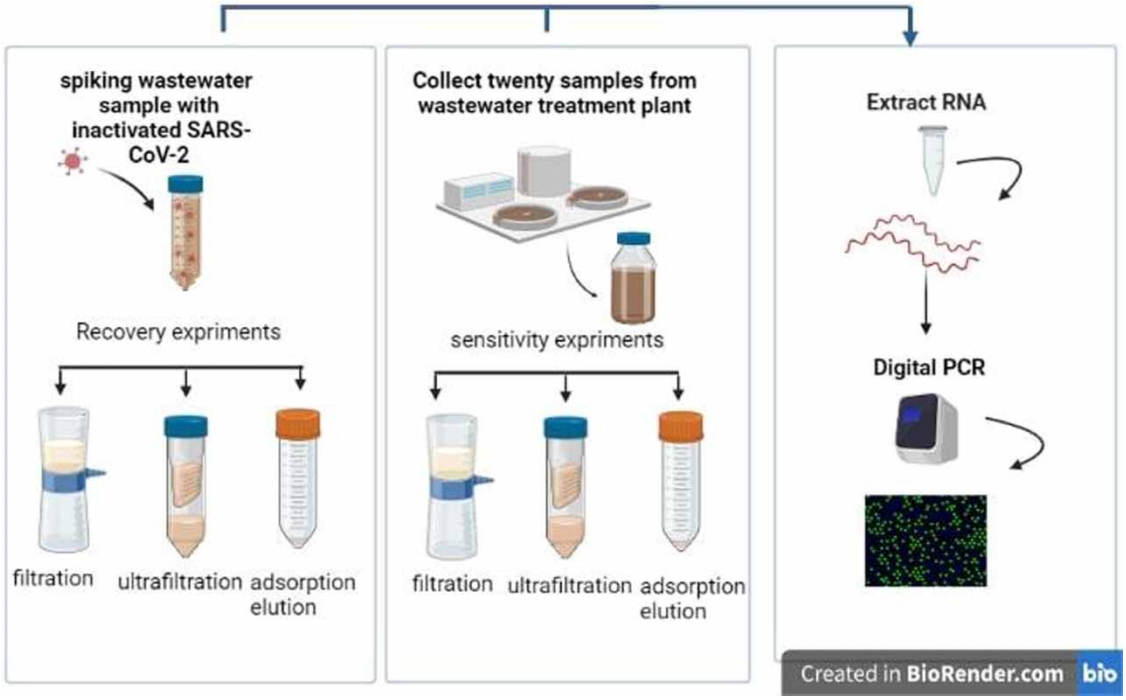
Wastewater-based epidemiology (WBE) could be useful as an early warning system for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic spread. Viruses are highly diluted in wastewater. Therefore, a virus concentration step is needed for SARS-CoV-2 wastewater detection. We tested the efficiency of three wastewater viral concentration methods: ultrafiltration (UF), electronegative membrane filtration and aluminum hydroxide adsorption–elution. We spiked wastewater with inactivated SARS-CoV-2 and we collected 20 other wastewater samples from five sites in Tunisia. Samples were concentrated by the three methods and SARS-CoV-2 was quantified by reverse transcription digital PCR (RT-dPCR). The most efficient method was UF with a mean SARS-CoV-2 recovery of 54.03 $\pm$ 8.25. Moreover, this method provided significantly greater mean concentration and virus detection ability (95%) than the two other methods. The second-most efficient method used electronegative membrane filtration with a mean SARS-CoV-2 recovery of 25.59 $\pm$ 5.04% and the least efficient method was aluminum hydroxide adsorption–elution. This study suggests that the UF method provides rapid and straightforward recovery of SARS-CoV-2 in wastewater.

Key words: concentration methods, COVID-19, SARS-CoV-2, wastewater-based epidemiology (WBE), wastewater virus

HIGHLIGHTS

- Spiked wastewater with inactivated SARS-CoV-2 was used to compare the recovery of three viral concentration methods.
- Untreated municipal wastewater samples collected from five sites in Tunisia were used to compare the detection sensitivity of the three viral concentration methods.
- UF showed the best recovery and virus detection ability in wastewater samples.

GRAPHICAL ABSTRACT



INTRODUCTION

Coronavirus disease 2019 (COVID-19) is an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that has emerged in Wuhan, Hubei Province, China, in December 2019 and was declared a pandemic in March 2020 by the World Health Organization (WHO).

