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Communication

Environmental surveillance of SARS-CoV-2 RNA in wastewater systems and related environments in Wuhan: April to May of 2020

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A B S T R A C T

Wastewater-based epidemiology (WBE) has emerged as an effective environmental surveillance tool in monitoring fecal-oral pathogen infections within a community. Congruently, SARS-CoV-2, the etiologic agent of COVID-19, has been demonstrated to infect gastrointestinal tissues, and be shed in feces. In the present study, SARS-CoV-2 RNA was concentrated from wastewater, sludge, surface water, ground water, sediment, and soil samples of municipal and hospital wastewater systems and related environments in Wuhan during the COVID-19 middle and low risk periods, and the viral RNA copies quantified using reverse transcription quantitative polymerase chain reaction (RT-qPCR). From the findings of this study, during the middle risk period, one influent sample and three secondary effluents collected from waste water treatment plant 2, as well as two samples from Jinyintan Hospital wastewater system influent were SARS-CoV-2 RNA positive. One sludge sample collected from Guanggu Branch of Tongji Hospital, which was obtained during the low risk period, was also positive for SARS-CoV-2 RNA. These study findings demonstrate the significance of WBE in continuous surveillance of SARS-CoV-2 at the community level, even when the...
Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent for coronavirus disease 2019 (COVID-19), a current public health crisis of global concern (Zhou et al., 2020). COVID-19 was declared a pandemic by World Health Organization (WHO) on 11th March 2020 (Cucinotta and Vanelli, 2020), after its first identification in Wuhan, China, and subsequent reports in many other regions and countries. COVID-19 is primarily transmitted via respiratory droplets that people cough, sneeze or exhale, and may also be spread via fomites (Jayaweera et al., 2020; Wang and Du, 2020). Moreover, current efforts in the mitigation and prevention of the spread and transmission of SARS-CoV-2 have been focused on the adoption of non-pharmacological intervention strategies.

In recent times, the assessment of different substances in wastewater has offered vital qualitative or quantitative knowledge on certain populations in a given wastewater catchment, particularly on the drug usage and distribution of drug resistant pathogenic genes within the environment. Moreover, this developing potential application has been suggested as a probable adoption in the field of infectious diseases to track and understand the distribution of disease biomarkers (Choi et al., 2018). Over time, environmental microbiologists have investigated pathogens in wastewater systems, as a public health surveillance tool known as wastewater-based epidemiology (WBE) (Sinclair et al., 2008; Daughton, 2018; Xagoraraki and O’Brien, 2020). WBE has been applied in surveillance of numerous fecal-oral viruses, foodborne and waterborne pathogens that infected persons typically excrete in high concentration (Katayama et al., 2008; Iaconelli et al., 2017; Bisseux et al., 2018; Tandukar et al., 2020). WBE has also been applied in investigating other viruses beyond enteric fecal-oral route, since viral shedding involves different body fluids ultimately discharged into the wastewater systems (La Rosa et al., 2020).

Therefore, in sight of this global COVID-19 pandemic, scientists from various parts of the world have adopted this surveillance system in detecting SARS-CoV-2 in wastewater systems (Ahmed et al., 2020; Chavarria-Miró et al., 2020; Haramoto et al., 2020; Kocamemi et al., 2020b; La Rosa et al., 2020; Rimoldi et al., 2020; Shcheran et al., 2020). Additionally, in China, a team of scientists from Tongji University investigated the presence of SARS-CoV-2 RNA in wastewater discharged from designated hospitals, municipal networks, and downstream influent into a wastewater treatment plant in Shanghai. In their study findings, SARS-CoV-2 RNA positive samples were detected from wastewater discharges of the designated hospitals (http://www.water8848.com/news/202003/30/123415.html).

Due to the outbreak of SARS-CoV-2 in Wuhan, stringent quarantine measures were enforced throughout the city on January 23, 2020 by the local government. After 11 weeks of lockdown, traffic control measures began to be officially lifted on April 8, and life started getting back to normal. As of 7th April, there were 574 confirmed cases and 673 cases under medical surveillance in Wuhan (http://wjw.wuhan.gov.cn/sy/). Indeed, Hubei Provincial center for Disease Control and Prevention reviewed the emergency response level for COVID-19 in Wuhan, and it was lowered from high to medium risk on 25th March 2020, and thereafter further lowered from medium to low risk on 12th April. Currently, there are no published reports on tracking of SARS-CoV-2 in wastewater and related environment in Wuhan during or after the epidemic.

1. Materials and methods

1.1. Sample collection and processing

Between the months of April and May 2020, a total of 216 samples that covered middle and low risk periods were collected from various points. Collection priority was given to wastewater treatment plants (samples of municipal wastewater system, designated hospitals for COVID-19 (samples of hospital wastewater system), and the environment close to them. These locations were responsible for handling wastewater that may have been directly or indirectly associated with COVID-19 patients. Environmental samples (from lakes and rivers) were collected based on the logic that once the wastewater has been thoroughly cleansed from the wastewater systems, it is discharged back into the environment (streams, rivers, or lakes). Except for the ground water (GW), influent, primary treatment effluent (PE), secondary effluent (SE), final effluent (FE), surplus sludge (SS), concentrated sludge (CS), de-watered sludge (DS), surface water (SW), lake sediment (LS), and soil samples were mainly collected in two districts: with the first one being around Leishenshan Hospital wastewater treatment plant 1 (WWTP1) in Hengshan district, and the other one being around Jinyintan Hospital wastewater treatment plant 2 (WWTP2) in Dongxiu district (Fig. 1).

