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Moore swab performs equal to composite and outperforms grab sampling for SARS-CoV-2 monitoring in wastewater

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HIGHLIGHTS
• The first report of sewage monitoring for SARS-CoV-2 in Iran is presented.
• We highlighted the importance of having a suitable and user-friendly sampling method in real-time wastewater surveillance.
• It is quite easy for public health authorities to use Moore swabs for more effective investigation of disease.
• CDC ‘N’ assay better revealed the presence of virus in sewage samples as compared to ‘ORF1ab’.
• Virus copy numbers showed clear differences between advantaged and disadvantaged areas.

GRAPHICAL ABSTRACT

ABSTRACT
Wastewater-based epidemiology (WBE) approaches to detect SARS-CoV-2 in municipal wastewater can provide unique information on the incidence or prevalence of COVID-19 in community. However, there are several technical challenges coupled with sewage sampling for SARS-CoV-2, including intermittent shedding of viruses, sampling time, volume, and frequency. Sampling schemes thus may need to be tailored to reach out highly sensitive, accurate, and reliable results. Herein, we compared the accuracy and threshold cycle (Ct) profiles of SARS-CoV-2 in Moore swabs, composite (16-h), and grab samples taken from sewage manholes (n = 17) at the Middle Eastern city of Tehran, Iran, on two occasions (November 2020 and May 2021). Samples were concentrated by polyethylene glycol precipitation and the corresponding Ct values for CDC ‘N’ and ‘ORF1ab’ assays were derived by means of real time RT-qPCR. Overall, the Moore swabs performed equal to samples composited over 16 h for qualitative monitoring, and 34/34 (100%) were positive for SARS-CoV-2. The ‘N’ assay showed the highest detection frequency as compared to ‘ORF1ab’. The mean Moore swab Ct profiles were more consistent with 16 h composite sampling as compared with corresponding grab samples, providing hints as to the best sampling protocol to adopt when planning a sewage monitoring campaign particularly under WBE. Furthermore, our analyses on
1. Introduction

By late May 2021, the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), responsible for COVID-19 pandemic, had caused >168,462,786 confirmed cases and >3,499,893 deaths (CSSE, 2021). The clinical spectrum of SARS-CoV-2 infection is wide, encompassing asymptomatic infection and flu-like symptoms with most affected patients suffering from fever, dry cough, and shortness of breath; but some become seriously ill requiring admission to hospital and even die (Ali and Alharbi, 2020; Kim et al., 2020a [preprint]; Zhou et al., 2020). These symptoms generally appear within 2–14 days following exposure to the infectious virus (Chen et al., 2020a; He et al., 2020). Infection is most notable for causing respiratory illness, there are reports on persisting symptoms and unexpected gastrointestinal dysfunction following SARS-CoV-2 infection in an increasing number of patients causing diarrhea, vomiting, and other gastrointestinal symptoms (Cheung et al., 2020; Redd et al., 2020; Xiao et al., 2020), as previously observed in SARS outbreak (Leung et al., 2003).

Urban wastewater is an accessible and economical source of real-time information and could serve as pooled epidemiological data demonstrating if the infectious agent is circulating in the human population because infected individuals excrete high levels of virus and continue to shed for up to 100 days, even after symptom resolution (Chen et al., 2020b; Peng et al., 2020; Wang et al., 2020; Young et al., 2020). Wastewater surveillance, in an approach known as wastewater-based epidemiology (WBE), coupled with the current routes of clinical testing for the virus is thus sought to detect the occurrence or re-emergence of disease at the local or regional scale (Medema et al., 2020). The approach indeed sheds light on the infection trends among the population, in part because it captures both asymptomatic and symptomatic infections (Gerrity et al., 2021). An analysis of the data available in the literature already depicts that 48 to 67% of infected patients excrete SARS-CoV-2 in their stool (Cheung et al., 2020; Parasa et al., 2020; Steinfeld et al., 2015; Wong et al., 2020), whereby the levels of shedding are notably independent of COVID-19 severity (Zheng et al., 2020). It seems thus a reasonable consideration that SARS-CoV-2 titers in wastewater is proportional to the number of affected patients within a catchment. Indeed, a significant proportion of infected individuals may be asymptomatic, meaning that they are infected but show no symptoms of illness during the period of infection. A study in the U.S. estimated that 0.1%–5% of all fecal samples were positive for SARS-CoV-2 in the testing period whereas the reported number of clinically confirmed COVID-19 cases was 0.026% (Wu et al., 2020b). Preliminary estimates of asymptomatic infected individuals range from 17.9 to 30.8% (Chau et al., 2020; Mizumoto et al., 2020; Yang et al., 2020). As a ‘pooled’ sample, therefore, wastewater provides a broad representation of community health and may reflect the virus circulation among human populations with just a small number of samples (Gerrity et al., 2021). Indeed, wastewater fingerprinting may provide early warning signs, identification of highly affected areas in a community, detecting re-emergence of infectious agent, and even documenting post-vaccination campaigns tracing the virus mutation rate; however, more information on methodological decisions and surveillance costs will be required to fully assess the effectiveness of this form of surveillance.

