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Detection of SARS-CoV-2 RNA in hospital wastewater from a low COVID-19 disease prevalence area

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HIGHLIGHTS

• The first study in Slovenia that reports the detection of SARS-CoV-2 in untreated wastewater.
• 66.7% (10/15) of untreated hospital wastewater samples tested positive for at least one SARS-CoV-2 RT-qPCR target.
• SARS-CoV-2 RNA was detected in untreated hospital wastewater when only one COVID-19 patient was hospitalized.

ABSTRACT

Previous studies on SARS-CoV and MERS-CoV reported the detection of viral RNA in the stool of both symptomatic and asymptomatic individuals. These clinical observations suggest that municipal and hospital wastewater from affected communities may contain SARS-CoV-2 RNA. Recent studies have also reported the presence of SARS-CoV-2 RNA in human feces. Wastewater-based epidemiology (WBE) is a promising approach to understand the prevalence of viruses in a given catchment population, as wastewater contains viruses from symptomatic and asymptomatic individuals. The current study reports the first detection of SARS-CoV-2 RNA in untreated wastewater in Slovenia. Two sizes of centrifugal filters were tested: 30 kDa and 10 kDa AMICON® Ultra-15 Centrifugal Filters, where 10 kDa resulted in a higher concentration factor and higher recovery efficiency. The results in hospital wastewater show that WBE can be used for monitoring COVID-19 and could be applied in municipal wastewater treatment plants as a potential complementary tool for public health monitoring at population level.

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ARTICLE INFO

Article history:
Received 22 September 2020
Received in revised form 19 October 2020
Accepted 20 October 2020
Available online 28 October 2020

Editor: Damia Barcelo

Keywords:
SARS-CoV-2
COVID-19
Wastewater
tqPCR
Wastewater-based epidemiology
Virology

1. Introduction

Coronavirus Disease 2019 (COVID-19) is an ongoing global pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Symptoms of COVID-19 patients include dry cough, myalgia, fatigue, fever, shortness of breath, diarrhea, anosmia and ageusia (Chen et al., 2020; Guan et al., 2020; Huang et al., 2020; Sharifian-Dorche et al., 2020; Wang et al., 2020). SARS-CoV-2 has spread to almost all countries and territories of the world with more than 30 million confirmed cases and 950,000 deaths (WHO, 2020).

Previous studies on SARS-CoV and MERS-CoV reported the detection of viral RNA in the stool of both symptomatic and asymptomatic individuals (Corman et al., 2016; Leung et al., 2003). Recent studies also...
reported the presence of SARS-CoV-2 RNA in human stool (Cai et al., 2020; Gao et al., 2020; Tang et al., 2020). These clinical observations suggest that municipal and hospital wastewater from affected communities may contain SARS-CoV-2 viral particles and/or RNA (Ahmed et al., 2020a). Wastewater-based epidemiology (WBE) is a promising approach to understand the prevalence of viruses in a given catchment population, as wastewater contains viruses from symptomatic and asymptomatic individuals. WBE is particularly important for early warning of disease outbreaks and information on the effectiveness of public health interventions, as previously demonstrated for enteric viruses such as norovirus, rotavirus, hepatitis A virus and poliovirus (Asghar et al., 2014; Gonçalves et al., 2018; Hellmér et al., 2014). When it was first proposed to track SARS-CoV-2 RNA, the prevailing scientific opinion was that the virus can be released into wastewater at insufficiently high rates and that both the viral particles and its RNA may be too unstable to be detected in wastewater (Medema et al., 2020). Nevertheless, three preliminary studies successfully report the detection of SARS-CoV-2 RNA in wastewater in the Netherlands, the United States of America and Australia (Ahmed et al., 2020a; Lodder and de Roda Husman, 2020; Medema et al., 2020; Sherchan et al., 2020).

