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Wastewater Based Surveillance of SARS-CoV-2: Challenges and Perspective from a Canadian Inter-laboratory Study

The current rapidly rising 5th wave of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), dominated by the omicron variant, has clearly shown the challenges in monitoring, and tracing clinical cases of infection. Omicron has been identified by the World Health Organization (WHO) as a variant of concern (VOC) on November 26th of 2021, because of the reported higher risk of transmission, reinfection, and potential to escape immunity (WHO 2022; Pulliam et al., 2021). As of January 13th, 2022, the WHO has reported over 312.8 million total cases of SARS-CoV-2, and over 5.5 million deaths, with cases continuing to rise at an unprecedented rate (WHO, 2022). To date, SARS-CoV-2 detection in the community is primarily through clinical testing that involves the gold standard process of nasopharyngeal swab sample collection followed by real time quantitative polymerase chain reaction (RT-qPCR) detection. However, omicron's rapid transmission has clearly shown that clinical testing alone cannot meet the needs for monitoring and tracing SARS-CoV-2 infection in communities. With the exponential increase in daily SARS-CoV-2 cases of omicron, many countries are struggling to clinically diagnose every infected patient and trace contacts. Public health districts and health care facilities are once again overwhelmed with this wave of the pandemic. Furthermore, many presymptomatic, asymptomatic, and mild cases significantly contribute to the spread and are undetected. Consequently, capacity-constrained clinical testing, as currently exists with omicron wave, systematically underestimates the prevalence of the SARS-CoV-2 pandemic.

This has led health care organizations and governments to re-emphasise the necessity and power of wastewater based surveillance (WBS) in community monitoring. Organizations such as the International Water Association (2020), the Global Institute for Water Security (2022), and the Canadian Water Network (2021) have meticulously been working towards using WBS to better understand the spread and community trends of the SARS-CoV-2 pandemic. To date, there have been over 200 research groups in 50 countries across the globe involved in WBS of SARS-CoV-2. (Naughton et al., 2021). Furthermore, there has been successful early detection of the omicron variant in wastewater samples (Lee et al., 2021) although Hrudey and Conant (2022) have described the practical limits for expectations about early warning with WBS as well describing its overall strengths and limitations. This emerging recognition and importance of WBS of SARS-CoV-2 has led us to highlight an inter-laboratory study published by Chik et al. (2021).

WBS is a useful surveillance tool that complements clinical testing of SARS-CoV-2 within a community. Unlike the clinical testing process, WBS minimizes population bias as it provides insight into a broader spectrum of SARS-CoV-2 case presentations and patient populations within a community (Michael-Kordatou et al., 2020). Although SARS-CoV-2 is a respiratory virus, several studies have demonstrated that up to 89% of infected patients shed SARS-CoV-2 viral particles in their feces as early as one day after infection (Wolfel et al., 2020; Zhang et al., 2020; Gupta et al., 2020). SARS-CoV-2 viral particles in wastewater are not infectious as determined from viral culture studies, demonstrating that the complex wastewater matrix and disinfection methods at drinking and wastewater plants are sufficient to inactivate SARS-CoV-2 (Zhao et al., 2022). Therefore, the presence of SARS-CoV-2 viral particles in wastewater makes it a useful source for community surveillance of SARS-CoV-2.

Successful monitoring of SARS-CoV-2 in wastewater systems is challenging to achieve for many reasons (Kantor et al., 2021; Pecson et al., 2021). Despite a great deal of research efforts, no standardization of methods for viral RNA concentration, extraction, and quantification has been implemented for WBS of SARS-CoV-2, which is limiting the application of WBS. Variation in results from inter-laboratory studies cannot be attributed to a single explanation (Kantor et al., 2021; Pecson et al., 2021). One explanation is due to the complex wastewater matrix. Many biological and chemical compounds, such as PCR inhibitors, exist in wastewater at variable concentrations, and the matrix can cause poor viral recovery, detection, and reproducibility. Other explanations include viral particle degradation, loss of viral particles during concentration, and RNA degradation during extraction (Kantor et al., 2021). A recent review by Kumblathan et al. (2021) outlined common methodologies used for WBS of SARS-CoV-2 and critically re-

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viewed the challenges associated with WBS sampling, enrichment of viral particles, RNA extraction, and subsequent RNA detection. Enrichment methods include polyethylene glycol precipitation, aqueous two-phase partitioning, electronegative membranes, ultrafiltration and ultracentrifugation, and flocculation with a beef extract solution. RNA extraction and purification methods include phenol-chloroform purification, and solid-phase RNA purification via silica membrane or silica beads. RNA detection involves RT-qPCR, in which viral RNA is converted to complementary DNA (cDNA) by the reverse transcriptase enzyme, then exponentially amplified and detected by fluorescent probes (Feng et al., 2020).

