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Investigation of the recovery efficiency of CeUF method through RT-qPCR quantification of inactivated SARS-CoV-2 in untreated wastewater

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ABSTRACT

Wastewater-based disease monitoring is an early warning system and a surveillance tool for infectious disease outbreaks regarding pathogens with pandemic potential. This study aimed at investigating the recovery efficiency of centrifugal ultrafiltration (CeUF), which is one of the most-used virus concentration methods, for inactive severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) added to wastewater. Inactivated SARS-CoV-2 was inoculated into untreated wastewater at different concentrations (4×10^3, 8×10^3, 16×10^3, 24×10^3 and 32×10^3 gene copy/μL) and concentrated through ultrafiltration with a disposable centrifugal filter device. Total nucleic acids in concentrated filtrates were extracted and isolated by an automated system. In isolates, total RNA was measured by a UV/VIS spectrophotometer, and the recovered virus was quantified by RT-qPCR with two gene regions (N1 and N2). The recovery rates were between 11% and 17.8% (mean 15.1%, CV below 15%). While there were positive correlations among the inoculated virus, total RNA and recovered virus, there was no correlation and linearity between the recovery rates. Despite limited recovery rates, CeUF integrated with RT-qPCR quantification can be a valid assay for monitoring SARS-CoV-2 in wastewater, and an early warning system.

Keywords: Coronavirus; RT-qPCR; SARS-CoV-2; Ultrafiltration; Wastewater

1. Introduction

Severe acute respiratory syndrome coronavirus of 2019 (SARS-CoV-2) causing severe acute respiratory syndrome disease (Covid-19) was first detected on December 12, 2019, in Wuhan, China. It was recognized as a pandemic by the World Health Organization (WHO) on March 11, 2020, since it spread around the world in a brief time and caused millions of human deaths [1]. Coronaviruses (CoVs) that cause Covid-19 disease are part of a large family of viruses which can infect animals and humans. Surrounded by a fatty protein layer called an envelope, coronaviruses are large, positive polarity, single-stranded RNA viruses with protein ridges on the surface. Genomic research on some bats and live animals in the seafood market in the province of Wuhan associated SARS-CoV-2 with bats or bat droppings causing the contamination in the market and in the environment [2].

SARS-CoV-2 negatively affects many physiological systems, the respiratory system in particular. Symptoms such as fever, dry cough, November pain, fatigue and diarrhea are observed in sick individuals [3]. The main routes of transmission of the SARS-CoV-2 are respiratory droplets and direct contact. The World Health Organization reported that the SARS-CoV-2 virus can be excreted with feces without any sign of diarrhea or intestinal infection [1]. The Water Environment Federation (WEF) reported that SARS-CoV-2 can be transmitted via the fecal-oral route, and that

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diarrhea was observed in some patients in the early stages of infection and the virus was detected in stool analyses [4].

Water is an important transmission route for microorganisms. During the Covid-19 outbreak, scientists suspected both food and water supplies of being the transmission route. So, by employing several methods, researchers focused on and analyzed the presence of the virus gene in surface, river, drink, bottled water, as well as wastewater [5–7]. The findings from these environmental samples, wastewater in particular, suggested that quantitative measurements and monitoring of SARS-CoV-2 could provide information about SARS-CoV-2 distribution for the virus epidemiology and be an early warning system [8–11].

Because of inorganic contaminants and debris in environmental samples, wastewater in particular, filtration, concentration, and isolation methods are crucial to qualify and quantify the virus gene [5,12]. Murine hepatitis virus (MHV) and SARS-CoV-2 in Coronaviridae family had been reported to adsorb to the pellet, debris, and particles in untreated wastewater. This adsorption affected the concentration and recovery efficiency of the virus from wastewater [5,12,13]. Electronegative membrane filtration, PEG (polyethylene glycol) precipitation, centrifugal ultrafiltration (CeUF) and ultrafiltration are the most common methods to concentrate and recover the virus from liquid matrixes. During the Covid-19 outbreak, CeUF has become prominent and been preferred in many studies, because it is a fast method with a few steps [5,9,14]. This study aimed at investigating the recovery efficiency of CeUF method by RT-qPCR quantification of inactivated SARS-CoV-2 inoculated into untreated wastewater.