A concentration step is usually employed as a preceding preparative step prior to molecular detection of viral DNA or RNA from wastewaters. SARS-CoV-2 RNA has been concentrated from wastewater by PEG precipitation (Wu et al., 2020), electronmagnetic filters, ultracentrifugation membrane filters (Medema et al., 2020; Sherchan et al., 2020), ultracentrifugation (Wurtzler et al., 2020), Al(OH)₃ adsorption-precipitation (Randazzo et al., 2020) and by the adaptation of the standard WHO protocol for Poliovirus surveillance (WHO, 2002).

The current study reports the first detection of SARS-CoV-2 RNA in untreated hospital wastewater in Slovenia. The results show that WBE is a potential tool that could be used as an early warning for COVID-19 and could be applied in municipal wastewater treatment plants as a potential complementary tool for public health monitoring at population level.

2. Materials and methods

2.1. Nasopharyngeal swab samples positive for SARS-CoV-2

Anonymous positive nasopharyngeal swab specimens for SARS-CoV-2 were collected from patients diagnosed with COVID-19 in the Department for Public Health Microbiology Ljubljana, National Laboratory for Health, Environment and Food. These native samples were used to spike the wastewater samples to test the performance of the concentration step and serially diluted 10-fold to 1 × 10⁵ followed by RT-qPCR detection in triplicates. A standard curve was obtained by plotting the Cq values for each dilution against the Log₁₀ dilution for RdrP and E genes and used to calculate the recovery efficiency and concentration factor for each gene, and relative quantification of the water samples tested.

2.2. Sampling of wastewater

One liter of untreated hospital wastewater samples per day were collected from June 1 to 15, 2020 (15 wastewater samples) from a pumping station representing the Department of Infectious Diseases, University Medical Center Ljubljana catchment area, starting when no positive COVID-19 patients were admitted to the hospital. As part of the main hospital complex in Slovenia, the Department of Infectious Diseases has 10 beds in the intensive care unit, 68 beds for adult patients on 3 wards and 51 beds for children and accompanying adults. The sampling personnel wore standard personal protective face equipment during wastewater sampling, including long trousers, steel-capped boots, hard hats, face mask, safety goggles and gloves. Samples were collected using a refrigerated automatic sampler (Avalanche, Teledyne Ecolab USA), resulting in a 24-h cumulative sample (set to sample 70 ml every 10 min). The samples were transported to the laboratory on ice and stored at −70 °C until further analysis.

2.3. Sample concentration

Wastewater samples were mixed and 100 ml of each sample was used for the concentration with 30 kDa and 10 kDa Amicon® Ultra 15 Centrifugal Filters (Merck KGaA, Germany). The samples were pre-filtered with a glass fiber filter membrane with 0.7 μm pore size (Sartorius AG, Germany). The centrifugal devices had a sample volume of 20 ml, therefore, the 100 ml sample was divided evenly and centrifuged five times at 4000 xg for 20 min. A fraction was collected before the concentration step (BC), the sample that passed through the filter (FC), and the concentrated sample that remained on the filter (E). Each fraction was stored at −70 °C until further analysis.

2.4. Viral RNA extraction

170 μl of wastewater samples were centrifuged for 10 min at 2000 rpm to form a pellet of larger particles that could interfere with the extraction process. 140 μl of each supernatant was used for viral RNA extraction using the automated nucleic acid extraction instrument QIAacube Connect (QIAGEN, USA) and QIAamp® Viral RNA Mini Kit (QIAGEN USA) according to the manufacturer’s instructions. The procedure resulted in 50 μl extracted RNA. A Negative Control of Isolation (NCI) was used with each viral RNA extraction set and prepared in the same manner as a sample, except that nuclease-free water was added in place of the sample. The NCI was used to monitor possible cross-contamination during viral RNA extraction.