Similar to other viruses, sewage monitoring for SARS-CoV-2 RNA encompass a number of steps, including sample collection, concentration/enrichment, laboratory assay, data normalization and interpretation. Despite the flood of reported SARS-CoV-2 RNA detections/enumerations in wastewater, there are undoubtedly some critical challenges that may seriously affect the monitoring results (Ahmed et al., 2020a; Lu et al., 2020; Medema et al., 2020; Wu et al., 2020b). Any effort to apply WBE within a drainage basin thus have to overcome these challenges in order to accurately reflect the real-time situation of pandemic. Among these challenges is the heterogeneous nature of wastewater, which in turn causes this type of research jeopardized by a statistically representative sampling of sewage. It is just as important to take a representative sample as it is to analyze the sample correctly. The limits of data variability are potentially influenced by sampling time, volume, and frequency, among others; so that detected amounts of RNA should be cautiously compared among studies. Thus, to obtain accurate results with statistical significance, applying standardized sampling protocols before the subsequent concentration/enrichment and detection/enumeration of virus particles is a must-have for sewage surveillance. A comparison of studies dealing with sewage surveillance for SARS-CoV-2 suggests that the employed sampling methods primarily focused on small (Randazzo et al., 2020a; Randazzo et al., 2020b) and large grab samples (Ahmed et al., 2020b), as well as flow or time-proportional composite samples (Sherchan et al., 2020; Westhaus et al., 2020). Grab sampling has been frequently applied in a considerable number of related studies due partly to its ease of use, low cost, and convenience for wastewater collection (Ahmed et al., 2020c; Wu et al., 2020a [preprint]). Nevertheless, since different parts of desired catchment area are not sampled on the same day, uncertainty is quite possible about the validity of RNA loading comparisons among different dates. This is more evidenced in the regular monitoring of urban sewer networks. Indeed, given the low concentration and dynamic nature of SARS-CoV-2 shedding in wastewater, recent attempts have also applied a more representative sampling approach by taking 24-h composite samples (Nemudryi et al., 2020; Saguti et al., 2020). However, taking 24-h composite samples (which necessitate the implementation of a composite autosampler and processing of large volumes of raw wastewater) often neither are feasible nor affordable because of resources constraints and onsite regulations particularly in under-developed communities. Classic environmental monitoring tools such as Moore swabs can be used to track infectious virion and associated viral fragments. The Moore swab is a revived environmental sampling tool whereby a gauze pad tied with string is suspended in flowing water or wastewater. The method has been used for decades in public health and environmental surveillance all around the world to detect and isolate enteric infectious agents (e.g. Salmonella typhi and paratyphi (Sears et al., 1986; Sikorski and Levine, 2020), Vibrio cholera (Barrett et al., 1980), Escherichia coli O157:H7 (Sodio et al., 2013), coxsackie viruses (Kelly, 1953), poliovirus (Holt, 1960), norovirus (Tian et al., 2018; Tian et al., 2017), etc). Uncertainties concerning with the types of sewage samples are indeed compounded by recent findings that point to high variabilities with regard to the volume of processed samples (Alygizakis et al., 2020). Therefore, the lack of standard protocols for virus sampling, sample preparation (i.e. concentration/enrichment procedures), and analysis (i.e. target gene regions) in untreated wastewater or effluents as well as...
onsite sampling limitations means that existing studies are not directly comparable (Alygizakis et al., 2020).

The aims of the current study were to: (i) investigate the occurrence of SARS-CoV-2 in a highly affected municipal sewage system, (ii) assess the effect of different sampling techniques on the abundance and viral load of SARS-CoV-2 collected from the same sewage samples, (iii) compare detection frequencies of target gene regions (CDC ‘N’ and ‘ORF1ab’), and (iv) elucidate spatio-temporal variations in the prevalence of SARS-CoV-2. The sampling strategy was based on three different sampling methods that were applied at 17 sites (sewage manholes) on two occasions (November 2020 and May 2021): a) simple grab samples (1 L), b) high-frequency time-proportional 16-h composite samples (8 L), and c) Moore swab samples. With the economic and practical restrictions in developing and under-developed countries and even in developed world coupled with limited health care resources, the present study compares the relative merits of Moore swab as a potential tool for assessing and managing the pandemic. To the best of our knowledge, combining assessment of different sampling protocols, together with using the Moore swab has not been previously attempted.