Currently most laboratories around the world do not have identical standard operating procedures (SOPs) or analytical equipment for wastewater surveillance of SARS-CoV-2, thus the lack of WBS method standardization can result in variation in results. To identify key factors contributing to variation of inter-laboratory results and develop an applicable approach for Canadian wastewater monitoring of SARS-CoV-2, Chik et al. (2021) collaborated with eight laboratories in Canada, each with documented experience in analyzing wastewater for SARS-CoV-2. This study examined inter- and intra-laboratory variability when a common set of wastewater samples spiked with SARS-CoV-2 surrogates were analyzed. They identified the key factors affecting quantitative measurements of SARS-CoV-2 genetic markers in wastewater samples to provide a basis for improved WBS of SARS-CoV-2 for surveillance of community infections in Canada.

Grab wastewater samples were collected from a wastewater treatment plant in Winnipeg, Manitoba, Canada, where, at the time of sampling, there were 85 active SARS-CoV-2 cases in a served population of ~750,000, identified by the clinical standard RT-PCR method. Composite wastewater samples are preferred for WBS because they can give a better representation of SARS-CoV-2 concentration variation over time. For the purpose of this study, grab samples were sufficient to provide an authentic wastewater matrix for spiking to evaluate recoveries, performance of each laboratory protocol, and to identify the key factors contributing to variations in results. The raw wastewater samples were spiked with SARS-CoV-2 surrogates – either gamma-irradiated inactivated SARS-CoV-2, or human coronavirus strain 229E (HCoV-229E) – at low and high concentrations, with no-spike wastewater aliquots provided as blank solutions. The samples were all shipped to participating labs across Canada under identical conditions (4°C). The eight participating laboratories then applied different concentration and extraction methods with varying quality assurance and quality controls, which were reported anonymously by Chik et al. (2021). All the laboratories detected SARS-CoV-2 and surrogate RNA using RT-qPCR, however, the thermocycle parameters varied across the laboratories. Additionally, the laboratories, used different protocols for enrichment of the viral particles and RNA extraction. Therefore, it remains difficult to determine which protocol is the most efficient.

Regardless of different protocols, each of the eight experienced laboratories in the Chik et al. (2021) study provided consistent results. All laboratories reported SARS-CoV-2 RNA concentration estimates within 1.0-log10 range for each of the spiked conditions. The results demonstrated some inter-laboratory variability in the RNA copy number reported. However, there was less variation with the high spike condition as opposed to the low spike condition, suggesting that variations are dependent on the sensitivity of the methods used. This is likely due to the cycle threshold (Ct) values observed for low-spike conditions approaching the sensitivity limit and thus not within the linear range of PCR amplification. Nonetheless, a clear distinction was found between the high-spike conditions and low-spike conditions, indicating each laboratory was consistent in their procedures. Furthermore, the Chik et al. (2021) study provided evidence that a wide range of methods used in WBS for SARS-CoV-2 are reliable. Although all 8 labs used different combinations of enrichment, extraction, and detection platforms, they all successfully distinguished low and high spike conditions, presented similar estimates for each condition, of which all estimates were within one order of magnitude. This is useful for ongoing wastewater monitoring as the trends observed from WBS data can be used to compare and analyze with clinically reported cases, as well as estimate prevalence. Wastewater surveillance can also be used to monitor for future outbreaks and detect variants, potentially before they are even clinically reported. This was recently demonstrated by a study done by Public Health France where omicron was detected in wastewater when only two cases of the new variant had been clinically reported (Ferré et al., 2022).