2.5. Detection and quantification of E and RdrP genes by RT-qPCR

SARS-CoV-2 RNA was detected by targeting the genes E and RdRP, as described by Corman et al., 2020, and as perfumed for more than 50% of the clinical samples in Slovenia for COVID-19 diagnostics (performed at the National laboratory of Health, Environment and Food, Slovenia). For both assays, 20 μl reactions, including 5 μl of extracted viral RNA, 4 μl of 5 x mastermix and 0.1 μl of RT enzyme (both Roche LightCycler® Multiplex RNA Virus Master), 0.5 μl LightMix® Modular SARS-Cov-2 primers and probes mix for the respective gene (E gene and RdRP, both TIB MOLBIOL) and 10.4 μl of PCR grade water were used for the RT-qPCR reaction. The PCR was performed with an RQ6000 or RQG PCR cycler (QIAGEN USA). Primer and probe sequences are shown in Table 1 (Corman et al., 2020). The cycle conditions for RT-qPCR assays were: 55 °C for 5 min for reverse transcription, followed by 95 °C for 5 min and 45 cycles of 95 °C for 5 s, 60 °C for 15 s and 72 °C for 15 s. All experiments were performed in three repetitions. A Negative Control (NC) and a Positive Control (PC) were added to each RT-qPCR to monitor the performance of the RT-qPCR. The quantification cycle (Cq) for each individual amplification was determined using the software Rotor-gene 1.7.87 (QIAGEN, Germany). For all calculations, the baseline
was automatically and the fluorescence threshold was manually set to 0.06 for both targets. A known concentration (2 ng) of luciferase (luc) RNA (Promega, Madison, USA) was added as an external control for the extraction procedure and to report potential PCR inhibitory effects inherent to the samples, according to Toplak et al., 2004.

2.6. Calculation of RdRP and E genes recovery efficiency and concentration factor

1 ml of anonymized positive nasopharyngeal swab specimens were spiked into 1 ml of a wastewater sample and incubated at room temperature at a rate of 500 rpm for 15 min. Total viral RNA was extracted from the spiked sample. The extracted RNA was 10-fold serially diluted in nuclelease-free water from neat $1 \times 10^6$ and assayed for RdRP and E genes with RT-qPCR in triplicate. A standard curve was obtained by plotting the Cq values for each dilution against the Log$_{10}$ dilution for each target (Fig. 1). Using the equation for the standard curve, the recovery efficiencies and concentration factors for RdRP and E gene were calculated as follows:

$$\text{RdRP gene Concentration Factor} = \frac{10^{\left(\frac{\text{Cq}_{\text{BC}} - \text{Cq}_{\text{E}}}{3.386}\right)}}{10}$$

$$\text{E gene Concentration Factor} = \frac{10^{\left(\frac{\text{Cq}_{\text{BC}} - \text{Cq}_{\text{E}}}{3.386}\right)}}{10}$$

$$\text{RdRP gene Recovery efficiency (\%)} = \frac{10^{\left(\frac{\text{Cq}_{\text{E}} - \text{Cq}_{\text{BC}}}{1.856}\right)}}{10} \times \frac{\text{E volume}}{\text{BC volume}} \times 100$$

$$\text{E gene Recovery efficiency (\%)} = \frac{10^{\left(\frac{\text{Cq}_{\text{E}} - \text{Cq}_{\text{BC}}}{1.856}\right)}}{10} \times \frac{\text{E volume}}{\text{BC volume}} \times 100$$

where E stands for the eluted sample remaining on the filter and BC for the sample before concentration.

3. Results

3.1. Performance of the concentration method

Based on the properties of SARS-CoV-2, especially the fact that it is a spherical particle with a diameter between 60 and 140 nm (Bar-On et al., 2020), and that centrifugal filters with the same properties have been successfully used to recover SARS-CoV-2 RNA from wastewater samples (Ahmed et al., 2020b), 30 kDa and 10 kDa AMICON® Ultra-15 Centrifugal Filters were tested.

The two size filters were tested by spiking hospital wastewaters with an anonymized positive clinical sample for SARS-CoV-2 (in triplicate) to hospital wastewater (1 ml of positive clinical sample in 99 ml in hospital wastewater). 20 ml of the spiked sample was added to the 30 kDa and 10 kDa AMICON® Ultra-15 Centrifugal Filters and centrifuged at 4000 x g for 20 min. The procedure was repeated 5 times for each sample until the 100 ml of the spiked sample had passed through the filter membrane. The resulting eluate had a volume of approximately 200 ± 30 μl. Considering the applicability of the method for WBE, the whole procedure was completed for 6 samples in approximately 120 min.

