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Short Communication

Survey of rapid development of environmental surveillance methods for SARS-CoV-2 detection in wastewater

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HIGHLIGHTS

• Environmental surveillance methods were rapidly applied to SARS-CoV-2 detection.
• Most respondents sampled influent wastewater from urban treatment plants.
• Frequent methods used were PEG, membrane filtration and centrifugal ultrafiltration.
• Molecular detection was commonly used, specifically RT-qPCR or ddRT-PCR.
• Variability in controls used and types suggests challenges comparing between groups.

ABSTRACT

Environmental surveillance as a part of wastewater-based epidemiology (WBE) of SARS-CoV-2 can provide an early, cost-effective, unbiased community-level indicator of circulating COVID-19 in a population. The objective of this study was to determine how widely SARS-CoV-2 detection in wastewater is being investigated and what methods are used. A survey was developed and distributed, with results showing that methods were rapidly applied to conduct SARS-CoV-2 WBE, primarily to test wastewater influent from large urban wastewater treatment plants. Additionally, most methods utilized small wastewater volumes and the primary concentration methods used were polyethylene glycol precipitation, membrane filtration and centrifugal ultrafiltration followed by nucleic acid extraction and assay for primarily nucleocapsid gene targets (N1, N2, and/or N3). Since this survey was performed, many laboratories have continued to optimize and implement a variety of methods for SARS-CoV-2 WBE. Method comparison studies completed since this survey was conducted will assist in developing WBE as a supplemental tool to support public health and policy decision making responses.

1. Introduction

SARS-CoV-2 is the enveloped virus responsible for the COVID-19 pandemic, infecting more than 76 million people and resulting in the death of over 1.6 million globally as of December 21, 2020 (https://news.google.com/covid19/map?hl=en-US&gl=US&ceid=US:en, n.d.). The virus belongs to the family Coronaviridae and genus betacoronavirus. SARS-CoV-2 is approximately 100–130 nm in size with a 30 kb positive sense RNA genome. The primary transmission route for the virus is via respiratory droplets (CDC, 2020), though recent evidence has also shown that the virus infects cells in the gastrointestinal tract and is readily shed in stool (Gu et al., 2020; Xiao et al., 2020).
Environmental surveillance is the monitoring of community health indicators of interest by periodically collecting and analyzing wastewater samples from sewers for the presence of chemical or microbiological targets. Environmental surveillance of wastewater is not a new area of research, and is now defined as part of wastewater-based epidemiology (WBE) (Bivins et al., 2020). It has been critically important in detecting the presence of wild and vaccine strains of poliovirus to support the World Health Organization (WHO) program of eradication of poliomyelitis (WHO, 2015; Asghar et al., 2014) and has also been used to investigate opioid use in communities (Keshaviah, 2017). What is new, however, is the exploration of its potential to provide an early, cost-effective, unbiased, integrated, community-level indication of (trends in) the presence of COVID-19 (International Water Research Summit on Environmental Surveillance of COVID-19 Indicators in Sewersheds, 2020; WHO, 2020; Randazzo et al., 2020a; Mallapaty, 2020).

As the pandemic unfolded the water sector rapidly engaged and responded to the crisis, seeking to increase knowledge and understanding of the virus and its potential pathways for infection. Early studies indicated that RNA indicative of the SARS-CoV-2 virus could be detected in wastewater and potentially serves as an early warning of infection levels in a community (Lodder and de Roda Husman, 2020; Medema et al., 2020; Nemudryi et al., 2020; Peccia et al., 2020; Randazzo et al., 2020b; Rosa et al., 2020; Wu et al., 2020; Ahmed et al., 2020a; Ahmed et al., 2020b). The International Summit held by The Water Research Foundation (WRF) in late April 2020, identified four potential use cases for wastewater surveillance data including:

1. Trends/changes in occurrence
2. Assessment of community prevalence
3. Risk Assessment
4. Viral evolution.

Results from a survey focused on WBE methods for SARS-CoV-2 are described here, representing a snapshot of how widely the methods are being investigated in the water sector and what methodological approaches are being used. This survey was conducted as SARS-CoV-2 is a novel enveloped viral pathogen, potentially requiring use of different sampling and processing techniques than routinely used for other viral pathogens and this has been explored by method comparison studies (Pecson et al., 2020; Philo et al., 2020; Ahmed et al., 2020c). Knowledge sharing and coordination of effort is essential to accelerate the development of effective methods for SARS-CoV-2 detection and support decision making and policy responses to COVID-19.

