SARS-CoV-2 sewage surveillance in low-income countries: potential and challenges

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ABSTRACT

This paper reviews the recent findings in the detection of SARS-CoV-2 in sewage samples. We discuss how wastewater-based surveillance (WBS) can be used as a complementary tool to help the fight against COVID-19 spread, particularly in low-income countries with low sewage coverage and where the testing coverage is deficient, such as Brazil. One of the major challenges on WBS is the use of different protocols to estimate the number of infected people in a community from the quantification of SARS-CoV-2 in wastewater. Therefore, we assembled and reviewed all the relevant data available to date about this topic. Virus concentration and detection methods were reviewed as well, and some of them can be performed in most of the microbiology and environmental engineering laboratories in low-income countries, as discussed. Moreover, the monitoring and sampling plan should represent the local reality. Thus, we suggest unique strategies for sewage sampling and monitoring in different sewerage network points and the slums, despite the possible logistics difficulties involved. Considering the low levels of sanitation in most urban agglomerates in Brazil, WBS can potentially assume a crucial role as a cost-effective strategy to monitor the circulation of the virus and assess the real prevalence of COVID-19.

Key words | COVID-19, poor sanitation coverage, SARS-CoV-2, sewage, virus concentration method, wastewater-based surveillance

HIGHLIGHTS

- The information available to date about SARS-CoV-2 detection in sewage samples was reviewed.
- Wastewater-based surveillance (WBS) can be used as a complementary tool to help in the fight against the spread of COVID-19.
- WBS can be a key tool to track SARS-CoV-2 circulation in low-income countries.
- Virus concentration methods were reviewed and can be performed in low-income countries.
- Strategies for WBS are unique for places with low sanitation coverage.

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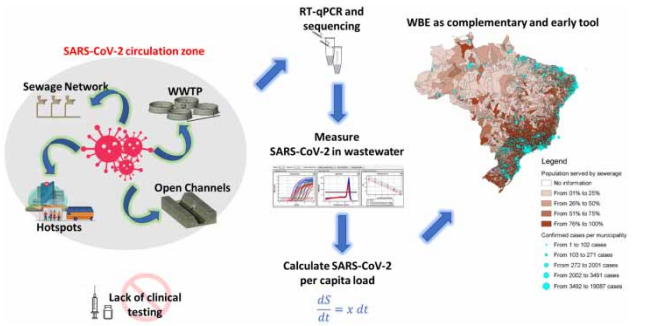
GRAPHICAL ABSTRACT



INTRODUCTION

Motivation on the monitoring of SARS-CoV-2 in wastewater

COVID-19, caused by the SARS-CoV-2 virus, is the most significant challenge healthcare systems have experienced in the 21st century. Asymptomatic patients represent a key factor for failing in control due to their high potential of unintentional disease spread (Bai *et al.* 2020; Li *et al.* 2020), leading many countries to adopt social distancing measures. Human feces can carry the SARS-CoV-2 virus for at least 5 weeks, even when the infected person does not show any COVID-19 symptoms (Wu *et al.* 2020b). This finding raises two main questions: (i) what is SARS-CoV-2 transmission potential through the fecal–oral pathway? and (ii) can sewage surveillance work as a complementary tool to monitor progression or decrease of viral spread in a community over time?

There is no report related to this virus viability and infectivity in domestic sewage or water samples to date. However, sewage surveillance was already recommended as a tool for COVID-19 monitoring in countries with a high level of sanitation (Mallapaty 2020). This approach finds support in the work of several authors, who highlighted the fecal–oral COVID-19 transmission as a possible or unlikely possible transmission route (Lodder & de Roda Husman 2020; Medema *et al.* 2020; Tang *et al.* 2020; Wang *et al.* 2020; Wu *et al.* 2020b; Xu *et al.* 2020). Recent studies have cultivated infectious virus from feces (Wang *et al.* 2020; Xiao *et al.* 2020; Yong Zhang *et al.* 2020) or urine (Sun *et al.* 2020). Besides, a previous study has shown that SARS-CoV can survive in stool samples for 4 days (Weber *et al.* 2016). Therefore, the possibility of fecal–oral transmission cannot be rejected, and prevention of transmission of SARS-CoV-2 from feces should be taken into consideration to control the spread of the virus (Qu *et al.* 2020; Xiao *et al.* 2020).