A fraction was collected before the concentration step (Before concentration - BC), the water that passed through the filter (Flow-through – FC), and the eluted sample that remained on the filter (Eluate – E). Aliquots of the collected fractions were used to evaluate the concentration step for both assays, as described in sections 2.4 and 2.5.

30 kDa and 10 kDa centrifugal filters have successfully concentrated and recovered SARS-CoV-2 RNA from wastewater (Table 2). The concentration factors ranged from 204-fold to 327-fold for the RdRP gene and from 129-fold to 242-fold for the E gene. The recovery efficiency ranged from 40.7% to 65.3% for the RdRP gene and from 25.9% to 48.4% for the E gene. Centrifugal filters with a cutoff of 10 kDa showed higher variability, with concentration factors ranging from 110-fold to 483-fold times for the RdRP gene and from 154-fold to 389-fold for the E gene. Similarly, the recovery efficiencies ranged from 22.0% to 96.5% for the RdRP gene and from 30.8% to 77.9% for the E gene.

3.2. Concentration, detection, and quantification of SARS-CoV-2 from untreated hospital wastewater

In this study SARS-CoV-2 was concentrated and detected from hospital wastewater samples. A total of 15 untreated hospital wastewater samples were tested for the presence of SARS-CoV-2 RNA. A sample was considered positive if the Cq was below 40 in at least 2 of 3 replicates and a clear amplification was observed in at least one of the two targets.

As summarized in Table 3, two samples were positive for both the RdRP and E genes (13.4%) without prior concentration step. The average Cqs were 36.22 and 37.03 for RdRP and 36.61 for the E gene. After concentration with 30 kDa AMICON® Ultra-15 centrifugal filters, two samples were positive for the RdRP gene (13.3%) and four were positive for the E gene (26.7%). For RdRP, the average Cqs ranged from 35.40 to 38.46 and for the E gene from 35.40 to 38.45. The highest number of positive samples was achieved when the concentration step was carried out with 10 kDa AMICON® Ultra-15 centrifugal filters. Ten samples were positive for the RdRP gene (66.7%) with average Cqs between

![Fig. 1](image-url)
In all samples. The RdRP target seems to be more sensitive than the E target, which is in accordance with the literature (Corman et al., 2020).

### 3.3. Comparison between the number of hospitalized patients diagnosed with COVID-19 and RT-qPCR quantification of SARS-CoV-2 in hospital wastewater

To assess the prospective use of SARS-CoV-2 WBE, data on the number of hospitalized patients diagnosed with COVID-19 in the Department of Infectious Diseases, University Medical Center, Ljubljana, were compared with the quantification of SARS-CoV-2 RNA from wastewater. At the time of sampling Ljubljana was a low-prevalence area with a maximum of 4 COVID-19 hospitalized patients in the largest infectious disease hospital in the country.

To better visualize and see the variations in the RT-qPCR results over time, the average Cq values obtained for each dilution and target (as described in section 2.6) were plotted against a hypothetical number of Log_{10} copies (10, 9, 8, 7 and 6). Using the equation established, hypothetical number of copies were calculated for each sample previously concentrated with 10 kDa AMICON® Ultra-15 centrifugal filters.

The number of hospitalized COVID-19 patients and the hypothetical number of copies for each target are shown in Fig. 2. The RdRP was positive from one day after the first hospitalization of one patient (4th of June). E gene was positive from the 8th of June (5 days after the first patient was hospitalized). The sample from the 5th of June was negative for RdRP, which may have been caused by the heavy rainfall recorded on that day, which may have diluted the sample collected, or by a variable shedding rate of the patients.