2. Materials and methods

2.1. Development and distribution

The Water Research Foundation (WRF) released a 42 question survey via social media to collect information on the development of methods for the detection of genes indicating the presence of SARS-CoV-2 in wastewater (Appendix A). The survey was distributed via targeted emails, professional organizations, LinkedIn, Instagram, Twitter and Facebook and was open from the 16th to 24th April 2020.

2.2. Responses

One hundred and sixty-nine (169) responses to this survey were received. The responses were reviewed to remove replicate responses based on replicate IP addresses (22), affiliations (13), and entries (7). This resulted in 127 responses. Of these, 32 (25.2%) respondents answered all of the questions and 95 (74.8%) provided partial responses.

3. Results and discussion

3.1. Respondent information

Responses were received from 35 countries with the highest response from the United States (50), followed by Australia (8), Canada (7), Japan (7) and India (5) (Table 1).

The survey was targeted and distributed to the water industry and responses came from a broad representation of organizations that contribute to this sector including academia (52.0%), industry and utilities (27.3%), state and federal government agencies (15.6%) and other groups including individuals, not-for-profit groups and consultants (11.7%). Respondents to the survey were mostly water industry individuals with technical expertise and identified their roles as either scientific, technical, operations or management responsibility suggesting they have familiarity with these methods and techniques (see Fig. 1).

Although the survey was conducted in mid-April 2020, more than half of the respondents 54.3% (38/70) were already sampling and testing wastewater for the presence of SARS-CoV-2 (42.9%) or other targets of interest (11.4%) for the purpose of WBE, and the other 45.7% (32/70) indicated that they planned to commence environmental surveillance as soon as they had methods ready.

The questions respondents were aiming to address with WBE and what they were going to use the data for widely varied with focus on compliance/policy (relaxation of social distancing measures), methods comparison and method optimization. Many focused on research questions about what could be learnt about SARS-CoV-2 using environmental surveillance to determine the relationship between genomic copies and prevalence estimations, to track temporal patterns of SARS-CoV-2 in wastewater, to determine if additional waves of infections can be predicted, and to inform models.

3.2. Sampling design

3.2.1. Locations

A question was posed to understand the types of locations sampled (question 7: urban, peri urban, rural, or other), indicating the majority of respondents already conducting environmental surveillance focused on urban sites (93.9%) with relatively few respondents additionally or solely conducting monitoring or peri urban (28.6%) or rural (12.2%) sites. The populations served by the urban locations tended to be very large, with 87.0% of respondents indicating samples were from sites with catchment areas with more than 50,000 (56.8%) and/or 500,000 (56.8%) people. Less than 10% of sampling was targeted to areas with <500 people. When this survey was conducted, COVID-19 incidence was highest in urban areas. This has changed in recent months with peri urban and rural areas now showing the greatest incidence of COVID-19 (CDCMMWR, 2020), suggesting increased WBE in rural areas may be needed.

3.2.2. Sampling scheme

With the rapid spread of SARS-CoV-2 and incubation period of 2–14 days (CDC, 2020), sampling frequency and type (grab vs. composite) is important for monitoring trends in virus circulation and type. Therefore, the survey sought to gain information on sampling frequency with most respondents (44.7%) indicating that they were sampling wastewater weekly. While 36.2% indicated they were sampling either fortnightly or monthly. The majority of samples collected were taken as grab samples (63.8%) and/or composite samples (72.3%). Where composite samples were taken, they were predominantly representative of a 24 h time period. Additionally, limited information is available on the persistence of this virus, therefore its survival in different matrices may vary making understanding of the matrices tested important. Although a variety were monitored including at various stages of effluent treatment, the
The majority of respondents indicated they were sampling raw sewage at the influent to the wastewater plant (85.4%). The next most tested matrices were secondary effluent (41.7%) and primary effluent (22.9%). Sampling at pumping stations within the sewer network represented 14.6% of responses. Other types of samples were also being tested by 33.3% of respondents including; primary solids, sludge, disinfected effluent and river water. Finally, the volume of sample being collected for analysis varied widely from as little as 50 mL up to as large as 100 L with larger sample volumes being applicable to treated effluents and cleaner water matrices and the smaller volumes typically being used for influent and primary effluent.

3.3. Shipping and storage

Virus survival could be affected by transportation and storage conditions and duration. As such, information was gathered about conditions used to assist future researchers. Of the 47 responses to question 17 about shipping conditions and time, a majority shipped samples on ice or with cold packs (36; 76.6%). Refrigeration was also infrequently used (3; 6.4%). A few respondents noted that shipping conditions were not applicable (7; 14.9%); it is unknown if this is due to samples not being shipped or not being transported on cold chain. Most samples were transported back to the laboratory in less than 4 h (16; 80%), with the remainder transported in 8 h (1; 5%) or overnight (3; 15%).

