Canadian Coalition on Wastewater-Related COVID-19 Research

Draft sample collection, processing and analysis protocol for a valid hypothesis-testing pilot study: Surveillance of community or institutional wastewater for SARS-CoV-2 to supplement clinical evidence about prevalence of infection

Context and objective

This draft guidance is specifically designed to support a pilot study in Canada to prove the concept of the ability of surveillance of wastewater for quantitative measurement of SARS-CoV-2 to track trends in community prevalence of COVID-19.

While this protocol is meant to enable a robust study that addresses the 8 design principles for the COVID-19 Wastewater Coalition pilot study, this protocol focuses on elaborating the elements that ensure principles #2, #3 and #4 in particular.

8 design principles for the COVID-19 Wastewater Coalition pilot study

1. Clearly define pilot monitoring program objectives
2. **Achieve rapid validation and adoption of a consistent Canadian sampling protocol**
3. **Confirm validity of wastewater analyses for SARS-CoV-2**
4. **Ensure potential for generalizability by fully understanding what samples represent**
5. Maximize value of results through strategic pilot project design
6. Maximize potential for productive collaboration with wastewater utilities
7. Maximize collaboration, cooperation and knowledge exchange
8. Consider ultimate use and ethics of data use in public health decision-making

The rapid and recent evolution of research methods worldwide since late March 2020 reflects the fact that the science of wastewater-based epidemiology is rapidly evolving to address the novel coronavirus SARS-CoV-2 and support actions for pandemic management. As a result, there is a variety different methods in current use and as yet no formally validated standard methods for this purpose. This guidance does not intend to establish an accepted standard method for Canada. Rather, it provides a recommended approach for the pilot study. The objective is to achieve a common approach to wastewater sampling and analysis in the pilot to support useful surveillance that will result in more comparable results across different locations and labs. The following advice is necessarily a product of the best judgement of an expert advisory group aiming to align a starting point for work in Canada. This advice builds on the practical experience of various researchers who have tackled this challenge.

Inevitably, methods will evolve as more is learned. Hence this protocol is provided as a draft document that can evolve as our experience increases. A set of flexible guidelines and generic controls are suggested to allow for evolution of testing methods and normalization of results. The national and international networks that have rapidly evolved in the face of this global pandemic have expressed strong interested in working collaboratively to foster the evolution towards a reasonably effective approach to gathering evidence for the stated purpose.

A practical approach for achieving comparability of PCR-based wastewater analyses

The proposed pilot surveillance project is seeking specifically to **track SARS-CoV-2 in municipal wastewater**, a matrix posing potential inhibition and variable recovery of the signal in the most commonly used analytical approach, RT-qPCR**.** There is currently no viable option for a single laboratory to process wastewater samples for multiple sites across Canada and whereas most labs are using RT-qPCR to detect SARS-CoV-2 in sewage samples, other labs are utilizing recently introduced digital-PCR instrumentation approaches such as ddPCR™. Each lab will necessarily adhere to different approaches and specific standard operating procedures (SOPs) for sample handling and analysis in line with their available infrastructure, experience and the requirements of their institution. Individual lab SOPs are typically very detailed and method/lab dependent so details may not be directly relevant to other labs although the analytical principles are applicable.

A focus on a common standardized QA/QC procedure

In recognition of some inevitable variability in sample preparation and analysis across the study labs, this protocol focuses on the recommendation for use of common standardized QA/QC procedures to allow, to the greatest degree possible, comparison of results among different labs following evolving procedures. For similar reasons the pilot study promotes the use of an inter-laboratory testing program as part of the program.

This document is the result of multiple inputs from many sources, including publications that are credited in the acknowledgements and reference citations.

Sampling considerations in study design

The final design of the pilot study will depend on the key criteria established with public health end-users and other partners, agreed length of the study, and level of resource support provided. This draft protocol includes some considerations to be addressed in the final study design related to sampling.