### 4. Discussion

Several studies have been conducted to detect and quantify SARS-CoV-2 RNA in untreated wastewater during the ongoing COVID-19 pandemic (Ahmed et al., 2020a; Kocamemi et al., 2020; Sherchan et al., 2020; Wu et al., 2020). Nevertheless, further studies are needed to test existing concentration methods used to concentrate and recover SARS-CoV-2 RNA and to investigate whether the presence of SARS-CoV-2 RNA in untreated municipal and hospital wastewater can be used as an early warning of COVID-19 infections in communities. In this study, the molecular detection of SARS-CoV-2 RNA was performed according to Corman et al., 2020. The assay was widely used for the detection of SARS-CoV-2 RNA in clinical samples. To the best of our knowledge, this is the first study in Slovenia in which SARS-CoV-2 RNA has been successfully detected in wastewater and especially in hospital wastewater.

Routine safe working practices (US EPA, 2020) have been applied that effectively protect sampling personnel from exposure to pathogens.
in wastewater and there is no evidence that SARS-CoV-2 has been transmitted via wastewater. Studies have shown that the fecal load of SARS-CoV-2 is not infectious (Woeielf et al., 2020). Therefore, the authors of this study suggest that wastewater sampling should be performed according to established safety guidelines.

Two sizes of centrifugal filters were tested: 30 kDa and 10 kDa AMICON® Ultra-15 Centrifugal Filters. The two filter sizes were chosen because of the size of SARS-CoV-2 (60 to 140 nm) and according to previous studies on viruses with similar properties (Ahmed et al., 2020a, 2020b; Bar-On et al., 2020). The choice of AMICON® Ultra-15 centrifugal filters instead of Centricon® Plus-70 centrifugal devices is due to the availability of the equipment. In previous studies (Sherchan et al., 2020) Centricon® Plus-70 was used, which allows the concentration of viruses from larger water volumes in one step. However, its use requires a centrifuge that can hold a volume of 70 ml, which is not generally available in clinical microbiology laboratories. The AMICON® Ultra-15 was able to hold a volume of up to 20 ml in a standard-sized centrifuge, making it easier to use in other laboratories with similar settings (Medema et al., 2020; Wu et al., 2020).

30 kDa and 10 kDa AMICON® Ultra-15 Centrifugal Filters successfully concentrated and recovered SARS-CoV-2 RNA from wastewater as shown in Table 2, where 10 kDa leads to a higher concentration factor and higher recovery efficiency, but also to higher variability. Ultrafiltration based on centrifugal filters seems to be an efficient method to recover SARS-CoV-2 RNA. These methods were previously used for the concentration of viruses from wastewater and environmental waters (Ijner et al., 2012; Rosario et al., 2009) and more recently for the concentration of SARS-CoV-2 RNA from untreated wastewater (Ahmed et al., 2020a; Kocamemi et al., 2020; Medema et al., 2020). In ultrafiltration with centrifugal filters, viruses are concentrated based on size exclusion, i.e. molecules smaller than the molecular weight cut-off are passed through the membrane by centrifugation, while larger molecules are retained and collected in the eluate fraction. The recovery efficiencies and concentration factors in this study are higher than those reported in previous studies with Centricon Plus-70 (Medema et al., 2020). The observed variation could be due to differences in filter design. The Centricon Plus-70 has a larger surface area for filtration compared to the AMICON® Ultra-15 and consequently more SARS-CoV-2 RNA could have been lost by adsorption to the membrane via hydrophobic binding and/or van der Waals interaction forces (Ijner et al., 2012). Ahmed et al., 2020a, 2020b showed a similar result by comparing 30 kDa AMICON® Ultra-15 and 10 kDa Centricon Plus-70 to concentrate murine hepatitis virus.

The main advantages of AMICON® Ultra-15 filters are the speed of the protocol, up to 1 h depending on the turbidity of the sample; the need for a generally available centrifuge; the comparable efficiency with other methods requiring more specific equipment and consumables; and the simplicity of the protocol. However, the low speed pre-filtration step required to remove large debris and cells prior to centrifugation is an important limitation. Pre-filtration can lead to loss of particle-associated viruses in the eluate. A previous study showed that the loss can be about 30% (Ahmed et al., 2020b). RT-qPCR inhibition has not yet been reported, but centrifugal filters can co-concentrate PCR inhibitors, especially when performed to concentrate viral RNA from large volumes of water samples. The SARS-CoV-2 RNA concentration factor and recovery rates probably vary depending on the properties of the wastewater, such as the concentration of total and dissolved suspended solids. Information on wastewater composition, environmental factors and weather, such as precipitation levels, are also important for WBE, as they can influence the performance of centrifugal filters (high turbidity) and heavy rainfall could dilute the sample (Gonçalves et al., 2018).