Although SARS-CoV-2 is predominantly a respiratory virus, it is also able to productively replicate in human gut enterocytes (Lamers *et al.* 2020; Bertrand *et al.* 2021) and the SARS-CoV-2 RNA has been detected in the stools of both symptomatic and asymptomatic patients (Gao *et al.* 2020; Xiao *et al.* 2020; Zhang *et al.* 2020; Bertrand *et al.* 2021), and in wastewater (Ahmed *et al.* 2020a; Randazzo *et al.* 2020; Jmii *et al.* 2021). Therefore, wastewater-based epidemiology (WBE) could be used as an early warning or surveillance system for infections or rises of the SARS-CoV-2 virus (Jafferali *et al.* 2021). WBE has been efficiently used for decades by health agencies and authorities to track trends in the general population for numerous diverse substances, including toxins and pesticides, as well as the prevalence of disease and illness (Nordgren *et al.* 2009; Been *et al.* 2014; Rousis *et al.* 2017; Yang *et al.* 2017; Du *et al.* 2018; D'Aoust *et al.* 2021). Hence, during the current pandemic, various investigations have concentrated on the identification of SARS-CoV-2 in wastewater (La Rosa *et al.* 2020; Medema *et al.* 2020; Randazzo *et al.* 2020; Jafferali *et al.* 2021).

Due to its low concentration in wastewater, the detection and quantification of SARS-CoV-2 by PCR require the concentration of the virus. Although numerous studies have utilized wastewater surveillance for SARS-CoV-2, current concentration methods vary widely and remain unstandardized. It is, however, obvious that there are variations among results due to differences between methodologies.

Several viral concentration methods used for the detection of SARS-CoV-2 in wastewater have been compared. These methods were based on various principles, including ultrafiltration (UF), filtration through electronegative membranes, ultracentrifugation of the wastewater and precipitation (using polyethylene glycol (PEG) and aluminum hydroxide) (Barril *et al.* 2021; Bertrand *et al.* 2021; Jafferali *et al.* 2021). From the limited published studies, it appears that the recovery efficiencies of these methods are highly variable. Therefore, further studies need to be conducted and validated to better evaluate the performance of viral concentration methods.

The purpose of the present study was to evaluate three previously published concentration methods: UF using the Vivaspin® Turbo 15 RC (100 kDa), electronegative membrane filtration and aluminum hydroxide adsorption–elution. The findings of this study will help researchers to choose an effective method to concentrate SARS-CoV-2 from wastewater.

MATERIALS AND METHODS

Wastewater sampling

Wastewater samples were collected from the entrance of a wastewater treatment plant (WWTP) located in the following five sites in Tunisia: Sidi Bouzid, Kairouan, Gabes, Monastir and Tunis. A 24-h composite sampling was performed using an auto-sampler; samples were collected in sterile glass containers kept at 4 °C upon arrival and concentrated within 24 h.

Spiking of wastewater

The wastewater samples used for spiking and concentration of SARS-CoV-2 were collected from the WWTP of the city of Sidi Bouzid. Aliquots of non-spiked wastewater samples were tested for SARS-CoV-2 by the methods described below and were found negative.

150 ml of wastewater was inoculated with 300 µl of a known number (4.5.104 GE/µl) of an inactivated SARS-CoV-2 (gamma irradiation. 50 KGy) diluted in 500 µl of 0.5% gelatin/phosphate buffered saline (PBSG). The spiked water sample was mixed and divided into three 50-ml samples.

Viral concentration

Spiked samples (50 ml each) and wastewater samples were concentrated by three different methods. The first third of the sample was centrifuged at 3,300 × *g* for 30 min at 4 °C in order to remove large and coarse particles and the supernatant was filtered through 100-kDa cut-off centrifugal ultrafilters (Sartorius) at 1,500 × *g* for 15 min (Megastar 1.6R bench top centrifuge). The second third of the sample was passed through 0.45-µm pore size, 47-mm diameter electronegative membrane (Sartorius) after addition of $MgCl_2$ to the sample to obtain a final concentration of 25 mM $MgCl_2$. Immediately after filtration, filters were stored in a −80 °C freezer until total nucleic extraction. Filters were extracted within 24 h. The last third of the sample was concentrated by the adsorption–elution method using aluminum hydroxide and beef extract as previously described (Jmii *et al.* 2021).

Viral RNA extraction

Viral RNA was extracted using the RNeasy PowerWater Kit (Qiagen) according to the manufacturer's instructions, with a slight modification. The RNA was eluted in 50 µl of RNase-free water, aliquoted and stored at −80 °C until used for viral RNA detection.