2. Materials and methods

2.1. Wastewater samples and collection sites

Wastewater samples were collected from influent streams (after grit removal) of three wastewater treatment plants (WWTP) in March 2021 in Istanbul, Turkey. Influent samples (n = 3) were collected as 24 h composite samples of 5 L. The temperature (9°C ± 3°C) and pH (7.36 ± 1.52) of the samples were logged at that moment. They were transferred to the laboratory at 4°C in 2 h. Within the same day, all samples were triplicate tested and confirmed to be negative for SARS-CoV-2 before use. Negative wastewater samples confirmed by RT-qPCR were pooled and kept at 4°C for virus inoculation.

2.2. Virus inoculation into wastewater

SARS-CoV-2 was cultivated and inactivated in the BSL-3 laboratory. Inactive virus stock solution (8×10^6 gene copies/μL) was separated from the residual cell debris through centrifugation, and the supernatant was stored in –80°C for further analysis. The dilutions of inactive virus stock solution were prepared in 50 mL (6 replicates) of untreated wastewater with a concentration of 25, 50, 100, 150 and 200 μL/50 mL (4×10^3, 8×10^3, 16×10^3, 24×10^3 and 32×10^3 GC/μL) in BSL-2 cabinet.

2.3. Filtration and concentration of virus by CeUF

We used the ultrafiltration method with a disposable centrifugal filter device (Centricon Plus-70, 10 kDa, Millipore, USA) for the concentration and recovery of the virus (Fig. 1). To remove glycerin as a humectant, the regenerated cellulose membrane filter of the device was pre-rinsed with the molecular grade water by centrifuging at 3,500 × g for 20 min. Then, each dilution spiked with the virus was filtered by CeUF at 3,500 × g for 20 min at 4°C. After filtration, the concentrate collection cup was placed on top of the filter cup, inverted, and centrifuged at 1,000 × g for 2 min to concentrate the filtrate. The concentrated filtrate of approximately 400 μL was recovered from each 50 mL dilution.

2.4. Extraction and isolation of total nucleic acids

The procedure of the isolation kit (ref: 03038505001, Roche Diagnostics GmbH, Germany) and MagNa Pure LC (LCPG 1170, Roche Diagnostics GmbH, Germany) instrument were used to isolate total nucleic acid from each dilution of 200 μL. Briefly, lysis/binding buffer lysed cells and released nucleic acids. For the digestion of proteins, the proteinase K enzyme was used. Then, the released nucleic acids bonded after the addition of MGP (magnetic glass

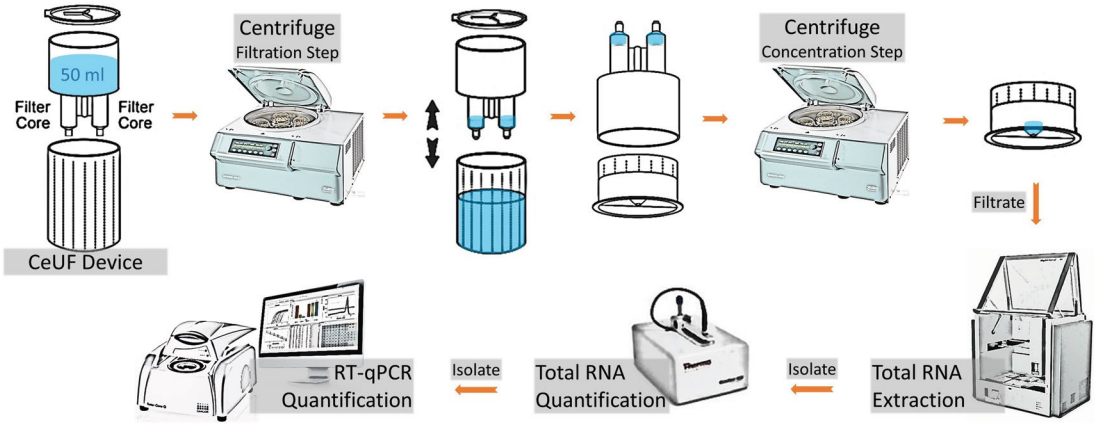

Fig. 1.

particles). MGPs with bound nucleic acids were magnetically separated from the residues and washed with wash buffers to remove such unbound substances like proteins (nucleases), cell membranes, PCR inhibitors and salt. The wash buffer containing residual debris was discarded. The purified nucleic acids were eluted at +70°C from the MGPs in a final volume of 50 μL and stored at +4°C. The quantifications of all samples were performed on the same day.