2. Materials and methods

2.1. Study area

This study was conducted in the capital and most populous city of Iran, Tehran. This metropolitan has a population of 8.694 million (Statistical Centre of Iran, 2016) and a density of 11,800 people km−2, with higher population densities in the midwest and mideast to southern areas. Tehran’s sewage project currently has a collection pipe network of almost 6000-km-long whereby about 684 thousand m3 of sewage is daily collected. So far just about 64% of Tehran residences are connected to the sewage pipe network while the remaining goes underground or flows over the surface and creates environmental hazards including groundwater pollution.

The first official announcement of COVID-19 in Tehran was reported on 12 February 2020 (Iran Ministry of Health and Medical Education, 2020), whereas the number of hospitalized PCR diagnosis has now been reached approaching 90,000 OR 100,000 in the city. Even though health care systems in this relatively well-equipped megacity are performing well, there will soon be a need to focus on populations in high populous, poor hygiene and sanitation residential areas that renders threat of COVID-19 transmission more plausible.

2.2. Sample collection and processing

Samples were collected on two separate occasions, November 2020 and May 2021. The first sampling run was conducted on two consecutive weekend days (Thursday and Friday) from 12 until 20 November 2020 at seventeen sewage manholes each representing different socio-demographic characteristics of area (population density, hygiene status, lifestyle, household income), as described in Fig. 1. On the second occasion, five months later, the same sewage manholes were sampled again on weekend days from 6 until 14 May 2021. Small grab samples (1 L) were collected in the early mornings, when defecation is most frequent in the general population compared with other times, while composite sampling was conducted manually throughout the course of a day.

For the grab sampling method, wastewater flow from a sewage manhole at each sampling point was sampled using separate stainless steel buckets and transported to laboratory on melting ice. The 16-h composite samples were collected manually. Briefly, about 8 L composite samples of urban sewage at each sampling site was collected by sampling wastewater every 60 min in a time-proportional mode (≈500 ml h−1), which was thence followed by pooling the subsamples. To avoid cross contamination, the buckets were washed with tap water between sampling events. The sampling was performed between 8:00 and 24:00. Sampling personnel wore standard personal protective equipment for wastewater sampling, such as long pants, capped boots, hard hats, safety glasses and gloves. Samples were maintained and transported on melting ice to the laboratory and stored at 4 °C until further analyses.

The Moore swabs were made by taking a piece of cotton gauze about 120 cm in length and 15 cm wide and folded eight times until an 8-ply square pad was formed (Fig. 2a), as described by Sikorski and Levine (2020). The square-pad was firmly tied by one end to a long piece of stout string around the center and sterilized in an autoclave. The gauze was then carefully placed into an autoclaved stainless steel wire sieve (Fig. 2b) and immersed in the flowing sewage. The string was attached suitably to a hook under the manhole cover and the gauze left in position for about 16 h (8:00–24:00). The swabs were then taken from the sewage and the sieves were meticulously removed. The sloppy swabs were stored in Ziploc bags, and delivered to the laboratory in a cooled sterile container. For swab processing, the swab was squeezed in a 500 mL sterile flask to collect all the trapped liquid out, and then was aseptically cut into small pieces and submerged in 100 mL elution buffer while gently shaking the swab for approximately 2 min. The liquid from the swab was merged with the initial squeezed sample. This step was repeated two more times before disposing the swab until the final total fluid volume of ~250 mL which was saved for further processing.

2.3. Virus concentration, RNA extraction, and quantitative Real-Time RT-qPCR

Viruses in sewage samples were concentrated by polyethylene glycol (PEG) as described by Jones and Johns (2009). The samples were analyzed in parallel by filtration and centrifugation. The latter method was found to be more sensitive than filtration for SARS-CoV-2 detection (data not presented here) and thus was followed in our study. About 40 mL of wastewater samples were centrifuged by Eppendorf 5810 R (Eppendorf, Hamburg, Germany) for 5 min at 4000 g. The clarified wastewater was mixed with NaCl (1.5 M NaCl) and PEG (50% w/v), and the mixture was left to stand overnight at 4 °C followed by recentrifugation at 15,000 g for 30 min. The supernatant was subsequently discarded and the bottom layer were re-suspended in 300 μL phosphate-buffered saline (PBS) and stored at −80 °C until analyzed.

