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Experimental Protocol for Detecting SARS-CoV-2 in Screenings and Grit Samples of Wastewater Treatment Plants

Ali Abdolahnejad [1], Mojtaba Pourakbar [1], Saber Raeghi [2], Amir Mohammadi [1], Behzad Ranjbar [3], Ali Behnami [1, 4]*

[1] *Department of Environmental Health Engineering, Maragheh University of Medical Sciences, Maragheh, Iran.*
[2] *Department of Laboratory Sciences, Maragheh University of Medical Sciences, Maragheh, Iran.*
[3] *Office of Waste Management Organization, Maragheh Municipality, Maragheh, Iran.*
[4] *Department of Environmental Health Engineering, Iran University of Medical Sciences, Tehran, Iran.*

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***Corresponding Author:**
Ali Behnami

Email:
ali.behnami64@gmail.com

Tel:
+989351632650

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ABSTRACT

Introduction: Although various liquid, solid, and gaseous streams of wastewater treatment plants (WWTPs) have been analyzed in many studies for the presence of SARS-CoV-2 RNA, no study was found to sample and detect SARS-CoV-2 RNA in screenings and grit samples separated from primary treatment units of WWTP. Hence, this study aims to provide an experimental protocol for sampling and extracting SARS-CoV-2 RNA from screenings and grits separated from WWTPs.

Materials and Methods: First, sampling was conducted to extract SARS-CoV-2 RNA from screenings and grit samples. After sample processing and viral RNA extraction, SARS-CoV-2 RNA detection was performed by one-step reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Results: Based on the results, SARS-CoV-2 RNA was successfully extracted from screenings and grit samples of the studied WWTP with concentrations of ($1.54 – 3.9 \times 10^4$) and ($0.8 – 2.3 \times 10^4$) genomic copies/L, respectively.

Conclusion: Considering the successfully isolation and detection of SARS-CoV-2 viral RNA in solid phase samples of WWTP, this method can be applied for extracting SARS-CoV-2 RNA and maybe other viruses from the screenings and grit samples of WWTPs in related studies.

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Introduction

The emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is considered a public health emergency of international concern [1]. SARS-CoV-2 symptoms are mainly fever, dry cough, tiredness, and loss of taste or smell, shortness of breath, muscle pains, sore throat, and conjunctivitis. However, human Corona Viruses (CoVs) are also known to cause gastrointestinal symptoms, such as nausea and diarrhea [2-5]. Although SARS-CoV-2 is considered to be transmitted via inhalation of contaminated aerosol/droplet, recent studies have confirmed the existence of SARS-CoV-2 viral RNA in the human stool samples [6, 7]. Therefore, SARS-CoV-2 RNA can enter the sewage collection network and finally wastewater treatment plant (WWTP) through fecal shedding and nasal/oral secretions of the infected patients [8]. Thus, wastewater can be considered a critical pathway to transmit SARS-CoV-2 viral particles into the environment, which poses a threat to WWTP staff and nearby residents [9]. Therefore, examining the wastewater for SARS-CoV-2 RNA could be a useful tool to find possible virus transmission routes through the WWTP [3, 10, 11]. SARS-CoV-2 RNA can enter into the environment

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through various streams of a WWTP, such as final effluent, disposed sludge, separated waste and grits, and bioaerosols. Several investigations have been conducted worldwide to investigate SARS-CoV-2 RNA presence in wastewater [2, 10, 12], biosolids [5, 13], and bioaerosols [8]. Tracking SARS-CoV-2 RNA in wastewater showed high affinity of the virus toward biological solids rather than liquid phase. Hence, sludge lines in a WWTP have been reported as a suitable spot for extracting and detecting SARS-CoV-2 viral RNA [5, 13]. Therefore, considering the results of previous studies, screenings and grits separated from primary treatment units can be also considered as an alternative to track viral RNA in a WWTP. However, no study has been conducted investigating the presence of SARS-CoV-2 RNA in screenings and grit samples separated from screening and grit removal units of a WWTP. In addition, a protocol describing the extraction of viral RNA from screenings and grit samples has not yet been reported elsewhere. Therefore, in this protocol article, a step-by-step protocol was provided for SARS-CoV-2 RNA extraction from screenings and grit samples collected from the studied WWTP.