2.5. Quantitation of total RNA

UV/VIS spectrophotometer (ND-1000, NanoDrop, Thermo Fisher Scientific, USA) was used to measure the total RNA. Before and after each measurement, the upper and lower optical surfaces of the micro-spectrophotometer were cleaned by bathing with sterile deionized water of 2 μL. And, both surfaces were wiped with a Kimwipe (Kimberly-Clark Professional, USA). Nucleic acid samples (six replicates) of 1 μL were used to measure total RNA. For the blank, sterile DNAse/RNAse free water was used. The absorption ratios of 260/280 were verified for the measurement quality.

2.6. RT-qPCR protocol

SARS-CoV-2 analyses were performed in 20 μL reaction volume (5 μL sample and 15 μL master mix) by using SARS-CoV-2 (2019 nCoV) real-time PCR diagnostic kit (Ref: KRM-136-002, V2, KrosQuanT, Turkey) recommended by the World Health Organization (WHO), China-CDC and USA-CDC [4]. The master mix contained two gene regions (N1 and N2) of SARS-CoV-2 virus. For N1 and N2 regions, forward primers, reverse primers and probes were designed as illustrated in Table 1. For the internal control, the human RNAse P gene was used. The RT-qPCR assays were performed using the Rotor-Gene Q (QIAGEN, Hilden, Germany). Thermal cycling conditions consisted of RT at 45°C for 10 min, denaturation and Taq polymerase activation at 95°C for 2 min and 45 cycles at 95°C for 10 s followed by 55°C for 30 s (data collection). RT-qPCR reactions were performed with six replicates for each sample. For initial screening stage of virus identification, the appropriate concentrations of reagents, temperature cycling and sufficient number of replication (39.8 cycles) were used. The whole analysis was performed with the positive and negative controls in the kit by generating the standard curve.

2.7. Data analysis

Data analysis (mean, standard deviation, CV; coefficient of variation and recovery) was made through the SPSS 21 statistical software (SPSS Inc., Chicago, IL, USA). Spearman's correlation coefficient was used to determine the correlations (two-tailed) among recovery rate (%), total RNA (ng/μL) and gene copies measurements (GC). The associations between the parameters were tested through linear regression analysis. The plots of linearity were generated with the Excel 2013 software (Microsoft, California, USA). Differences among data means were tested at a $P < 0.05$ level of significance.

3. Results and discussion

Wastewater matrix generally needs some pre-treatments (pre-centrifuging, manual filtration and removing debris, etc.) because of inhibitor factors such as debris, solid particles, and clogging, which affect the recovery rate of microorganisms [5,12,13,15]. Fores et al. [5] reported that SARS-CoV-2, MHV and MS2 were absorbed or retained by solid fractions of wastewater at the rates of 23%, 26% and 43%, respectively. Meanwhile, up to 30% of MHV inoculated into wastewater was shown to be lost by pre-treatment procedures [12,13]. Furthermore, Jones et al. [15] suggested that the sonication treatment of wastewater and pre-rinsing of filter device decreased the absorption of the virus to the solid fractions of water and increased the recovery efficiency. In this study, the filters of CeUF device were pre-rinsed before use, and untreated wastewater samples without solid particles were used to avoid the loss of virus and to observe the recovery efficiency of CeUF. The absorbance ratio of A260/A280 is an indicator of total nucleic acid purity in the isolate. Solid particles and debris cause an absorption at 280 nm and a reduction in the A260/A280 ratio. A ratio between 1.9 and 2.0 indicates that the isolated total RNA is highly purified [16]. The A260/A280 ratios were determined between 1.78 and 2.09 for all isolates of untreated influent wastewater samples in this study (Table 2). So, the total RNA in isolates (ng/μL) increased linearly with the increasing concentrations of the inoculated ($R^2 = 0.973$, Fig. 2, Table 3) and recovered virus gene copies as quantified by RT-qPCR ($P < 0.01$, Table 4). However, the content of total RNA in isolates did not significantly correlate ($P = 0.543$) and interact ($P = 0.196$) with the recovery rates (Table 4).

Table 1
Sequences of primers and TaqMan probe for the detection of SARS-CoV-2 in this study

Assay	Name	Function	Sequence (5′----3′)	Reference
2019-nCoV_N1	CDCN1-F	Forward primer	GACCCCAAAATCAGCGAAAT	[4]
	CDCN1-R	Reverse primer	TCTGGTTACTGCCAGTTGAATCTG	
	CDCN1-P	TaqMan probe	ACCCCGCATTACGTTTGGTGGACC-	
2019-nCoV_N2	CDCN2-F	Forward primer	TTACAAACATTGGCCGCAAA	[4]
	CDCN2-R	Reverse primer	GCGCGACATTCCGAAGAA	
	CDCN2-P	TaqMan probe	ACAATTTGCCCCCAGCGCTTCAG-	

All probes were labelled with 5′-FAM and Q1 3′.