Purification of RNA was carried out using the QIAamp® Viral RNA mini kit (Qiagen Company, Hilden, Germany — Catalog No. 52904) according to the manufacturer’s instructions. Briefly, 140 μL dissolved pellet after centrifugation was mixed with lysis buffer supplemented with carrier RNA. After binding on membranes nucleic acids were eluted in 60 μL elution buffer. Isolated RNA were stored at −80 °C.

RT-qPCR was performed for detection of SARS-CoV-2 using novel coronavirus nucleic acid diagnostic real time PCR kit (Sansure Biotech, China) containing primers and probes that target the ORF1ab (FAM channel) and N (ROX channel) genes and RNase P as an internal control. For one-step RT-qPCR, the reaction mixture contained 26 μL buffer, 4 μL enzyme reaction solution, and 10 μL isolated RNA. For each qPCR run, a series of three positive and negative controls were included. The RT-qPCR assays were performed using a Rotor-Gene Q MX (QIAGEN Hilden, Germany). All reactions were performed in four replicates with the following amplification conditions: reverse transcription at 50 °C for 30 min, cDNA initial denaturation at 95 °C for 3 min (1 cycle), denaturation at 95 °C for 15 s (45–50 cycles), annealing, extension and fluorescence measurement at 55 °C for 40 s and cooling at 25 °C for 10 s. The PCR runs were analyzed with Rotor-Gene Series software version 2.35. According to the study of the reference value, the Ct reference value for the target gene detected by this kit is 40 (Corman et al., 2020) and those with threshold cycles beyond 40 were discarded, which previous experience indicated to be probable false positives (i.e., Ct = 40 was the detection limit). Results were considered positive when samples with at least one target gene was amplified.
2.4. Reporting of results

Homogeneous reporting enables to draw global comparisons and assessment of results despite the use of different methods among most studies. Based on the type of result sought, currently available studies have reported the presence and quantity of SARS-CoV-2 RNA in sewage in two distinct ways: i) presence/absence of the virus in the form of Ct values delineated directly by the exploited quantitative PCR instrument, and ii) gene copies present in a unit volume of sample (relative quantification). The later is performed through the use of a quantitative calibration curve of Ct profiles against known concentrations of the virus. Total number of viral gene copies per certain volume of sample is thus

Fig. 1. Study area of Tehran, sewage network and location of 17 sampling points. Colors of bars (labeled with the sewage manhole ID) relate to different sampling methods and their height show the virus copy number. a) 12 until 20 November 2020, b) 6 until 14 May 2021.

Fig. 2. (a) Moore swab, and (b) Stainless-steel wire sieve.
carefully prepared, initially with the calculation of mean \( C_t \) from associated technical replicates, followed by the calculation of the relative quantity of gene copies and finally by the enumeration of gene copies per volume of samples alongside the performance of statistical analysis (Michael-Kordatou et al., 2020).

We employed qualitative measurement, and hence, increasing and decreasing viral load was measured based on the \( C_t \) value which may indicate disease burden and also a change in the prevalence of shedders in a sampled catchment area. Grab and composite sampling approaches are very clear in terms of the sample volume. There remains, however, debate on how to calculate the total number of viral gene copies per unit volume of wastewater, since even with concerted effort it seems impossible to reach the volume of wastewater directly contacted with the cotton swab when dipped. The successful recovery of viral fragments from the cotton tag and subsequent analysis of specific targets can also prove to be difficult. Addressing these problems calls out for flow inside the sewers and the mean velocity of wastewater coupled with swab immersion time over extended time periods and a sophisticated surveillance system. Our tailored function, thus, allows for a comparative analysis of sampled catchment areas in terms of \( C_t \) values to fully interpret the data.

2.5. Statistical analyses

All statistical analyses were conducted using SPSS software (Chicago, IL, USA) and data were reported as the mean ± standard error of mean. The Shapiro-Wilk test revealed that the distribution of data was statistically normal. Additionally, homogeneity of variance was significant, \( p < 0.05 \), underlying that the assumption of the normality or homogeneity has been met for given samples. Accordingly, One-way ANOVA analysis of variance was used to test for statistical differences among sampling methods comparing the detection of SARS-CoV-2 (\( C_t \) values) in urban sewage samples. A Student’s \( t \)-test was conducted to test for difference between target gene regions as well as temporal variations of SARS-CoV-2 prevalence in the community, with a \( p \)-value of 0.05 or lower signifying significance.