Virus viability information is available for other coronaviruses in domestic wastewater, such as the 229E (HCoV) and SARS-CoV, which remained active for 2.0–3.5 days at 23 °C (Gundy *et al.* 2009) and 14 days at 4 °C (Wang *et al.* 2005). A study using two surrogate coronaviruses, transmissible gastroenteritis (TGEV) and mouse hepatitis (MHV), showed that the time required for a 99% decrease of virus infectivity was 17–22 days in pure water or 7–9 days in pasteurized settled sewage, both at 25 °C (Casanova *et al.* 2009). The average sewage temperature in tropical countries is 24 °C (Dias *et al.* 2017), varying from 21 to 31 °C (Santos 2019). Thus, it is expected that the coronaviruses can, in fact, be viable for a couple to several days in tropical sewage. In many tropical countries, such as Brazil, where sanitation is poor (as further discussed) and exposure to open sewage is a risk, the potential transmission of SARS-CoV-2 via sewage should not be neglected.

Wastewater-based surveillance potential for SARS-CoV-2 monitoring

Early detection of enteric viruses and public health interventions has been made possible through the epidemiological

monitoring of wastewater. That is the case for noroviruses, rotaviruses, adenoviruses, and picornaviruses, such as hepatoviruses and enteroviruses (Blomqvist *et al.* 2012; Asghar *et al.* 2014; Hellmér *et al.* 2014; Miagostovich *et al.* 2014; Berchenko *et al.* 2017; Teixeira *et al.* 2017). In some cases, wastewater-based surveillance (WBS) enables one to estimate the number of infected people in a given region. Hence, it can be a powerful complementary tool when clinical testing is not widely available, as reported for COVID-19 in low-income countries such as India, Mexico, and Brazil (Worldometers 2020). Additionally, monitoring infection dynamics via wastewater reveals where infections are prevalent, supporting public health interventions. As a surveillance tool, WBS can be used as a noninvasive early warning tool to alert communities of new COVID-19 infections (Ahmed *et al.* 2020a; Medema *et al.* 2020; Peccia *et al.* 2020). Such surveillance can ultimately be used to determine testing areas to be targeted, where SARS-CoV-2 is persistent or in high concentration.

Different research groups around the world have already started or are planning to dive into COVID-19 sewage surveillance. So far, SARS-CoV-2 was detected in the sewage of the Netherlands (Lodder & de Roda Husman 2020; Medema *et al.* 2020), USA (Nemudryi *et al.* 2020; Peccia *et al.* 2020; Sherchan *et al.* 2020; Wu *et al.* 2020a), Australia (Ahmed *et al.* 2020a, 2020b), France (Wurtzer *et al.* 2020a, 2020b), Spain (Randazzo *et al.* 2020a, 2020b), Italy (La Rosa *et al.* 2020; Rimoldi *et al.* 2020), Israel (Bar-Or *et al.* 2020), Turkey (Kocamemi *et al.* 2020), and Japan (Haramoto *et al.* 2020; Hata *et al.* 2020). Most of these studies investigated the sewage (or primary sewage sludge – Peccia *et al.* (2020)) collected at the main wastewater treatment plant (WWTP) or more than one WWTP at the specific region to have an idea about the community health. One study (Bar-Or *et al.* 2020) collected samples from a hospital sewage and sewer network. In Brazil, some WBS initiatives have already started in the cities of Belo Horizonte, São Paulo, Brasília, and Niterói (Prado *et al.* 2020; Sodre *et al.* 2020).

This approach has been used in sewer served areas and WWTP. However, many questions still persist, such as (i) what should be the sampling strategy to be used in countries where many municipalities do not have sewage collection or where less than 50% of the sewage produced is collected? (ii) what is a good and feasible method to measure virus concentration in wastewater? (iii) what the best primer-probe set combination is? (iv) how to design a representative sampling approach? (iv) what is the virus concentration range to be expected in untreated and treated wastewater? (v) how to estimate the number of infected people in the community based on the detection of SARS-CoV-2 in wastewater? and (vi) how could WBS help in the fight of COVID-19 in low-income countries such as Brazil? Therefore, the objective of this review is to assemble all relevant data on SARS-CoV-2 concentration methods, molecular assays used to quantify the virus in sewage samples, sampling approaches, and sample volume to be collected to support WBS in low-income countries not always covered by centralized sanitation systems, such as Brazil. The information provided here will guide researchers to look for representative samples and generate results that can contribute to understanding virus circulation, potentially helping on the setup of public policies to control the spread of COVID-19.