Materials and Methods

Study area and sampling

Samples were taken from Maragheh WWTP located in north-west of Iran. The studied WWTP consists of two different modules: a conventional activated sludge (CAS) system and a sequencing batch reactor (SBR). In the studied plant, the primary treatment unit is the same for both CAS and SBR systems. Screenings and grit samples were collected from screening and grit removal units, respectively (Figure 1). In this study, four sampling runs were carried out over a period of 4 months from 28 December 2020 to 13 April 2021. Time-proportional, 12 h composite wastewater samples were collected at 4-hourly intervals [14]. In total, eight composite samples were collected during four sampling periods. At each sampling period, 2 L of solid waste and grit samples were collected, preserved on ice (4 °C), and transported to the Maragheh Cellular-Molecular diagnostics laboratory for viral RNA extraction and detection.

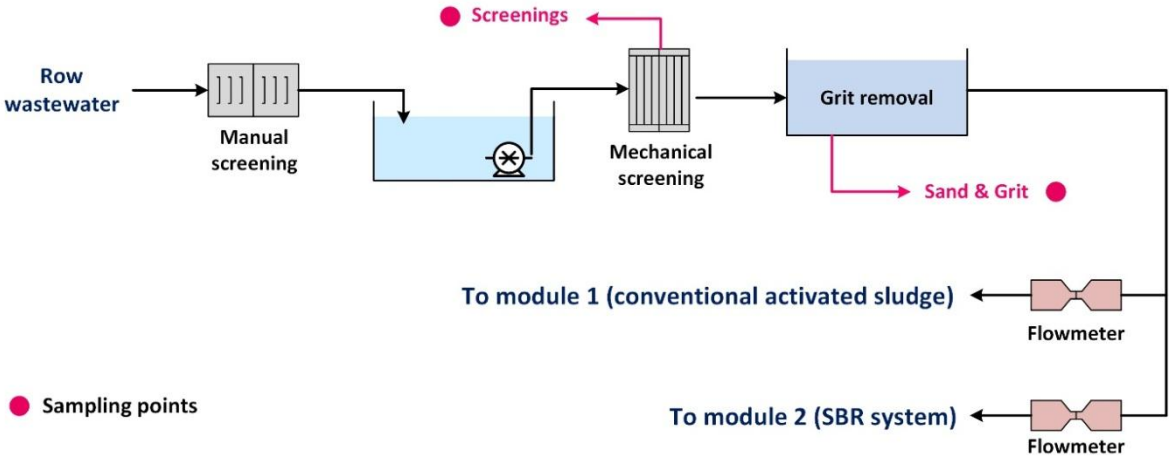

Figure 1: Sampling points of the studied WWTP

Sample processing

Upon arrival at the Cellular-Molecular diagnostics laboratory, the following steps were performed for SARS CoV-2 RNA extraction from solid waste and grit samples:

First, the collected screenings and grit samples were transported into the vessel coupled with mesh tray and washed many times with deionized water to transfer SARS CoV-2 viral RNAs from the solid phase into the liquid phase. Then, 200 mL of each prepared sample was removed and centrifuged at 3500 rpm for 30 min to eliminate the large floating particles. After that, aluminum hydroxide adsorption-precipitation method as explained in reference criteria [15] was used to concentrate SARS-CoV-2 viral RNA in the samples. Finally, the

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concentrated samples were stored at –80 °C prior to being processed and analyzed for RNA extraction. The regents and tools used for the extraction and detection of SARS-CoV-2 are summarized in Table 1.

Figure 2 schematically describes all the steps conducted for recovery, extraction, and detection of SARS-CoV-2 viral RNA in solid waste and grit samples.

Table 1: The regents and equipment used in extraction and detection of SARS-CoV-2

Process	Reagents/tools
SARS-CoV-2RNA extraction	Proteinase k Elution buffer Lysis buffer Collection Tube (2ml) Ethanol (Merck-Millipore) Nuclease-free tips (Rnase/Dnase free) Sodium chloride solution (Sigma-Aldrich)
SARS-CoV-2 detection	Multiplex One Step q-Real Time PCR kit (Pishtazteb®, Iran) RdRp region Specific Primers/ FAM Probe N Gene Specific Primers/ HEX Probe RNase P Endogenous Control Primers/ ROX Probe Positive Control Template One Step q-RT PCR Master Mix (5X) Resuspension Buffer DEPC-H_2O served as negative control (−)
Equipment	Micropipettes (Eppendorf, P-10, P-100 and P-1000) Water bath (Thermo-Scientific) Mic Real-Time PCR System NanoDrop 2000 (Thermo ScientificTM) Microcentrifuge (Hettich MIKRO 200-R) Vortex Mixer (ONiLAB) Class 2 biological safety cabinet

SARS-CoV-2 extraction

SARS-CoV-2 viral RNA extraction and one-step reverse transcription quantitative polymerase chain reaction (RT-qPCR) were carried out based on the following steps at Maragheh Cellular-Molecular diagnostics laboratory, a specialized center assigned to SARS-CoV-2 diagnosis.