The samples were transported to the laboratory through cold-chain transportation and processed within 6 hr of collection. For water samples, 50 mL of raw samples were first centrifuged at 5000 × g for 15 min to remove the debris. As previously described elsewhere, for the sludge, sediment, or soil, 10 g sample was added to 50 mL of phosphate buffered solution (PBS), then the tube was vortexed for 1 min and centrifuged at 5000 × g for 15 min to remove debris. After centrifugation, 3.2 g polyethylene glycol (PEG) 8000 (8%, W/V, Millipore...
Sigma, USA) and 0.9 g NaCl (0.3 mol/L, Millipore Sigma, USA) were added to 40 mL supernatant either from water, sludge, sediment, or soil sample (Miura et al., 2011). The suspension was mixed vigorously and incubated overnight at 4°C. The supernatant was discarded after centrifugation at 9000 × g for 30 min at 4°C. The pellet was resuspended in 1 mL of PBS. The samples were finally aliquoted and stored at −20°C for subsequent RNA extraction and virus isolation.

1.2. RNA extraction and RT-qPCR

Viral RNA was extracted from 200 μL concentrated samples from the above step by using Direct-zol RNA Kit (Zymo Research, USA), as per the manufacturer’s guidelines in a biosafety level-2 laboratory. Thereafter, reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed using two primer-probe sets targeting the receptor binding domain (RBD2) and open reading frame 1ab (ORF1ab) genes (Table 1). Reverse transcription droplet digital polymerase chain reaction (RT-ddPCR) was used to determine the concentration of the standard RNA samples with 10⁰–10⁻¹ FFU/mL (10-fold serial dilutions) of SARS-CoV-2, which was performed and used in plotting the standard curve (Appendix A Fig. S1). The regression equation of standard curve is displayed in Eq. (1):

\[ y = -0.3097x + 13.92 \quad R^2 = 0.9995 \] (1)

where, x is the threshold cycle and y is the logarithm of RNA samples’ concentrations (copies/mL), respectively; R² is the coefficient of correlation obtained for the standard curve, as previously described elsewhere (Nyaruaba et al., 2020). RT-qPCR reactions were performed on a CFX96 Touch Real-time PCR Detection System (Bio-Rad, USA) using a PrimeScript RT-qPCR Kit (Takara, China), under the following thermocycling conditions: 50°C for 15 min, 95°C for 15 min, followed by 40

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Table 1 - List of primers and probes used to detect SARS-CoV-2.

| Target | Primer Sequence 5’ to 3’ | Reference |
|--------|--------------------------|-----------|
| RBD2   | Forward: CAATGTTTAAACAGCCACAGG | Nyaruaba et al., 2020 |
|        | Reverse: CTCAAGTTGCTTGAGATACAG |          |
|        | Probe: ACACGACATGAGTAGGCTGCAATG |          |
| ORF1ab | Forward: CCCTGTTGTGTTTACATTAA | National Institute For Viral Disease Control and Prevention, 2020 |
|        | Reverse: ACGATTGTCATACAGCTGA |          |
|        | Probe: CCAGCTCGCGTATGTGGAAAGT |          |

RBD2: receptor binding domain 2; ORF1ab: open reading frame 1ab.
cycles of 95°C for 15 sec and reading at 55°C for 40 sec respectively. The positive and negative controls were included in each run. Both ORF1ab and RBD2 targets were used for screening. The test samples were considered positive for SARS-CoV-2 RNA with a Ct < 37, and the suspicious samples (Ct 37–40) were considered positive if the cycle threshold curve crossed the threshold line of both ORF1ab and RBD2 targets within 40 cycles in the repeated experiment.

1.3. Viral cell culture

Viral cell culture was performed to verify if the RT-qPCR positive SARS-CoV-2 samples are infectious. In a biosafety level 3 laboratory, Vero E6 cells were grown on Dulbecco’s Modified Eagle Medium (DMEM) (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin, in 5% CO₂ at 37°C, in 24-well plates with 1 × 10⁵ cells per well. For all the samples detected positive by RT-qPCR, 100 μL of the originally concentrated sample was inoculated into Vero E6 cells and incubated for 1 hr at 37°C. Thereafter, the inoculum was discarded and 500 μL DMEM, supplemented with 2% FBS and 1% penicillin/streptomycin was added to each well. 250 μL supernatant was collected on day 7 post inoculation, followed by 3 blind passages. Cytopathic effect (CPE) was observed daily. RT-qPCR was used in detection and quantification of SARS-CoV-2 RNA presence in the collected supernatant samples.