3. Results and discussion

3.1. Method comparisons

In the context of the global effort to combat COVID-19, sewage monitoring represents an affordable, convenient and practical program which supplement the current clinical surveillance systems. As with any analytical assessment, sampling protocols are of key importance for wastewater based monitoring of COVID-19, since a wide variety of methods can be used to investigate infectious virion and/or associated viral fragments. The literature already includes several reports of SARS-CoV-2 RNA presence and abundance in wastewater and virus circulation in defined geographic regions (Haramoto et al., 2020; Sherchan et al., 2020). These investigations, primarily conducted for research purposes, have used a variety of sampling approaches in the absence of standardized operating procedures (Alygizakis et al., 2020). In addition, some have reasonably proposed to increase the sampling volume especially in the wake of uneven distribution of virus in untreated wastewater (Haramoto et al., 2020; Mlejnko et al., 2020), making it challenging to draw direct comparisons between data from different studies.

For a clearer comparison and to assess whether these conventional procedures (grab versus composite) minimize the sources of uncertainties, particularly associated with small grab sampling method, Moore swab samples were indeed collected in the present study on two separate occasions (November 2020 and May 2021). Besides the presence/absence assessment of SARS-CoV-2 RNA in sewage samples, \( C_t \) values were also worked out to facilitate comparison between applied sampling methods and the results are summarized in Table 1. Notwithstanding of the sampling method employed, all tested samples were positive for SARS-CoV-2 according to at least one of the two SARS-CoV-2 viral gene targets (i.e. N or ORF1ab). Among them, totally 16 out of 34 grab samples (47%) were positive for both targeted gene region assays. Likewise, 23 out of 34 composite samples (67%) depicted positive. While there observed some inconsistency with the detection of SARS-CoV-2 in the sewage samples, the sensitivity of composite samples showed to be superbly better than corresponding grabs. On the other hand, the Moore swab samples were positive for SARS-CoV-2 in all of sampling sites (100%). These results seem to indicate the relative merits of the Moore swab as a sensitive and specific method for SARS-CoV-2 detection in sewage.

Based on the detection frequency (n detected/n tested), Moore swabs resulted in 31 out of 34 (for ORF1ab) and 34 out of 34 (for N) positivity to RT-PCR as compared with the 16-h composite (23 and 29 out of 34 for ORF1ab and N targets, respectively). The superiority of swabs is more pronounced as compared with the grab samples (16/34 and 24/34, respectively) (Table 2, and Fig. 3). In samples from sewage manholes 3, 4, 5, 10 and 11 covering the north and east parts of the city, most of grab samples were negative. However, most of the 16-h composite samples were positive for SARS-CoV-2, which indeed coincided with those observed for placed swabs.

| Manhole no. | Grab samples | Composite (16-h) samples | Moore swab samples |
|------------|--------------|--------------------------|-------------------|
|            | Nov. 2020 | May 2021 | Nov. 2020 | May 2021 | Nov. 2020 | May 2021 | Nov. 2020 | May 2021 |
|            | N | ORF1ab | N | ORF1ab | N | ORF1ab | N | ORF1ab |
| 1          | 34.00 | 36.00 | ND | ND | 31.44 | 32.44 | ND | ND |
| 2          | 37.00 | 39.00 | 33.66 | 34.34 | 34.08 | 38.37 | 31.38 | 31.15 |
| 3          | ND | ND | 35.68 | ND | 35.51 | 37.21 | 31.04 | 33.37 |
| 4          | ND | ND | 32.12 | 33.78 | ND | 34.19 | ND | 34.34 |
| 5          | ND | ND | 34.19 | ND | 32.01 | 34.14 | 36.16 | ND |
| 6          | 34.10 | 35.00 | ND | ND | 31.50 | 34.05 | 32.26 | 31.41 |
| 7          | 38.00 | 34.87 | 32.96 | 33.41 | 34.40 | ND | 32.90 | ND |
| 8          | ND | ND | 34.71 | 35.32 | 34.40 | ND | 32.90 | ND |
| 9          | 40.00 | 34.56 | 33.06 | 34.14 | 31.43 | 34.23 | 30.40 | 33.16 |
| 10         | ND | ND | 35.67 | 36.83 | ND | ND | 32.16 | 35.46 |
| 11         | ND | ND | 33.77 | ND | 34.43 | ND | 33.36 | 33.71 |
| 12         | 31.50 | ND | 33.06 | 35.33 | 31.02 | 33.69 | 33.84 | 35.40 |
| 13         | 36.78 | 39.00 | ND | ND | 35.88 | 38.8 | 33.68 | ND |
| 14         | 37.00 | ND | 35.67 | ND | 33.48 | 35.52 | 34.54 | ND |
| 15         | ND | ND | 33.69 | ND | ND | 33.07 | 37.60 | ND |
| 16         | 37.80 | 40.00 | 34.12 | ND | 37.67 | 37.81 | 32.39 | 33.81 |
| 17         | 33.98 | 37.00 | 32.94 | 36.66 | 33.56 | 35.27 | 32.77 | 32.93 |