There were 42 responses to question 18 specifically about sample storage temperature, indicating that storage in a refrigerator (25; 59.5%), –20 °C freezer (20; 42.6%), or –80 °C freezer (17; 40.8%) were all commonly used. As anticipated, notes suggested that storage temperatures depended upon purpose of sample (for processing or archiving) and length of storage. Of the 47 total responses to question 18, two indicated that they use preservatives during sample storage (4.3%). Of the few responses received about sample storage times, samples were either processed immediately (3; 25%), within 10 h (3; 25%), 24 h (3; 25%), or 48 h (1; 8.3%), with 2 respondents indicating the time varied or was unknown.

3.4. Sample processing

Processing of samples for SARS-CoV-2 detection involves multiples steps such as preliminary treatment, primary concentration, secondary concentration, purification, extraction and/or detection (Fig. 2). Not all steps are used for every processing method. This section will detail the different steps and techniques used in each.

3.4.1. Preliminary treatment

Viruses can partition to solids in wastewater, and this is more pronounced for enveloped than non-enveloped viruses (Ye et al., 2016). Therefore, it was anticipated that some laboratories may be dissociating the viruses from the solids prior to treatment to improve their detection of SARS-CoV-2 or removing the solids for separate processing. A majority of 36 respondents to question 19 indicated they do not dissociate the viruses from solids in suspension (26; 72.2%). Of those performing virus dissociation, methods used included filtration, elution, sonication, and centrifugation (Fig. 3). Of the 35 responses to question 20 about preliminary solids removal, a majority of those indicated that they do conduct solids removal (25; 71.4%) either via centrifugation (14; 40.0%) or filtration (17; 48.6%), with six respondents saving solids for analysis. Analysis of these separated solids was conducted primarily by direct nucleic acid extraction (8) and/or by acid adsorption/elution (2), amino acid buffer extraction (1), and Vertrel™ purification (1).

3.4.2. Primary concentration

Information was sought about the types of methods used for concentration of SARS-CoV-2 as many have been used over the years for WBE of other viral pathogens (WHO, 2015; Fagnant et al., 2018; Calgua et al., 2013; Ahmed et al., 2015). Some methods may be more appropriate for certain use cases than others (depending on sample type, sample characteristics, anticipated viral load, cost, virus recovery, etc.) and at the time this survey was conducted method comparisons for SARS-CoV-2 concentration were not available. A majority (86.5%) of the 37 respondents answering question 21 conduct primary concentration while only 13.5% do not. Polyethylene glycol (PEG) precipitation is the most frequently used primary concentration method, followed closely by membrane filtration (Fig. 4). There were 5 respondents who conduct primary concentration, but not secondary concentration. Of these, the primary concentration methods used included membrane filtration (1), centrifugal ultrafiltration with centricons (3), PEG precipitation (2), and skim milk flocculation (1). The most frequently processed sample volume was 100 mL or less, yielding a final volume after concentration of 10 mL or less for most respondents (Fig. 5).

Of the 16 responses to question 22 about recovery efficiency, the most frequent response indicated that the recovery efficiency is unknown (7; 43.8%). Other responses stated that the recovery was ≥20% (1; 6.3%), ≥40% (2; 12.5%), ≥90% (4; 25.0%) or varied (2; 12.5%).
3.4.3. Secondary concentration

Approximately half of the 32 respondents to question 23 conduct secondary concentration (46.9%) and half do not (53.1%). The most frequently used secondary concentration method was centrifugal ultrafiltration, followed by PEG precipitation and skim milk flocculation (Fig. 6).

Similar to the responses for primary concentration, a majority of the 7 responses to question 24 about recovery efficiency indicated that the recovery efficiency is unknown (4; 57.1%). Other responses stated that the recovery was <50% (2; 28.6%) or ≥70% (1; 14.3%).

3.4.4. Puriﬁcation

There were 29 responses to question 25 about the use of puriﬁcation, indicating most respondents do not conduct puriﬁcation of the samples (26; 89.7%). Of the three respondents who do, two use Vertrel™ and one uses Vertrel™ and Sephadex™.