2. Results and discussion

From the findings of this study, during the middle risk period, positive samples were detected both in municipal and hospital wastewater systems. One of the three influent samples from WWTP2 tested positive and quantified as 7.4 × 10³ copies/L, and two samples from Jinyintan Hospital wastewater system influents (3.8 × 10³ and 9.3 × 10³ copies/L) were determined as SARS-CoV-2 RNA positive. Compared to previous studies, the abundance of SARS-CoV-2 RNA in influent samples was in the low range in general (Kocamemi et al., 2020a; Wu et al., 2020a), which is consistent with our results. Interestingly, three samples of six SE samples (5.3 × 10³, 1.0 × 10⁴, and 2.3 × 10⁴ copies/L) of WWTP2 tested positive for SARS-CoV-2 RNA, and the RNA copies in SE samples were higher than those in the influent samples. We suspect that the sediments at bottom of wastewater collection pipes (from communities or hospitals) contained SARS-CoV-2, which may have entered the wastewater treatment plant after continuous washing. Since there is no primary sedimentation tank in the WWTP2, SARS-CoV-2 is blocked and concentrated by the biochemical treatment system and further released into the SE. During the low risk period, only one positive sludge sample (1.4 × 10⁴ copies/L) was detected in wastewater system handling wastes from hospitals designated for COVID-19 patients (Fig. 2). This was consistent with the COVID-19 risk level in Wuhan at the time. All of the tested FE, SW, GW, LS, and soil samples were SARS-CoV-2 RNA negative in both the middle and low risk periods. Additionally, attempted viral isolation for all positive samples detected by RT-qPCR was not successful. This illustrates that the disinfection process of drinking water and wastewater plants could effectively inactivate SARS-CoV-2. At the same time, tested samples from environmental sites far away from the COVID-19 designated hospitals were also SARS-CoV-2 negative, which further supports that Wuhan was undergoing a low risk of COVID-19 at that time.
Since the first detection of SARS-CoV-2 in feces (Zhang et al., 2020), it became clear that human wastewater might contain the novel coronavirus. Hence, WBE of SARS-CoV-2 is crucial because it has been suggested that aerosolization of virus-containing faces might pose a threat in its spread and transmission (Wu et al., 2020b). However, it is not yet clear whether SARS-CoV-2 is viable under environmental conditions that could facilitate fecal-oral transmission (Lodder and de Rota Husman, 2020). Moreover, evidence exists of potential community spread, with the virus spreading easily and sustainably in the community in some affected geographic areas, including China (MacKenzie and Smith, 2020; Wu and McGoogan, 2020).

Based on recently conducted studies, SARS-CoV-2 RNA surveillance in wastewater is a useful WBE surveillance approach, the public health risk associated with water cycle is unclear since viral particle infectivity in wastewater and feces is yet to be determined in addition to its probable fecal-oral transmission. Indeed, a recently conducted study has inferred that risk of infection from wastewater and river is insignificant due to the low success rate in cell culture of SARS-CoV-2 from water samples in spite of the high RNA copies (Rimoldi et al., 2020).

From our findings, this study explored the use, sensitivity, and probable reliability of environmental surveillance in the detection of emerging disease outbreaks in the population. The sampling was done in late April, when the COVID-19 epidemic was on a decline in Wuhan, post-lockdown, and massive epidemiological containment measures had been implemented prior. Therefore, the detection of SARS-CoV-2 viral RNA in the tested samples is not surprising because the samples were collected from the designated hospitals and surrounding wastewater treatment plants. This points to the sensitivity of WBE in tracking the pathogen and the need for its adoption in pathogen detection, even under a low prevalence record of human illnesses. This phenomenon, in which a virus can be detected in wastewater in spite of the low prevalence record of human illnesses, might be linked with the capability of wastewater-based surveillance to estimate after careful epidemiological models, mild, subclinical, or asymptomatic cases (La Rosa et al., 2020). These infected individuals shed viruses into local wastewater systems and contribute to virus circulation while remaining substantially undetectable by clinical surveillance, a phenomenon known as the “surveillance pyramid” (Martinez Wassaf et al., 2014). Crude wastewater is a comprehensive and macro indicator. In addition to feces, the domestic wastewater produced by patients with COVID-19 infection also contains a large number of oropharyngeal secretions, nasal secretions, etc., and also has a high viral load (Shi et al., 2020), which increases the amount of virus detection in the wastewater. However, one limitation of this study was that data for the high-risk period was lacking. This was because surveillance and detection of SARS-CoV-2 in residents was a prime priority due to the peak in COVID-19 infection in Wuhan. Moreover, there was no regulation that guided WWTPs on periodic collection and storage of water samples over a period of time.

3. Conclusions

The detection of SARS-CoV-2 RNA in various wastewater systems and related environmental samples in Wuhan shows the significance of WBE in continuous surveillance of SARS-CoV-2 at the community level, under low prevalence record of human illnesses, in contrast to clinical surveillance. This application is principally useful in remote communities and confined populations where mass sampling for the entire population may not be easily achievable at the onset due to inadequate resources or existence of asymptomatic patients. However, effective sampling techniques are of great essence for achieving accurate results in WBE.

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Appendix A Supplementary data

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jes.2021.05.005.

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