Strategic selection of communities or institutions

A core objective of the initial pilot project, addressed by this draft protocol, is to validate the sampling and analytical procedures used in the pilot sufficiently that there is confidence in applying them to provide useful data to public health decision-makers. The intent of the proof-

of-concept pilot is to first test the hypothesis that wastewater surveillance can provide useful insights that would enable development of a more expanded surveillance program; potentially enabling implementation of a longer-term wastewater-based surveillance program that provides a wider geographic coverage.

For the immediate purpose of this initial pilot study — i.e., validating the ability to usefully augment population COVID-19 surveillance through wastewater monitoring — the spectrum of communities or institutions selected for sampling should include a range of different anticipated levels of COVID-19 in the community to test the range and efficacy of the technique. This should include communities with a known level of infection high enough that detection of SARS-CoV-2 is expected and at least one community that has very low levels of reported infections, such that detection of SARS-CoV-2 through wastewater is expected to be challenging. Including communities with levels of infection that fall in between these two boundary conditions is desirable to provide evidence of the quantitative ability of the techniques to discriminate high from medium and low infection burdens, and lend more confidence for the strength of the technique to effectively define trends over a range of concentrations that might be experienced by a community due to the rise and fall of infection rates anticipated during outbreaks, secondary transmissions and recoveries.

Frequency of sampling

To maximize cooperation with wastewater utilities, sampling should take advantage of using a portion of samples that the utility already collects for its own purposes. Where possible, refrigerated autosamplers should be used, or samplers than enable addition of ice to the storage mechanism to keep samples cool. For the purposes of the pilot project, as many samples as can be realistically analyzed in a timely manner should be obtained. These considerations must reflect the capability of the lab to process samples as soon as they are received so that sample timing must consider only taking samples on days when they can be processed by the lab to a degree sufficient to achieve sample stability (enrichment/extraction) on the next day (until alternative plant preservation methods are validated). Because the pilot project is aimed at assessing trends in the titer of SARS-CoV-2 as they may reflect trends in community infection with COVID-19, **more than 2 samples per week** will likely be necessary for the initial phases of the study. Once the local schedule is determined, it should remain consistent for the duration of the project.

Duration of sampling program

The pilot project will need to include a minimum initial sampling phase of **at least 3 weeks** of sampling, assuming 3 samples per week can be obtained. If less frequent sampling is necessary, then longer duration will be necessary. Ultimately, the nature and quality of results obtained in the initial phase of pilot project will dictate how long the initial sampling program must be maintained.

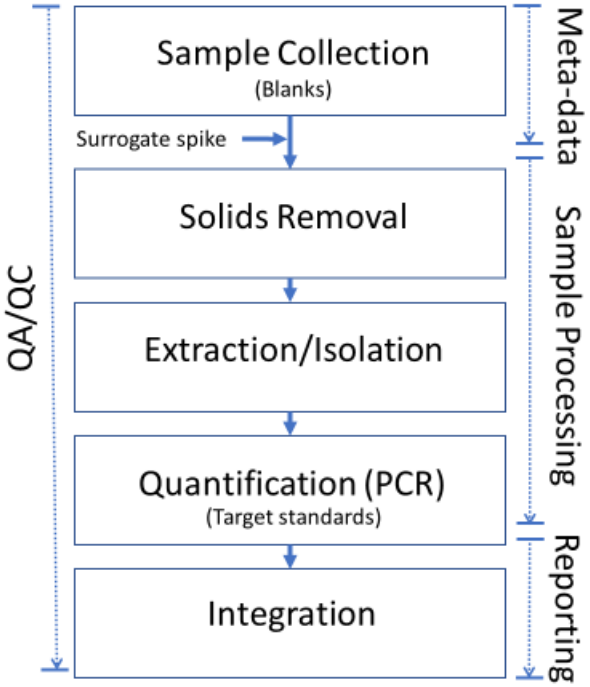

Figure 1. General outline of the sample/analysis protocol.