Table 2
Results of total RNA measured by the UV/VIS spectrophotometer

Inoculated virus (GC/μL)	Total RNA (mean ± SD) (ng/μL)	Absorbance (nm)		
		A260	A280	A260/A280
4,000	3.95 ± 0.26	0.098	0.047	2.09
8,000	8.45 ± 1.07	0.147	0.079	1.86
16,000	14.09 ± 2.34	0.202	0.107	1.89
24,000	22.74 ± 4.13	0.196	0.110	1.78
32,000	25.05 ± 1.47	0.198	0.102	1.94

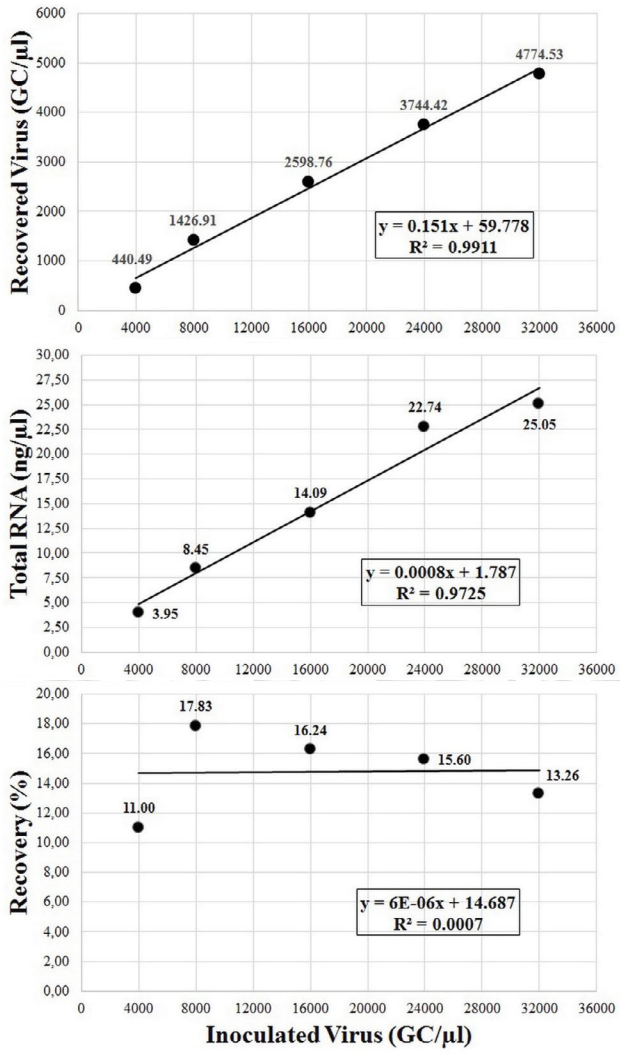

Fig. 2.

Due to the advantages of one-step, minimum equipment and small sample volume, the ultrafiltration devices and methods were preferred to concentrate SARS-CoV-2 and other viral pathogens from the solutions such as ground, surface, drinking waters, and wastewaters. To control the process and the concentrating performance of the devices in wastewater studies, bacteriophage MS2 and an enveloped RNA virus such as murine hepatitis virus (MHV) are frequently used as a surrogate for SARS-CoV-2 [5,12,13,17–19]. In these previous studies, the recovery rates for these enveloped viruses were determined in a very wide range (22%–96%). Ye et al. [13] suggested the CeUF had higher recovery rates for MHV (25.1% ± 3.6%) than methods of PEG precipitation and ultracentrifugation. Fores et al. [5] found the recovery efficiency of CeUF as 34 (±22.71%) and 24.07 (±14.48%) for MS2 and MHV, respectively. Similarly, Ahmed et al. [12] determined the recovery rates of 56% (±32.3%) and 28.0% (±9.10%) for MHV as a surrogate for SARS-CoV-2 from wastewater by CeUF devices.