METHODS

This review was designed based on the questions raised when we started working on WBS, and those that were sent to us from research groups and government agencies also willing to start conducting WBS, about the more appropriate virus concentration method to be used for virus (SARS-CoV-2) detection and quantification from sewage samples. Therefore, we searched the literature for articles related to SARS-CoV and SARS-CoV-2 detection in sewage and water samples by molecular methods published until 30 June 2020. Some articles about enteric virus quantification in sewage samples using molecular methods were included as well.

RESULTS

Methods and conditions reported to date to detect different viruses, including SARS-CoV-2 in sewage samples via RT-qPCR

Table 1 shows the information available about the detection of different viruses in sewage samples, including SARS and SARS-CoV-2 in untreated and treated wastewater.

Table 1 | Methods and conditions used to detect different viruses, including SARS and SARS-CoV-2 in sewage samples via RT-qPCR

Reference/country/virus	Sampling	Sample volume and virus concentration method	Assay and primers used	Virus detected?	Virus concentration (copies of viral genome/100 mL)	RT-qPCR results confirmation
Lodder & de Roda Husman (2005)/ Netherlands/ NoV, RV[a]	Sewage pumping engine station Not reported	10,000 mL raw sewage and 200–400 L treated sewage Modified conventional adsorption–elution method	Norovirus specific primers (RNA gene-Orf1). Primers for VP6 gene of rotavirus for RT-PCR	Yes, in raw sewage Yes, in treated sewage (1.8 log removal for noroviruses and 0.2 log for rotaviruses)	Not reported for RT-PCR	Sequencing the RT-PCR products after cloning
Symonds *et al.* (2014)/Bolivia/ NoV, RV and PMMoV[a]	Raw sewage and treated effluent Composite samples of 24 h	60 mL Modified adsorption–elution method	Primers for norovirus genotype I (NoVGI), rotavirus (RV) group A, and PMMoV	Yes, in raw and treated samples	1.0×10^2–1.0×10^5 for NoVGI, RV; 1.0×10^5–1.0×10^6 for PPMoV	Not reported
WHO (2014)/ Brazil/ Enterovirus C	Airport Not reported	Not reported	Not reported	Yes, at raw sewage	Not reported	Confirmed by virus isolation and sequencing
Vecchia *et al.* (2012)/Brazil/ AdV, EV, RV and TTV[b]	1 WWTP Grab samples	500 mL Adsorption on electronegative membrane and elution with NaOH	AdV – Hexon (VTB2-HAdVC), EV – 5′UTR (ENT-F1), RV – VP6 (ROTAFEEVALE), TTV – ORF2	Yes, at raw sewage	Not reported	Not reported
Heijnen & Medema (2011)/ Netherlands/ Influenza A	1 WWTP Grab samples	1 L Ultrafiltration	Matrix protein gene and Neuramidase gene (panN1)	Yes, at raw sewage and No, at treated effluent	2.6×10^4	RT-qPCR products were cloned and sequenced by Sanger
Teixeira *et al.* (2017)/Brazil/ Norovirus	Sewage and surface waters	2,000 mL Adsorption–elution method followed by ultrafitration (Amicon ultra-15)	Norovirus detection using GI and GII primers and probes separately	Yes, in lakes and raw sewage/ No, in treated water	Not reported	Direct sequencing of the amplicons
Wang *et al.* (2005)/ China/ SARS-CoV	Hospital and domestic sewage Grab samples	2,500 mL and 25,000–50,000 mL after chlorine disinfection Electropositive particle adsorption method	Three primers sets: Cor-p-F2, Cor-p-F3, and Cor-p-R1	Yes, in hospital sewage Yes, in sewage after chlorine disinfection	Not reported	Electrophoresis to confirm amplicon size and sequencing of the amplicons
Medema *et al.* (2020)/ Netherlands/ SARS-CoV-2	3 WWTP Composite samples of 24 h	100–200 mL Ultrafiltration (Centricon® Plus-70, 10 kDa)	Nucleocapsid (N) gene (N1, N2, and N3) and Envelope (E) gene	Yes, at raw sewage No, at treated effluent	Not reported	Electrophoresis to confirm amplicon size

(*continued*)