The Chik et al. (2021) study also gives valuable insight into some factors that may contribute to the inter and intra-study variation. One such factor is due to the different SOPs and standards used. Pecson et al. (2021) evaluated thirty-six WBS of SARS-CoV-2 SOPs across laboratories in the United States to determine reproducibility of SARS-CoV-2 concentrations in wastewater. As expected, the most consistent results were found when using the same SOP in the same laboratory, indicating that consistency in SOPs for WBS of SARS-CoV-2 is an important factor. The laboratories in the Chik et al. (2021) study used different standards for quantification, and anonymously reported whether an RNA or plasmid DNA standard was used. This is important to note that using plasmid DNA as a calibration standard as opposed to an RNA standard can result in two orders of magnitude variability, likely due to DNA being supercoiled during early stages of RT-qPCR (Chik et al., 2021; Hou et al., 2010; Kumblathan et al., 2021). Regardless, in both the Chik et al. (2021) and Pecson et al. (2021) studies, laboratories were reporting relatively similar orders of magnitude of RNA copy numbers.

Variation can result from the use of different surrogate viruses that may partition differently in wastewater between the solid and aqueous phase. In the wastewater system, the SARS-CoV-2 viral particle is transported by fecal matter and then released into the wastewater matrix where they may adsorb onto solids (Peng et al., 2020). In the Chik et al. (2021) study, surrogates were spiked into the post-grit grab wastewater sample which may not contain many solids, so there may be bias towards the aqueous phase (Buonerba et al., 2021; D'Aoust et al., 2021). Current methods are focused heavily on the aqueous phase, which may contribute to better recovery for this study. One potential method for precipitating SARS-CoV-2 from the solid phase is to use a beef

extract solution (Mlejnkova et al., 2020). The viral particles separated from the solid phase should be combined with the aqueous phase supernatant (Kumblathan et al., 2021). Future studies should investigate each of the aqueous and solid phases individually to determine the relative abundance and partition characteristics of SARS-CoV-2 and surrogates in each phase. When viral particles in the solid phase are sufficiently high, the combined recovery from aqueous and solid phases of real wastewater samples should provide better estimate of the viral load. This also indicates the need to develop new methods capable of efficiently recovering viruses in wastewater samples which contain both aqueous and solid phases.

The presence of PCR inhibitors in wastewater samples is another factor contributing to the variations of results. Due to the complex wastewater matrix, there is potential for PCR inhibition from proteins, fats, carbohydrates, polyphenols, metal ions, and RNAses (Ahmed et al., 2020; Schrader et al., 2012). Therefore, false negatives are a challenge when quantifying SARS-CoV-2 with qPCR. Quality assurance and control optimization steps should be taken into consideration to help alleviate any PCR inhibition downstream. PCR inhibition should be monitored using appropriate reference and recovery controls. Steps may also be taken to alleviate PCR inhibition. This may include optimizing RNA extraction methods, so they are more efficient for wastewater samples. Sample dilution is another option; however, this may result in a loss of sensitivity. Studies have also reported the use of heat and chemical treatment, as well as the use of inhibitor tolerant polymerases for environmental samples (Ahmed et al., 2022; Schrader et al., 2012).

Finally, this Canadian inter-laboratory study by Chik et al. (2021) demonstrates the consistency of results within the established laboratories and the ability to provide reliable trends of SARS-CoV-2 variants in wastewater samples. This suggests that WBS data can assist to estimate trends in the prevalence of SARS-CoV-2 and complement clinical data. The combination of clinical and WBS data will provide critical information which may lead to better public health measures for the on-going control of the SARS-CoV-2 pandemic. Although substantial progress has been made in WBS of SARS-CoV-2, this study also identified important factors causing variation which limits some applicability of WBS data. Regardless, the comparison of occurrence and trends of the variants in different communities can still be valid. The currently limited application of WBS in public health surveillance and policy making highlights the needs to modernize and standardize WBS for future monitoring of infectious agents. These knowledge gaps demand the need for future research and development towards establishing a standardized sampling protocol that considers various parameters in different sewer systems, developing simplified and robust sample preparation and detection techniques that can provide sensitive and reliable data, and establishing validated surrogates that can be normalized to estimate prevalence of infection. Current WBS of SARS-CoV-2 studies and the ongoing public health crisis have demonstrated the WBS as another powerful tool for community surveillance, control of infection by variants, and signaling of future infectious disease outbreaks.

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