Sample collection process

The wastewater utility SOP must be followed with necessary PPE and sample chain of custody protection for up to a 1 L sample volume. Specific operational protocols for each site should be written as each will differ in details, but general principles will be similar. Some staff may require training on proper collection and personal protection including protocols on the use of social distancing, clean gloves, coveralls/lab coats, masks, eye protection, etc.

The lab will normally work with a microbiological water sample of appropriate volume to the method being used, for each day of sampling. If available volumes are greater than minimum required for the method, they should be split into aliquots that may be used for inter-laboratory testing, other QA/QC measures or archival storage. Samples held, if stored for more than 24 hours, should be frozen to -20°C and if the intent is to store or archive samples for extended periods (> 1 to 2 weeks), they should ideally be frozen at -80°C. The impacts of freezing raw wastewater samples on method results is not yet known. When possible, the raw wastewater samples should be processed and the extracts frozen instead of raw samples, to improve stability. Freezing and thawing cycles must be avoided. This can be aided by taking larger samples and

splitting a given sample, or preferably the enriched/concentrated, or extracted samples, into aliquots to enable freezing of split samples for later analysis or for inter-lab studies.

Sample container preparation
The sample bottles, containers and anything used for collection must be sterilized in advance (inside and outside) as per normal procedures for microbiological sampling (Baird & Bridgewater, 2017). Use of new pre-cleaned bottles is highly recommended.

The sample bottles will be provided to the field/municipal staff in a container that has been pre-cleaned (e.g., sterilized using 10% bleach — taking care to not leave a residual — or 70% ethanol). The bottles should all be pre-labelled and data sheets included (along with sterilized pencil/pen). The cooler (or other container that can be sterilized and contain any spill) should also be pre-cleaned using 10% bleach or 70% ethanol).

Sample type (grab or composite)
24h composite samples, collected in refrigerated sampling devices, are strongly preferred. Once collected, samples should be kept cool (4-8^{o}C) and should be dark composite samples collected in equal time/volume intervals that are as small as possible (e.g., 10 minutes). The composite should be collected in a pre-cleaned polypropylene/polycarbonate container (this will depend on the sampler and plant SOP for microbiological sampling). Specific volumes, time of collection, name of person doing collections, type of sampler, sample container, etc., should be recorded. For the purpose of the pilot, composite samples are preferred. Grab samples can be used for specific purposes (checking some aspect of methods).

Sample container
The samples can be collected in individual sample bottles or collected at the site in a larger container and then split into aliquots in the lab. The container material can differ, but durability and transportation feasibility should govern. However, blanks should be handled in the same way as samples. Preference should be to use new supplier-pre-cleaned/sterilized containers to avoid any opportunity for contamination. Sample containers should not be over-filled and 10-20 mL space should remain at the top (this is to avoid spillage later in the lab and/or accommodate expansion upon freezing should the sample be archived).

Sample volume
The volume of the sample collection containers should be standardized. Use of replicated (3 or more) 250 mL polypropylene bottles (or 50 mL Falcon Tube containers) are convenient and provide lots of options but some labs may require greater volumes per aliquot.

Sampling location
The optimal location will depend on specific work to evaluate method sensitivity for quantification of SARS-CoV-2. The sample collected should be whole influent to the treatment

plant. The sample location will likely be dependent on the normal sampling location(s) of the treatment plant. The specifics of this location should be documented and represent the incoming sewage to the best degree possible. Sampling after the larger solids (screening) and grit chamber treatment is recommended. The possibility has been demonstrated that primary sludge (i.e., the solids settled from raw sewage in a primary sedimentation treatment) can show a strong signal for SARS-CoV-2 (Peccia et al., 2020); however, for the pilot study whole homogenous influent will be used. If the participating lab has the capacity, processing of primary sludge should be considered for gaining additional insights.

Travel/field blanks

A field blank sample with sterile water should be opened at the site of collection for about the same amount of time and transported with all sample bottles. The caps of these travel/field blank bottles should be opened at the site for a similar time to the other bottles, recapped and treated like the other samples. Where sampling must be done by utility personnel, the capacity limitations of those personnel to take on additional duties must be acknowledged.