In north-eastern France during the Covid-19 outbreak in 2020, Bertrand et al. [9] quantified the RdRp_IP4 and envelope protein genes of SARS-CoV-2 in wastewater by using CeUF (Centricon Plus-70). The recovery rates were 64.1% (±50.2%) for RdRp_IP4 and 0% for envelope protein gene (E). With an additional RNA purification by an inhibitor removal kit, the recovery rate could have increased to 45.0% (±44.6%) for E gene of SARS-CoV-2. In the Netherlands, Medema et al. [17] concentrated SARS-CoV-2 from untreated wastewater with CeUF device, and the recovery efficiency was 73% (±50%) with a correlation coefficient of 0.997, 0.992, and 0.987 for N1, N2, and N3 genes of SARS-CoV-2, respectively. Gonçalves et al. [14] used two CeUFs (Amicon Ultra-15) to concentrate SARS-CoV-2 from hospital and untreated wastewaters (n = 6). The recovery efficiency of CeUFs was 22%–96% for RdRP gene and 25%–77% for E gene [9]. Moreover, Gerrity et al. [20] recovered bovine coronavirus at a rate of 17%–93% (±38%) with CeUF.

After the isolation and total RNA measurements in the isolates, recovered gene copies of SARS-CoV-2 and recovery rate was quantified and shown in Table 5. The recovered virus (gene copy/μL) increased linearly as the inoculated virus concentration increased (R^2 = 0.991, Fig. 2). The inoculated virus was recovered at 11%–17.8% by CeUF (15.1% ± 2.53% overall recovery). The highest recovery rate (17.8% and 16.2% respectively) were detected in the wastewater samples inoculated with 8×10^4 and 16×10^4 of virus gene copies. As the inoculated virus concentrations increased, the recovery rate did not increase linearly (R^2 = 0.0007, Fig. 2, Table 3). So, a significant correlation ($P > 0.05$) and regression (P = 0.196) were not observed with the recovery rate (Table 4). The equation between the inoculated virus gene copies and recovery rate was as follows (Fig. 2):

Recovery rate = 6×10^{-6} × inoculated virus (gene copy/μL) – 14.687

Table 3
Parameters of linearity measurements

Items	Recovered virus (GC/μL)	Total RNA (ng/μL)	% Recovery
N of measurement	6	6	6
R^2	0.991	0.973	0.0007
Slope	0.151	8×10^{-4}	6×10^{-6}
Slope SD	0.019	5×10^{3}	3×10^{2}
Intercept	59.77	1.787	14.687
Intercept SD	19.67	1.33	1.27

Table 4
Correlation and regression coefficients between measured parameters

Items	Correlation coefficients						Regression coefficients (β)	
	Recovered virus (GC/μL)	*P*	Total RNA (ng/μL)	*P*	Recovery %	*P*	R^2	*P*
Recovered virus (GC/μL)	1	–	0.896	<0.01	0.059	0.758	0.930	<0.01
Total RNA (ng/μL)	0.896	<0.01	1	–	0.116	0.543	0.902	<0.01
Recovery %	0.059	0.758	0.116	0.543	1	–	0.474	0.196

Table 5
Results of recovery efficiency of ultrafiltration by RT-qPCR quantification

Inoculated virus (GC/μL)	Recovered virus (mean ± SD) (GC/μL)	Ct	CV (%)	Recovery (%)	Overall recovery (%±SD)
4,000	440.49 ± 22.31	17.47	5.1	11.0	
8,000	1,426.9 ± 98.87	15.60	6.9	17.8	
16,000	2,598.8 ± 215.1	14.45	8.3	16.2	15.1 ± 2.53
24,000	3,744.4 ± 452.9	14.20	12.1	15.6	
32,000	4,774.3 ± 575.3	13.74	12.0	14.9	

The coefficient of variation (CV) indicates the precision of the repeated measurements and the precision (% CV) should not exceed 15% in method validation. However, limited replicates of samples and high standard deviation cause an increase in the CV [21,22]. In this study, the coefficients of variation (CV) were calculated as below 15% for all RT-qPCR quantification of SAR-CoV-2 in six replicates (Table 5).

4. Conclusion

This study aimed at determining the detailed efficiency of CeUF by using inactive authentic SARS-CoV-2 at different concentrations. In conclusion, despite the limited recovery efficiency, CeUF might be a rapid and up-to-date method for monitoring the wastewater-based epidemiology of pathogens such as SARS-CoV-2 for the early warning system.

Conflict of interest statement

The authors have no competing or conflict of interests in submitting this article.

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