3.5. Extraction and assay

3.5.1. Nucleic acid extraction

Nucleic acid extraction was performed by a majority of the 31 respondents (28; 90.3%) (question 29), with 65.2% (15/23) of respondents using manual extraction and 39.1% (9/23) using automated extraction (question 30). A variety of extraction protocols or kits were used including Trizol extractions, PEG extractions,
PureLink™ Viral RNA/DNA Mini Kit, NucliSENS® easyMag®, biomeMérieux, Zymo extraction kits, and Qiagen extraction kits (RNeasy PowerMicrobiome, RNeasy PowerSoil Total RNA, RNeasy PowerWater, QIAamp Viral RNA Mini, modified QIAamp Fast DNA Stool, and QIAamp UltraSens Virus). The amount of the sample extracted ranged from ≤140 μL to the entire sample via extraction of a filter and/or pellet with 85% (17/20) of respondents extracting 1 mL or less of the concentrated sample. The extracted eluate volume ranged from 30 μL to ≤3 mL with 95.5% (21/22) of respondents eluting in 100 μL or less. Additionally, the average concentration factor from nucleic acid extraction was 6.5× and ranged from 1.4× to 20×. Twenty respondents indicated that they use an RNA factor from nucleic acid extraction was 6.5× and ranged from 1.4× to 20×. Twenty respondents indicated that they use an RNA extraction control (question 31). The types used included inactivated SARS-CoV-2, norovirus G1L, murine norovirus, pepper mild mottle virus, hepatitis G, bacteriophage MS2, mouse lung DNA, salmon DNA, West Nile virus armored RNA, and non-target or surrogate RNA. Recovery efficiencies reported by four groups ranged from 5% to 90%, 20–80%, 75%, and 80–90% (question 31).

3.5.2. Detection
Choosing an appropriate detection method is crucial to obtaining usable results for the desired research or policy question, though some methods have limitations for use. For example, tissue culture will supply information on viable SARS-CoV-2, but is limited to laboratories with appropriate resources and containment facilities. Molecular detection is commonly used, but low levels of SARS-CoV-2 in samples can make quantification challenging. Understanding the techniques utilized can help future researchers identify those appropriate for their use case. A majority of the 31 respondents obtained quantitative detection results using RT-qPCR, ddRT-PCR, or tissue culture (28; 90.3%) rather than presence/absence results using RT-qPCR (7; 22.6%) (question 32). Of the detection methods asked about, molecular methods were the most frequently used (24/30; 80%; question 34) followed by minION or Sanger sequencing (4/21; 19%; question 36) and tissue culture using plaque assay, TCID50, or MPN (most probable number) methods (3/25; 12%; question 33).

Thirty (30) respondents answered question 34, with 66.7% (20) indicating that they use TaqMan RT-qPCR, 10% (3) use SYBR RT-qPCR, 16.7% (5) use ddRT-PCR, and 10% (3) use another or unspecified type of RT-qPCR. A majority of respondents use a one-step RT-PCR method (70%; 14/20) rather than a two-step method (30%; 6/20). The volume per reaction ranged from 5 to 40 μL, with most using a volume of 20 or 25 μL (61.1%; 13/18). The volume of sample per reaction ranged from 1 to 10 μL, with most using a volume of 5 μL (57.9%; 11/19) This resulted in an average amount of sample volume to reaction volume of 28%, with a median of 25%, minimum of 10%, and maximum of 80%. The number of replicate reactions ranged from 1 to 10 per sample, with most respondents using duplicates or triplicates (72.2%; 13/18). Respondents obtained primers and probes from Integrated DNA Technologies, Sigma-Aldrich, Bio-Rad, LGC Biosearch Technologies, ThermoFisher Scientific, and Metabion. Reagents used included those from Applied Biosystems™ (TaqMan® Fast Virus 1-Step Master Mix, TaqMan® Environmental MasterMix 2.0, TaqPath™ 1-Step RT–qPCR Master Mix, TaqPath 1-Step Multiplex Master Mix, and AgPath-ID™ One-Step RT-PCR Reagents), Qiagen (QuantiTect® Probe PCR Kit and QuantiFast Pathogen – IC Kit), Bio-Rad (Reliance One-Step Multiplex RT-qPCR Supermix and One-Step RT-ddPCR Advanced Kit for Probes), and Invitrogen (SuperScript™ III One-Step RT-PCR System with Platinum™ Taq). Instruments used included those from Bio-Rad (CFX and QX200), ABI (StepOnePlus, QuantStudio 5 and 7, and 7500), Roche (LightCycler® 480 II), Rotor-Gene (3000 and Q). Of the 14 responses about the molecular methods targeted, the genes included Nucleocapsid (N) (10; 71.4%), Envelope protein (E) (5; 35.7%), RNA-dependent RNA polymerase (RdRP) (2; 14.3%), and Open reading frame (orf1a) (1; 7.1%). Three responses did not specify a target or
targeted organisms other than SARS-CoV-2 (21.4%). Standard curves were generated using DNA standards (gBlocks, synthetic DNA, or plasmids) or RNA standards (RNA transcripts) (question 35). Information about the limit of detection (LOD) for RT-qPCR and ddPCR methods was provided by 15 respondents in question 37, with 6 stating the LOD was unknown or under determination. The LOD ranged from 1 to 100 genome copies per reaction or 0.0007 to 2 genome copies per μL.