Table 1 | continued

Reference/country/virus	Sampling	Sample volume and virus concentration method	Assay and primers used	Virus detected?	Virus concentration (copies of viral genome/100 mL)	RT-qPCR results confirmation
Lodder & de Roda Husman (2020)/ Netherlands/ SARS-CoV-2	1 WWTP Composite samples of 24 h	10 L Not reported	Not reported	Yes, raw sewage	Not reported	Not reported
Ahmed *et al.* (2020a)/ Australia/ SARS-CoV-2	2 WWTP and 1 pumping station Automated sampler and grab samples	100–200 mL Adsorption-direct RNA extraction using electronegative membrane (Method A). Ultrafiltration using the Centricon® Plus-70 filter (Method B)	N protein (N_Sarbeco assay and NIID_2019-nCOV_N assay)	Yes, at raw sewage	1.9–12	RT-qPCR products were sequenced by Sanger and Illumina sequencing
Wu *et al.* (2020a)/ USA/ SARS-CoV-2	1 WWTP Composite samples of 24 h	40 mL Filtration through 0.2 μM membrane followed by precipitation with PEG	Nucleocapsid (N) gene (N1, N2, and N3)	Yes, at raw sewage	1.0×10^4	Confirmed by amplification of 150 bp amplicon with S gene primers
Nemudryi *et al.* (2020)/USA/ SARS-CoV-2	1 WWTP Composite samples of 24 h and grab samples	500 mL Filtration through 0.45 μm membrane followed by ultrafiltration using the CorningSpin-X UF filter	Nucleocapsid (N) gene (N1 and N2)	Yes, at raw sewage	$10–1.0 \times 10^2$	Confirmed by amplification of 10 regions of virus genome and sequencing the products
Sherchan *et al.* (2020)/USA/ SARS-CoV-2	2 WWTP Automated sampler and grab samples	1 L Ultrafiltration (Centricon® Plus-70) (Method A), Adsorption–elution method using electronegative membrane (Method B)	Nucleocapsid (N) gene (N1 and N2)	Yes, at raw sewage and No, at treated effluent	$3.1–7.5 \times 10^2$	Not reported
Wurtzer *et al.* (2020a)/France/ SARS-CoV-2	3 WWTP Not reported	11 mL Ultracentrifugation	Envelope (E) gene	Yes, at raw sewage and Yes, at treated effluent	$1.0 \times 10^3–1.0 \times 10^5$ in sewage and 2 log reduction in treated effluent	Confirmed by amplification of the RNA polymerase-RNA region
Randazzo *et al.* (2020a)/Spain/ SARS-CoV-2	3 WWTP Not reported	200 mL. Aluminum hydroxide adsorption–precipitation method with 3% beef extract	Nucleocapsid (N) gene (N1, N2)	Yes, at raw sewage and No, at treated effluent	$1.0 \times 10^4–1.0 \times 10^5$	Not reported

(*continued*)

Table 1 | continued

Reference/country/virus	Sampling	Sample volume and virus concentration method	Assay and primers used	Virus detected?	Virus concentration (copies of viral genome/100 mL)	RT-qPCR results confirmation
Randazzo *et al.* (2020b)/Spain/SARS-CoV-2	6 WWTP Not reported	200 mL Aluminum hydroxide adsorption–precipitation method with 3% beef extract	Nucleocapsid (N) gene (N1, N2, and N3)	Yes, at raw sewage, Yes, at secondary treated effluent and No, at tertiary treated effluent	10 to 1.0×10^4	Not reported
La Rosa *et al.* (2020)/Italy/SARS-CoV-2	3 WWTP Composite samples of 24 h	250 mL PEG/dextran concentration method	ORF1ab assay for nested PCR; primers for RdRP gene for RT-qPCR	Yes, at raw sewage	No positive results were obtained by RT-qPCR	Electrophoresis to confirm amplicon size and sequencing of the amplicons
Rimoldi *et al.* (2020)/Italy/SARS-CoV-2	3 WWTP and surface waters Grab samples	500 mL Filtration through glass fiber filters followed by filtration through 0.2 μm filters	Nucleocapsid (N) gene (N1, N2, and N3), Envelope (E) gene, and ORF1ab assay	Yes, at raw sewage, No, at tertiary treated effluent; Yes, at surface waters	Not reported	Genome sequencing and phylogenetic analysis of the isolated virus
Haramoto *et al.* (2020)/Japan/SARS-CoV-2	WWTP and river samples Not reported	200–5,000 mL Adsorption–elution method using electronegative membrane; Adsorption-direct RNA extraction method	N protein (N_Sarbeco and NIID_2019-nCOV_N assays), N1 and N2 assays, ORF1a and S protein assays	No, at influent wastewater No, at river waters Yes, at secondary treated effluent	1.0×10^2	Not reported
Hata *et al.* (2020)/Japan/SARS-CoV-2	4 WWTP Grab samples	80 mL PEG (10%) precipitation with sodium chloride (1 M)	N protein (NIID_2019-nCOV_N assay), Nucleocapsid (N) gene (N2 and N3)	Yes, at raw sewage	1.0×10^3	Electrophoresis to confirm amplicon size
Bar-Or *et al.* (2020)/Israel/SARS-CoV-2	11 WWTP, sewer network and hospital sewage Composite samples of 24 h	250 mL PEG or alum (20 mg/L) precipitation followed by ultrafiltration using Amicon ultra-15 (30 kDa)	Envelope (E) gene	Yes, at raw sewage	Not reported	Not reported