Sample spike — internal standard, positive control

In method development, the integrity of target analytes should be tested using blanks and matrix spikes. It may not be possible to routinely spike samples during field collections, but they should be spiked as early as possible. Ideally all samples (including the field blank but not the travel blank unless it is a different surrogate) should be spiked with a suitable surrogate as soon as possible in the process to ensure sample integrity and extraction efficiency.

Some studies have used MS2, phi 6 bacteriophage or bovine coronavirus spike surrogates, but seasonal flu coronaviruses (strain 229E [α-CoV] or OC43 [β-CoV]) may be more appropriate. Multiple surrogates can be used at different stages of sample collection, extraction and analysis (especially during method development). The appropriate surrogate should be selected for each process. Having an internal standard (spiked, positive control) is essential to establish the level of recovery that is being achieved in a given laboratory and is thus essential to allow for any meaningful comparison of results among participating labs.

It is also highly desirable to include analysis of a virus that is commonly found in sewage, such as the Pepper Mild Mottle virus (PMMoV) (Kitajima et al., 2020). A variety options are available and future updates will provide additional advice on this matter.

Sample transportation

Samples should be transported in precleaned secondary containment (e.g., cooler) with precleaned ice packs (ice is less desirable because of creating melt water) as soon as possible to

the laboratory for immediate processing/extraction if possible (less than 24 hours). This time should be standardized and recorded for each sample.

Sample storage
Samples should be processed immediately to a stage where they can be stabilized or frozen without impacting the RNA integrity.

Whole sewage samples should not be frozen unless there is no other option. If samples are frozen for storage this should be done at -80°C and if feasible should be spiked with a different quantified surrogate in advance, if possible, to assess process losses.

Sample processing

Handling of samples
Wastewater samples should be handled in the lab using sterile techniques. All surfaces should be sterilized in advance and regularly during sample handling. Personnel should use appropriate PPE (lab coats, gloves, goggles) and follow local specific SOPs to ensure personal safety as well as sample integrity.

The trace levels of the viral fragments in wastewater necessitates that cross-contamination control be rigorous. The separation of tasks into separate areas/rooms is highly desirable. In addition, precautions must be observed to minimize the risk of cross contamination when setting up PCR reactions:

- The sample needs to be processed in an environment free of RNase.
- PCR should be performed in a dedicated 'clean' area which is free from DNA contamination.
- All stocks of pipette tips, microcentrifuge tubes, etc. to be used should be DNase, RNase and nuclease-free, and stored in a dust free environment. The use of filter tips is advisable.
- Pipettes designated solely for PCR should be used to set up the reaction.
- Wear RNase and DNA free gloves at all times.
- Prepare the PCR master mix in an environment (laboratory, BSC or PCR hood) which is DNA/RNA isolation and gel-electrophoresis-free lab.
- Template RNA should be added in a controlled clean environment such as a PCR hood and conventional precautions should be followed regarding laboratory workspaces to avoid sample contamination.
- Decontaminate instruments and laboratory areas before and after isolation (e.g., 10% bleach or 70% ethanol).
- For biosafety cabinet and PCR PRE-station, turn on the UV-light for at least 15 minutes.

Disinfection of the sample container
The outside of the container should be disinfected on arrival to the lab before it is opened. The bottles should be wiped down with a disinfectant (e.g., 70% ethanol).

Deactivation/sterilization
Samples can be pasteurized/deactivated to enhance lab safety by pasteurization (e.g., 60°C for 90 minutes) (Biobot Analytics, 2020; La Rosa et al., 2020).

As per laboratory SOP for any biohazard, all materials used and residuals should be placed within biohazard bags and autoclaved and/or incinerated.

Pre-treatment to remove solids
The removal of large particles can be done using centrifugation (slow speeds) and/or filtration. This should be done consistently within the study.

pH Adjustment: In some protocols, whole influent samples can be adjusted to pH 10.5 with carbonate buffer, vortexed to aid desorption of virions.