ND: not detected.
The Ct values determined for raw wastewater using the RT-PCR and various sampling methods are comparable, as typically shown in Fig. 4. It is interesting to note that almost in all 34 samples of this study Moore swabs outperformed 16-h composite campaign, with respective Ct values of 33.38 ± 0.31 and 32.23 ± 0.29 (targeting N gene) (Table 2, and Fig. 3). Although the literature on SARS-CoV-2 RNA Ct values shows heterogeneous results, the findings observed in this study mirror those of the previous studies (Queensland (Australia) (Ahmed et al., 2020a), Amsterdam, Den Haag, Utrecht, Apeldoorn, Amersfoort, Schiphol, Tilburg (The Netherlands) (Medema et al., 2020), Murcia (Spain) (Randazzo et al., 2020b), Nevada (Gerrity et al., 2021), and Louisiana (USA) (Sherchan et al., 2020) – also see Section 3.2). Significantly lower Ct values detected by the Moore swab sampling (pointing towards higher levels of SARS-CoV-2) may reflect its superbly enhanced trapping activity. These values were markedly lower compared to those noticed using the grab sampling (34.98 ± 0.35) (Table 2, and Fig. 3), which possibly undermine the relative advantages of the last sampling method. Moreover, there was a significant difference in Ct values for Moore swab samples as compared to grab (p < 0.001) and 16-h composite (p = 0.04) samples. Overall, the low standard deviation that characterized the Moore swabs implies that microorganism infiltration appreciably lowers the error relative to the grab sampling. In contrast to just a snapshot in time as in grab sampling, Moore swabs act as continuous filters to “trap” microorganisms and their associated fragments. The greater variability inherent in the grab samples was further noticed by the higher average coefficient of variation (CV, 5.06%) compared with that of 16-h composite (5.55%) and the Moore swab samples (5.41%). Results from grab sampling may be used for systematic surveillance purposes; however, the method lacks the accuracy and detection of positive signals and thus outbreak responses when the prevalence of affected individuals is low or the virus is highly diluted in the flowing sewage. Continuous filtration thus helps to alleviate the limitations of transiently present pathogens such as human-restricted SARS-CoV-2 and the effects of dilution which is valuable to obtain the near real-time concentration of viruses detected in wastewater. Accordingly, Moore swabs coupled with composite samples, though characterized by much more difficulties, can detect sub-clinical, pre-symptomatic, and even asymptomatic carriers who do not seek medical care but can harbor SARS-CoV-2 in their gastrointestinal tract and intermittently excrete virus in faeces. The results of the 16-h composite samples almost agree with the Moore swab samples in terms of Ct values and relative abundance of positive signal.

3.2. Comparison of target gene regions

Few studies have specifically sought to the relative merits of target gene regions on SARS-CoV-2 tracing in wastewater; most reported on wastewater occurrence of virus. Relying on the results of Moore’s swab method and to obtain a broader picture, a conventional RT-PCR from two regions of virus was worked out in our study to confirm the presence of SARS-CoV-2 in sewage samples. As summarized in Table 2, all samples (17 out of 17) were positive for ‘N’ gene region during the first sampling occasion (November 2020), whereas of the 17 tested samples, 15 (88%) showed positive for ‘ORF1ab’ and in two remaining samples led to false negatives. Congruently, this pattern was observed in the second sampling occasion (May 2021), where detection frequency kept almost the similar outcomes except for one more positivity (i.e. 16 out of 17) for ‘ORF1ab’. While there observed some inconsistency with the detection of SARS-CoV-2 in the sewage samples, the sensitivity of ‘N’ outperformed ‘ORF1ab’. Given the Ct value differences, these results suggest that ‘N’ is more sensitive (10–40 fold) for SARS-CoV-2 detection in sewage, as compared with ‘ORF1ab’. Three N gene assays (N1, N2, and N3) each detecting a various region of the nucleocapsid (N) gene have been proposed by US CDC and their specificity against other viruses, comprising human coronaviruses, has been reported (Corman et al., 2020). Very recently, the ‘ORF1ab’ gene assay has also been used as a confirmatory testing (specific to SARS-CoV-2) in some studies concerning with sewage surveillance to estimate the infection incidence within the population. In addition, the E gene assay is used as a screening tool because it detects all viruses from the Sarbecovirus subgenus (e.g., SARS-CoV, SARS-CoV-2, and related bata viruses) (Arora et al., 2020; Kumar et al., 2020; Rimoldi et al., 2020). Although firm evidence is lacking, a combination of SARS-CoV-2 molecular tracing protocols in sewage samples put substantial differences for amplification of the viral RNA with different primer/probes. For instance, compared with the results for the sensitivity of primer/probe sets on SARS-CoV-2 RNA in clinical samples reported by US FDA, Medema et al. (2020) affirmed a higher frequency of positive amplification targeting ‘N’ genes while other regions of gene did not track down