Digital PCR (dPCR)

The dPCR assay was performed following the manufacturer's instructions (Qiagen, Germany) using the QIAcuity One, 5plex, the Qiacuity One Step Viral RT-PCR Kit (Qiagen) and the SARS-CoV-2 N1 + N2 assay kit (Qiagen), for detection and quantification of SARS-CoV-2 (N1 and N2, published by Centers for Disease Control and Prevention (CDC)) in spiked and wastewater samples. Briefly, 40 µl of reaction mix was prepared for each reaction. It comprised 10 µl of master mix, 800 nM forward primer, 800 nM reverse primer, 250 nM probe, 0.4 µl of 100× multiplex reverse transcription mix, 7.6 µl RNase-free water and 20 µl of template RNA.

Following assembly in the pre-plate, reaction mixtures were transferred into a QIAcuity Nanoplate 26 k 24-well and loaded onto the QIAcuity One, 5plex. The reverse transcription digital PCR (RT-dPCR) workflow includes (i) a priming and rolling step to fill and then seal the reaction chamber partitions; (ii) an amplification step following this cycling protocol: 40 min at 50 °C for reverse transcription, 2 min at 95 °C for enzyme activation, 5 s at 95 °C for denaturation and 30 s at 60 °C for annealing/extension in 40 cycles and (iii) an imaging step completed by reading in the FAM channel. The entire workflow, comprising the three steps, takes about 2 h. Data were analyzed using the QIAcuity Software Suite V1.2 and expressed as copies/µl.

Statistical analysis

The average value and standard deviation (SD) were calculated. Student's *t*-test was used for comparison and values of $P < 0.05$ were deemed significant.

RESULTS

Three concentration procedures; UF using the Vivaspin® Turbo 15 RC (100 kDa), filtration through a 0.45-μm pore size.47-mm diameter electronegative membrane (Sartorius) and adsorption–elution using aluminum hydroxide and beef extract were compared.

The yields of the three viral concentration methods were first determined by spiking inactivated SARS-CoV-2 in wastewater. Results showed that the three methods were able to concentrate the seeded wastewater samples as reported in Table 1.

The UF method provided the highest mean SARS-CoV-2 recovery of 54.03 ± 8.25. The second-highest mean recovery was for filtration through the electronegative membrane method (25.59 ± 5.04) (Table 1). The difference of the average was statistically significant ($P < 0.05$).

Next, we compared the mean concentration and virus detection ability in 20 untreated wastewater samples collected from five sites in Tunisia, taking the three concentration methods into account. As presented in Figure 1, the detection of the virus was highly dependent on the used concentration method.

Nineteen of 20 samples tested positive for SARS-CoV-2 with N1-N2-gene primers. SARS-CoV-2 RNA was detected in 19, 17 and 12 samples concentrated by UF, filtration through an electronegative membrane and aluminum hydroxide adsorption–elution method, respectively. The mean ± SD SARS-CoV-2 concentrations in wastewater samples for each concentration method used in this study are shown in Figure 1.

Table 1 | Mean(±SD) of SARS-CoV-2 recovered through each concentration method and recovery efficiency of SARS-CoV-2 using three different viral concentration methods from spiked wastewater

Concentration methods	Mean ± SD SARS-COV-2 concentration (10^5 copies/l of SARS-COV-2 recovered)	Mean ± SD of percentage recovery of SARS-COV-2
Ultrafiltration	48.63 ± 7.42	54.03 ± 8.25
Filtration through a electronegative membrane	23.03 ± 4.53	25.59 ± 5.04
Aluminum hydroxide adsorption–elution	13.9 ± 3.27	15.44 ± 3.64

Note: SD, standard deviation.