First, 200 µL of each concentrated sample was used for SARS-CoV-2 viral RNA extraction using the RNJia Virus Kit (ROJETechnologies, Yazd, Iran), according to manufacturer protocol [16]. Then, 200 µL of each sample was removed and vortexed for about 5 min. Lysis buffer containing RNA carrier and proteinase K was added to the sample and vortexed again for 15 s and incubated at room temperature (~ 22 °C) for about 10 min. After that, 280 µL of pure ethanol (99.99%) was added to the mixture and vortexed again for 15 s. The resulting solution was passed through the separating column as follows: First, the homogenate was injected into the RNA separating column and centrifuged at 8000 rpm for 1 min allowing RNA to be isolated. Second, the columns were washed in two stages using washing solution 1 and 2, and centrifuged again at 14000 rpm for 3 min. Then, the columns were transported to the RNA collecting tubes, 50 - 70 µL elution buffer was added, and centrifuged at 8000 rpm for 1 min.

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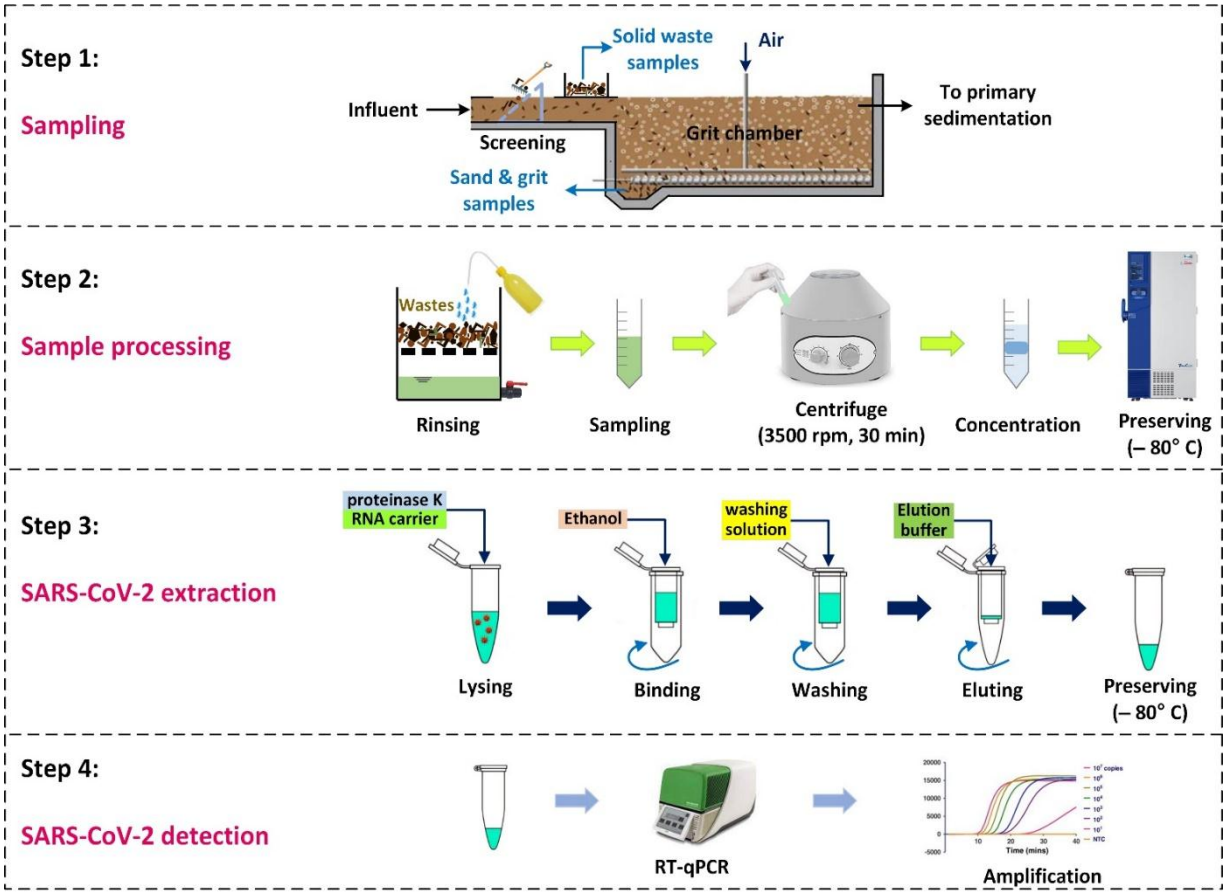