![Fig. 3](image_url) Percent positivity for SARS-CoV-2: Moore swab showed highest percent positivity for N and ORF1ab. Both genes had roughly 50 and 70% positive with grab and 16-h composite methods, respectively.
SARS-CoV-2 in wastewater samples. Moreover, ‘N’ outperformed ‘E’ primers. The expression of ‘ORF1ab’ requires ribosomal frameshifting, implying that it is produced at significantly lower levels as compared to N-encoded functions and sub-genomic RNA (Barra et al., 2020). Therefore, in the infected samples, the ‘ORF1ab’ copy number is lower than ‘N’. On the other hand, the ‘N’ target is highly expressed because its sequence is present in almost all sub-genomic RNA (Barra et al., 2020; Kumar et al., 2020; Manupati et al., 2020[preprint]; Mlejnkova et al., 2020). This fact also corroborates with the better diagnostic capability of ‘N’ compared to ‘ORF1ab’. Accordingly, in case of SARS-CoV-2, RT-PCR targeting ‘N’ gene showed lower Ct value and more reproducible detection than ‘ORF1ab’, which might reflect the different RNA abundance and/or stability. Also, the use of multi-gene reactions cause the minimum detectable range to increase drastically, and low rate of viral loads in cases where the shading of virus is low, are not detectable and results can be false negatives. In contrast to our findings, however, Rimoldi et al. (2020) reported that the ‘ORF1ab’ region resulted in the most sensitive region for RNA amplification and detection.

Amplification cycles of SARS-CoV-2 RNA ranged from 29.59 to 40.00 in the wastewater. Although reported Ct values of SARS-CoV-2 in untreated wastewater around the world are highly variable (22 to 40) (Kumar et al., 2020; Manupati et al., 2020[preprint]; Mlejnkova et al., 2020), the results of the current study are consistent with other recent studies. For example, one of the lowest Ct values for SARS-CoV-2 in wastewater, an average of 24, was reported by Manupati et al. (2020) [preprint]. However, it is worth bearing in mind that high titers of wastewater, an average of 24, was reported by Manupati et al. (2020) [preprint]. This variability in approaches makes direct comparison of data sets challenging, an issue compounded by the lack of standard protocols for virus enrichment (Philo et al., 2020).

3.3. Temporal and spatial variability in prevalence of SARS-CoV-2

Identification of accurate vulnerable population in a community or at certain public facilities (e.g., institutions, schools, hospitals, prisons, public/private buildings, or ports and terminals), as a complementary approach to clinical diagnosis, may inform epidemiological investigators to estimate the presence and prevalence of COVID-19 in the sampled region and help differentiate response deployment patterns. SARS-CoV-2 excreted from undiagnosed but active cases can be traced in sewage and thus WBE potentially predicts the overall picture of epidemic status, without the risk of such biases. Such information may eventually be used to locate the highly affected areas and target localized appropriate actions, including provision of health warnings, implementation of strict lockdown measures and individual diagnostic testing.

Seventeen sewage manholes (sampling points), each covering one part of the city were sampled on two occasions during the ongoing pandemic (Fig. 1). Since there were distinct fluctuations in observed Ct values, possible local and temporal differences in disease prevalence within the city were investigated along a seven-month period. At collection point 12, covering the south part of the city, the lowest Ct values (30.26) (corresponding to the highest concentration of SARS-CoV-2 copies) was observed in November 2020. It is worth noting that these parts coincide with rundown areas of city with relatively high population density. On the other hand, sewage samples from collection points 3 and 6, covering the north of the city, showed the highest threshold cycles (35.94 and 35.9 in November 2020 and May 2021, respectively). These points coincides with built up areas (Fig. 1).