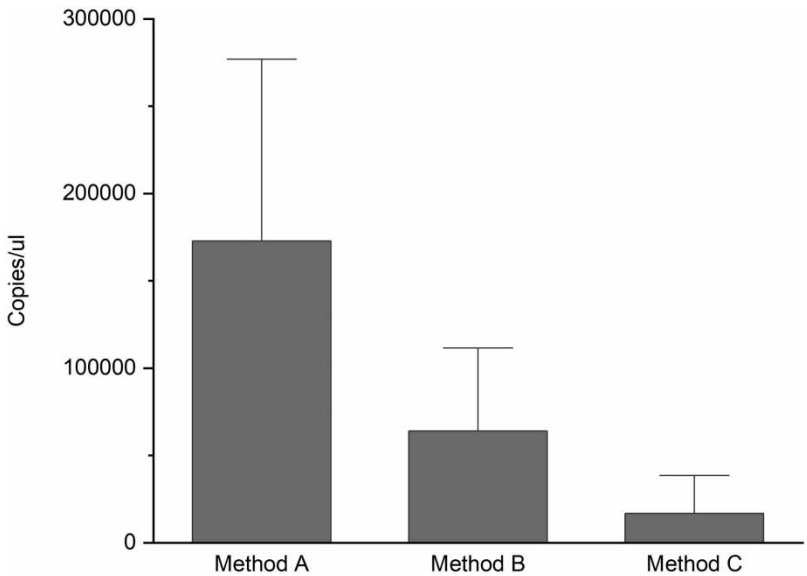


Figure 1 | Mean ± SD SARS-CoV-2 concentration in wastewater samples using the UF method (method A), filtration through an electronegative membrane (method B), and the aluminum hydroxide adsorption–elution method (method C). The error bar represents SD.

The UF method provided a significantly greater mean concentration than the electronegative membrane filtration method and the aluminum hydroxide adsorption–elution method.

The mean concentrations of SARS-CoV-2 were $1.7. \pm 2.08 \times 10^5$, $6.39 \pm 9.5 \times 10^4$ and $1.6 \pm 4.3 \times 10^4$ copies/l by UF, electronegative membrane filtration and the aluminum hydroxide adsorption–elution, respectively. The difference of the mean concentration was statistically significant ($P < 0.001$).

DISCUSSION

Since March 2020, several concentration methods such as UF, precipitation with PEG, electronegative or electropositive membrane filtration, aluminum hydroxide adsorption–elution and skimmed-milk flocculation (SMF) have been applied for the detection of SARS-CoV-2 in wastewater (Ahmed *et al.* 2020c; Jmii *et al.* 2021; Mousazadeh *et al.* 2021). Nevertheless, there is no standardized approach.

UF, electronegative membrane filtration and aluminum hydroxide adsorption–elution are the most common techniques for the concentration of SARS-CoV-2 RNA from wastewater (41, 13 and 8%, respectively) (Ahmed *et al.* 2020c; Mousazadeh *et al.* 2021).

In the current study, these three methods were used for the concentration of SARS-CoV-2. First, these methods were evaluated for their recovery of SARS-CoV-2 from spiked wastewater. The UF method provided the highest mean SARS-COV-2 recovery of 54.03 ± 8.25. Moreover, 95% of the 20 wastewater samples collected from five sites in Tunisia tested positive for SARS-CoV-2 by UF, providing a significantly greater mean concentration of the positive wastewater samples than the two other methods.

Our results confirm previous studies showing that the best concentration method was UF. Thus, this method showed a better performance in terms of recovery efficiency and capacity for viral detection (Bertrand *et al.* 2021; Jafferali *et al.* 2021; Pino *et al.* 2021).

Filtration through the electronegative membrane method has the second-highest mean recovery and means SARS-CoV-2 concentration in wastewater samples. Moreover, 85% of wastewater samples tested positive.

Ahmed *et al.* evaluated the efficiencies of murine hepatitis virus (MHV) (an enveloped and positive-sense single-stranded RNA Betacoronavirus, which belongs to the same genus as SARS-CoV-2) recovery from wastewater using seven wastewater viral concentration methods including UF and electronegative membrane filtration methods. The best mean MHV recovery was achieved by electronegative membrane filtration through 0.45-μm pore size, 47-mm diameter electronegative membranes (Ahmed *et al.* 2020b).

This can be explained by the structural differences between the envelopes of MHV and SARS-CoV-2 that may influence the recovery after the enrichment of water samples. The aluminum hydroxide adsorption–elution method has the least favorable SARS-CoV-2 recovery and mean SARS-CoV-2 concentration in wastewater samples. Compared with the two other methods, the aluminum hydroxide adsorption–elution method has the lowest virus detection ability (60%) in wastewater samples collected from the five sites in Tunisia. This is attributed to the fact that this method requires acidification of the sample. Thus, it has been reported that sample acidification might affect virus integrity and infectivity (Sabatino & Maier 1980; Abdelzaher *et al.* 2008) and that, unlike other enteric viruses, coronavirus may be sensitive to low pH (Ahmed *et al.* 2020b).

To summarize, our study reports that the three methods were able to concentrate SARS-CoV-2, but UF using the Vivaspin® Turbo 15 RC (100 kDa) provides better recovery and virus detection ability in wastewater samples compared to filtration through 0.45-μm pore size, 47-mm diameter electronegative membrane and adsorption–elution using aluminum hydroxide and beef extract. Our study contributes to further knowledge of the quantitative efficiency of methods to concentrate SARS-CoV-2 from wastewater.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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