Centrifuge: Whole influent samples (either pH-adjusted or not) can be spun at low speed (e.g., 600 g for 10 minutes, although this varies considerably) and the supernatant decanted for further processing.

Filtration: Whole effluent can be passed through sequential filters of different pore size with a final filters pore size of 0.2 microns (µm) to remove solids.

RNA extraction/isolation
There are several approaches that can be applied to the RNA extraction. Caution should be used here as the method needs to be matched to the clean-up/isolation stages. There are a variety of commercial kits available for RNA extraction and clean-up. Several approaches include but are not limited to:

Ultrafiltration: Sample can be concentrated using ultrafiltration (Centricon Plus-70 10 kDa) (Ahmed et al., 2020) and then prepared using a method of preference, such as a commercial RNA extraction kit. For example, one-step centrifugal method using Centricon Plus-70 (MWCO) developed previously for non-enveloped viral concentration of wastewater samples (Qiu et al., 2012). Viral RNA can be extracted using a variety of commercial kits.

Ultracentrifugation: Samples can be mixed in polyethylene glycol (PEG) and spun at high speed (e.g., 12,000 g for at least 30 minutes) to collect a pellet. The pellet is recovered in a buffer and then the RNA can be extracted using commercial kits (Wurtzer et al., 2020; Bar-Or et al., 2020), or the pellet is recovered in trizol and extracted with chloroform/isopropanol and sequential washing steps (Biobot Analytics, 2020; Wu et al., 2020).

Flocculation: Samples can be precipitated using various methods including AlCl3 at reduced pH followed by centrifugation. The sample can then be extracted with commercial RNA isolation kit (Randazzo et al., 2020).

Quantification of target(s)

The RNA can be detected using a one or two step RT-PCR. A two-step process isolates the RT step from the PCR step and may enable operator's further optimization of the RT process isolated from the PCR step. However, one-step PCR may reduce the sensitivity because of the additional dilution step.

Analysis should be done with at least three sample replicates and at least 3 technical replicates. Non template controls and internal controls are recommended to be run on each plate.

qPCR or dd-PCR validated target specific genes of SARS-CoV-2 should be used. These can differ but many studies on wastewater have used the nucleocapsid (N) gene N1 and N2 primers from SARS-CoV-2 and these should be included.

Standards curves are required for RT-qPCR and are recommended to be comprised of 10-fold dilutions (typically from 10 copies to 10^6 copies) of a SARS-CoV-2 RNA fragment. Results can be expressed as gene copy numbers and then expressed as per volume (e.g., mL) of original wastewater sample.

PCR inhibition

If there is a loss of recovery standard, the sample can be checked for PCR inhibition. An appropriate amount of a known DNA that is not expected to be in the influent can be added at a known amount to the sample and contrasted to non-matrix control.

QC/QA protocols

The reporting of the PCR results should be consistent with MIQE Guidelines (Bustin et al., 2009).

Recovery rate (%) of the selected spike (e.g., MS2, phi6, OC43/229E) introduced to the whole influent should be assessed as the amount of coronavirus spiked in the sample / amount of RNA detected in the baseline sample x 100 (e.g., Li et al., 2019). Some labs may use additional multiple spikes introduced at different stages to monitor various stages of the method. In addition to the method recovery (for surrogates and targets), it is preferable that recoveries be reported for each individual sample tested.

If any field or laboratory blanks for a set of samples shows a positive result above a preset threshold, then associated test samples should be considered questionable and results not used and/or samples rerun.

Sampling metadata considerations to support interpretation

Information to support compiled data on total testing numbers, positive and negative results, and temporal and spatial trends will be required to investigate correlation with SARS-CoV-2 in wastewater and community data on infection and potentially COVID-19 disease burden associated with the associated sewer-sheds.

As much corresponding data from the wastewater treatment as possible to support this analysis should be collected, including: flow (including precipitation as it affects combined sewers), influent pH, temperature, conductivity, suspended solids plus any other data concerning the influent raw sewage that is normally collected by the wastewater utility.

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