A majority of groups use controls (88.5%; 23) with 11.5% (3) stating the control asked about were not applicable (question 38; 26 respondents). More specifically, 76.9% (20) indicated that they use a positive control, 76.9% (20) use a negative control, 42.3% (11) use an internal control, 65.4% (17) use an inhibition control, 3.9% (1) use an RT efficiency control, and 3.9% (1) use a positive extraction control. Most positive controls used were SARS-CoV-2 in some form including a plasmid with the nucleocapsid gene, RNA transcripts, gBlocks, RNA extracts from inactivated SARS-CoV-2, and positive clinical samples. Negative controls generally consisted of negative extraction blanks, no template controls, and nuclease free water. Internal controls used included pepper mild mottle virus, murine norovirus, extracted norovirus GI, and Enterobacteriaceae, as well as non-target or non-viral RNA. Inhibition controls included hepatitis G, murine norovirus, West Nile virus, phocine herpesvirus, and MS2, as well as non-target or non-viral RNA.

3.6. Effective volume assayed

The effective volume assayed is important to consider when selecting a sample processing method. Larger effective volumes assayed can lead to increased likelihood of detection of a low concentration target but can also concentrate more inhibitors which can affect detection. Typically, 76.9% (20) of respondents indicated they use one and 34.8% indicating they just use one control.

Respondents were asked about the steps taken to validate their methods (question 40). Many indicated plans to complete this work but noted it was ongoing or in the planning stage. Techniques used or planned to use included recovery studies with spiked samples, analysis of negative and positive field samples, use of internal controls and matrix spikes, and sequencing.

4. Conclusions

This survey showed that the water sector quickly adapted methods to conduct environmental surveillance for SARS-CoV-2 and applied them primarily to test wastewater influent from large urban wastewater treatment plants. Future studies should likely expand WBE to peri urban and rural areas as the COVID-19 incidence is higher in those locations (as of December 15, 2020). Only small volumes were needed, and the primary methods of concentration were PEG, membrane filtration and centrifugal ultrafiltration. Comparison of these concentration methods in separate studies, indicated that there is no one best method and many will yield positive results (Pecson et al., 2020; Philo et al., 2020). Therefore, methods should be chosen based on the specific use case, supply chain availability, and resources. Assay for N gene targets was primarily accomplished via RT-qPCR. This should be further explored, as digital RT-PCR may lead to improved detection at lower concentrations and with greater inhibition present. Since the survey was conducted in mid-April 2020 many laboratories have continued to develop and implement WBE approaches for SARS-CoV-2. This data helped inform the WRF International Summit in late April 2020, which enabled global experts to determine best practices and rapidly guide the design of a methods comparison study. In June 2020, 92 laboratories indicated that they had methods developed and were willing to take part in a comparative evaluation of methods (WRF project 5089) (Pecson et al., 2020). As further research is conducted, it is vital to consider development or optimization of methods applicable for use in lower- and middle-income countries (LMIC), which are more resource limited. Finally, as SARS-CoV-2 WBE studies have continued and
methods improved over the past eight months since this survey was conducted, a follow up survey will be developed to determine optimized methods and focus on effective volumes processed, cost, and method effectiveness and applicability for LMICs. Comparative evaluation of methods will enable rapid identification of the most reliable and robust methodological approaches and increase the credibility of WBE as a supplemental tool to support public health and policy decision making responses to COVID-19.

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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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References

Ahmed, W., Harwood, V.J., Gyawali, P., Sidhu, J.P.S., Toze, S., 2015. Comparison of concentration methods for quantitative detection of sewage-associated viral markers in environmental waters. Appl. Environ. Microbiol. 81, 2042–2049.

Ahmed, W., et al., 2020a. First confirmed detection of SARS-CoV-2 in untreated wastewater in Australia: a proof of concept for the wastewater surveillance of COVID-19 in the community. Sci. Total Environ. 728, 138764.

Ahmed, W., et al., 2020b. Detection of SARS-CoV-2 RNA in commercial passenger aircraft and cruise ship wastewater: a surveillance tool for assessing the presence of COVID-19 infected travellers. J. Travel Med. 27.