Measurements taken at seventeen sewage manholes during the three sampling campaigns were also included in the analysis of data from two sampling occasions. Measurements were successful at all 17 sampling points during both November 2020 and May 2021 campaigns. As with the spatial distribution, however, Ct measurements from all sites revealed almost similar concentrations of SARS-CoV-2 RNA in November 2020 and May 2021 in the study area. This partially reflects negligible contrasts in the temporal variations of virus shedders within Tehran on these cross-sections. The respective Ct values mean
The positive SARS-CoV-2 RNA detection in wastewater confirms the presence of active infections most of them may be clinically undiagnosed. This statement is supported by a recent study in the U.S. (Wu et al., 2020b) in which the authors estimated that about 5% of all stool samples showed positive for SARS-CoV-2 in the time course of study whereas the number of clinically diagnosed cases was just 0.026%. Transmission by such asymptomatic and pre-symptomatic individuals may adversely affect the initial containment of pandemic. Our analyses on differences between low density advantaged areas and high density poorer areas also revealed somewhat lower Ct values for the latter. Although the virus copy numbers were higher in the disadvantaged southern to central areas of the city as compared to the wealthier northern areas, the minimum and mean Ct values were somewhat lower as compared to the reported developed communities all around the world (Ahmed et al., 2020a; Westhaus et al., 2020; Wu et al., 2020a [preprint]).

The spread of highly contagious COVID strain probably enhances with population size and density in a geographical area, which could be assigned to the closer contact in their homes as well as in public places. In an effort to further establish the local differences in our study, several researches indicate that lower income is linked with poorer health. The lower individuals’ income, the higher is their likelihood of infectious disease and death (Scarbrough et al., 2019). Therefore, it can be inferred that a higher risk of contracting an infectious disease may exist for those living in poverty, particularly across south regions in the study area (Fig. 1).

4. Conclusions

Wastewater is an ideal source of epidemiological information and provides a more accurate picture of population health. However, a wastewater-based model informed by intermittent shedding of target chemical or biological agent highlights the potential significance of taking representative samples of an entire event when interpreting wastewater surveillance data. Here, we characterized the effect of sampling scheme in the estimation of SARS-CoV-2 dynamics by collecting municipal sewage samples from a large populated area using different sampling methods (Moore swabs, 16-h time-proportional composite and simple grab samples). This is also the first report of sewage monitoring for SARS-CoV-2 in our country.

SARS-CoV-2 detection rates and targeted genes Ct values differed among sampling protocols, demonstrating method sensitivity which in turn affects the estimated total number of viral RNA copies in wastewater samples. Sewage samples generally showed a positivity to real time RT-qPCR during the study period with lowest Ct values for Moore swabs. For a clearer comparison, the swab monitoring corroborated the results of a great deal of the 16-h composite sampling; however, the relative detection rates were significantly lower and Ct profiles were higher using grab sampling method, presumably reflecting intra-daily variations in SARS-CoV-2 signal. These seem to indicate that grab sampling is a relatively inefficient methodology for capturing mean concentrations of virus in sewage systems subjected to intermittent fecal shedding, especially when it is restricted to small drainage areas (sub-sewersheds). Although it has not yet played any role in recent research on sewage monitoring for SARS-CoV-2, this study throws light on the superiority of Moore swab sampling to provide a more representative snapshot of viral agent in sewage. Indeed, CDC N gene region signal shows superior quantification of SARS-CoV-2 as compared to ORFlab.

Given the transient presence of SARS-CoV-2 in sewage coupled with the sensitivity, simplicity and affordability of Moore swab, this observation is highly relevant in resource poor communities and even in developed world facing the threat of COVID-19. The method may also be considered as a powerful strategy for COVID-19 surveillance in rural communities. However, there are still open methodological questions on how to derive correlations between recovered SARS-CoV-2 from municipal sewage and acute infected cases in the sampled region, as well as effective use of this revived surveillance tool concerning the sampling frequency, duration of immersion, and swab processing procedure that highlights the importance of standardizing this versatile technique.

CRediT authorship contribution statement

Mohammad Rafiee: Conceptualization, Methodology, Supervision, Data curation, Writing – review & editing. Siavash Isazadeh: Conceptualization, Writing – review & editing. Anoushiravan Mohseni-Bandpeiei: Writing – review & editing. Seyed Reza Mohhebbi: Methodology. Mahsa Jahangiri-rad: Writing – review & editing. Akbar Eslami: Writing – review & editing. Hossein Dabiri: Writing – review & editing. Kasra Rooostaei: Investigation. Mohammad Tanhaei: Methodology. Fatemeh Amereh: Writing – original draft, Investigation, Software, Data curation, Methodology, Formal analysis, Visualization.

Declaration of competing interest

The authors have no conflicts of interest to declare.

Acknowledgements

This work was supported by National Institute for Medical Research Development (NIMAD) under Grant No. 994141. We would like to acknowledge Tehran Sewerage Co. for assistance with sample collection. We would also like to acknowledge Research Institute for Gastroenterology and Liver Diseases that generously provided us with their laboratories.

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