Considering the advantages and disadvantages, AMICON® Ultra-15 Centrifugal Filters were successfully employed to concentrate SARS-CoV-2 RNA from wastewater and used to detect SARS-CoV-2 RNA from hospital wastewater over a period of 15 days. The 15-day sampling was used to determine the ability of AMICON® Ultra-15 Centrifugal Filters to concentrate SARS-CoV-2 RNA present in a range of hospital wastewaters of unknown turbidity and to determine its presence at the sampling site. Before a concentration step, 13.3% (2/15) of the samples were positive for SARS-CoV-2 RNA. After concentration with 30 kDa centrifugal filters 26.7% (4/15) were positive. 10 kDa centrifugal filters yielded the highest detection rate of 66.7% (10/15). These results show the necessity of a concentration step before molecular detection of SARS-CoV-2 RNA (Bofill-Mas and Rusiñol, 2020). Two samples were positive before concentration with 30 kDa AMICON® Ultra-15, but negative after concentration for at least one of the targets. Although this was not the case with 10 kDa centrifugal filters, in one of the samples (13. 6. 2020) the Cq value after concentration was higher for the E gene than before concentration. The observations suggest that centrifugal filters could concentrate PCR inhibitors and/or that pre-filtration caused a loss of particle-associated viruses, as previously suggested (Ahmed et al., 2020a). A further limitation of the study is that the infectivity of the viral particles cannot be assessed, as biosafety level 3 laboratory (BSL3) laboratory equipment is required for the isolation of SARS-CoV-2 in cell cultures. In the current study, the Cqs for both targets ranged from 29.65 to 38.12. The observed Cqs were slightly lower than in previous studies, where the Cqs ranged from 34 to 40 (Medema et al., 2020; Randazzo et al., 2020; Wu et al., 2020).

During the sampling period, the number of hospitalized patients with COVID-19 was low and ranged from 0 to 4. As shown in Fig. 2, SARS-CoV-2 RNA was detected in hospital wastewater when only one patient was hospitalized. The hypothetical number of copies were calculated according to the standard curve obtained from the spiked

![Fig. 2](image-url)
wastewater sample (section 2.6). The standard curve obtained is a limitation in the current study, as it may not mimic the potential association of SARS-CoV-2 RNA with particles. In addition, the variation in the hypothetical number of copies may be due to the composition of the wastewater, particularly turbidity, with the strong precipitation and/or differences in viral shedding rate of the patients. Current results show that surveillance of wastewater for SARS-CoV-2 RNA provides a useful epidemiological approach that can help to monitor the ongoing pandemic and support public health measures. The present study complements the increasing number of studies that are establishing an important link between wastewater surveillance and epidemiology COVID-19.

5. Conclusions

The present study is the first to report the detection of SARS-CoV-2 RNA in wastewater in Slovenia using RT-qPCR and it shows that viral concentration methods are an essential step to accurately detect SARS-CoV-2 RNA in wastewaters. The results from this study show that 10 kDa centrifugal filters can be a successful method to concentrate SARS-CoV-2 RNA from wastewaters. Further research and development are required to develop and optimize concentration methods and thus improve the detection sensitivity of SARS-CoV-2 RNA in wastewaters. SARS-CoV-2 RNA is likely particle associated which might be an important parameter when considering the concentration step to be employed. Further research is needed to evaluate the effect of particles in the concentration of SARS-CoV-2 RNA using AMICON centrifugal filters. The study was conducted in hospital wastewater as a proof of concept that WBE may represent a complementary approach to estimate the presence and prevalence of COVID-19 in a given community. WBE is especially important in cases where there is limited capacity for clinical testing and should be improved to be used in municipal wastewater corresponding to larger communities.

Credit author statement

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All the authors have read and approve the submitted manuscript.

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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