Figure 2: Stepwise steps for extracting and detecting SARS-CoV-2 RNA in screenings and grit samples separated from the studied WWTP

SARS-CoV-2 detection

Molecular detection of SARS-CoV-2 was performed using RT-qPCR assay. COVID-19 ONE-STEP RT-PCR kits (Pishtaz Teb Diagnostics, Tehran, Iran) was used for diagnosing SARS-CoV-2 genes in the prepared samples in accordance to the manufacturer's instructions. The primer and probe mixture of the kit is designed based on dual-target gene method targeting two different regions of the SARS-CoV-2 genome, specifically RdRp and N genes. The kit includes a solution containing probe and internal control primer (RNase P) for improving the accuracy of sampling process and preventing false negative results. Furthermore, the kit includes PCR negative (Diethyl pyrocarbonate (DEPC)-treated water) and positive controls. For each PCR run, 10 µL of prepared sample was added to 10 µL of master mix and primer probe mixture. The thermal cycling conditions for the RT-qPCR consisted of reverse transcription at 50 °C for 20 min and cDNA initial denaturation cycle at 95 °C for 3 min. It was followed by 45 cycles of denaturation at 94 °C for 10 s, and annealing and extension reaction at 55 °C for 40 s, and cooling at 25 °C for 10 s. The qPCR time-temperature protocol was in accordance with the kit's instructions.

Quality control/ Quality assurance

In present study, a negative control was used to investigate possible cross over contamination of the prepared samples during viral RNA extraction process. In order to improve the accuracy and also re-checking the samples, different SARS-CoV-2 detection kits were used. As the kits included Novel Coronavirus (2019-nCoV) Nucleic Acid Diagnostic Kit (Sansure Biotech, China), which targets N and ORF-1ab genes, and Novel Coronavirus (2019-nCoV) Real Time Multiplex

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RT-PCR Kit (Liferiver Bio-Tech, US), which targets ORF1ab, N, and E genes. Each sample was examined in triplicate. The interpretation of the RT-qPCR results was carried out as follows. The target gene (RdRp, N) with a Ct value lower or equal to 40 was considered positive for SARS-CoV-2 RNA. In this study, a sample was considered positive for SARS-CoV-2 RNA when at least two of the three corresponding replicates were positive. For quantification of SARS-CoV-2 RNA, 10-fold dilutions of SARS-CoV-2 positive control of the reference kit were used to construct the standard curves.

Ethical issue

The ethical issue of this research was IR.MARAGHEHPHC.REC.1399.024

Results

SARS-CoV-2 RNA detection in solid waste and grit samples

Table 2 illustrates the mean amplification cycles of SARS-CoV-2 RNA in collected screenings and grit samples. The SARS-CoV-2 RNA was successfully isolated and detected in the screenings and grit samples separated from the primary treatment units of the studied WWTP using the proposed protocol. As can be seen in Table 2, the separated screenings and grit samples were positive for SARS-CoV-2 RNA in half of the collected samples. Based on the results, SARS-CoV-2 genome concentration in the screenings and grit samples were (1.54 –3.9 × 10^4) and (0.8 – 2.3 × 10^4) genomic copies/L, respectively.

Table 2: Mean amplification cycles of SARS-CoV-2 RNA in screenings and grit samples of the studied WWTP

Sampling point	Sample type	Molecular target	12/28/2020	01/05/2021	01/13/2021	04/14/2021
Screening	Screenings	RdRp	-			-
		N	-			-
Grit removal	Sand and grit	RdRp	-			-
		N	-			-

* Results are reported for each of the two regions of the SARS-CoV-2 RNA; RdRp and N genes.
Abbreviation: white boxes = negative.

Ct scale: 30 31 32 33 34 35 36 37 38 39 40

The results revealed that a portion of the inlet SARS-CoV-2 RNA to the WWTP are attached to the solids and can be transmitted into the environment via waste disposal.

Conclusion

Considering that no available method was found in the literature illustrating virus recovery from screenings and grit samples, this study provides experimental protocol for SARS-CoV-2 RNA recovery from these samples. The results confirm the potential of the proposed method for extraction of SARS-CoV-2 RNA and maybe other viruses from the screenings and grit samples of WWTPs.

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Conflict of interest

The authors declare that there is no conflict of interest.

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