(*continued*)

Table 1 | continued

Reference/country/virus	Sampling	Sample volume and virus concentration method	Assay and primers used	Virus detected?	Virus concentration (copies of viral genome/100 mL)	RT-qPCR results confirmation
Prado *et al.* (2020)/ Brazil/ SARS-CoV-2	2 WWTP, sewer network and hospital sewage Composite samples of 10 h	1 L Ultracentrifugation method	Nucleocapsid (N) gene (N2)	Yes, at raw sewage	Not reported	Not reported
Fongaro *et al.* (2020)/Brazil/ SARS-CoV-2	Separate collection sewage system Grab samples	200 mL PEG precipitation followed by ultrafiltration using Centriprep YM 50	Nucleocapsid (N) gene (N1), RdRp gene, Spike glycoprotein (S) gene	Yes, at raw sewage	1.0×10^4–1.0×10^5	Not reported
Kocamemi *et al.* (2020)/Turkey/ SARS-CoV-2	7 WWTP Not reported	250 mL Ultrafiltration using Amicon ultra-15; Filtration through 0.2 μM membrane followed by PEG 8,000 precipitation	Primers and Taqman probe for SARS-CoV-2 RdRp gene	Yes, at raw sewage	1.0×10^2–1.0×10^3	Not reported
Ahmed *et al.* (2020b)/ Australia/ MHV[c] as surrogate for SARS-CoV-2	1 WWTP	50 mL Comparison of seven virus concentration methods; Best results using adsorption-direct RNA extraction method (at neutral pH, and with 25 mM $MgCL_2$) using electronegative membrane	MHV specific primers	Yes, untreated wastewater	Not reported	Not reported

[a]Norovirus (NoV), rotavirus (RV), and pepper mild mottle virus (PMMoV).
[b]Adenoviruses, enteroviruses, rotaviruses, and torque teno virus (TTV).
[c]Murine hepatitis virus (MHV).

So far, there is no standard or optimized protocol to be used for the SARS-CoV-2 concentration, genomic RNA extraction, and detection in wastewater, nor a consensus about the best target gene or primer-probe set to be used (Table 1). Although some researchers have mentioned that there is a need for a standard protocol (Kitajima *et al.* 2020) to recover and detect SARS-CoV-2 from environmental samples (including sewage) to be established, the diversity of conditions (e.g., temperature, social, economic, laboratory capacity, and qualified personnel) that are largely different between the countries hinders the standardization. Thus, specific and regionalized protocols might be necessary. These protocols should be validated, establishing, for instance, recovery efficiency, proper controls, and limits of detection.

Viruses concentration methods

Based on Table 1, choosing a protocol will depend on the equipment available in the laboratories, resources, and the

team's expertise. In Brazil, the high prices of the Centricon Plus-70 centrifugal filters (ranging from 30 to 40 US$ each unit – SIGMA (2020)) can hamper the use of this method for virus concentration, especially for the analysis of a high number of samples. Ultracentrifugation is a common method in the virology field for virus concentration (He & Jiang 2005). However, the ultracentrifuge is a costly instrument that is not commonly available in environmental engineering laboratories in low-income countries. On the other hand, the direct RNA extraction method using electronegative membranes, as described by Ahmed *et al.* (2020a), Haramoto *et al.* (2020), and filtration (using 0.2 μm filters) followed by precipitation with polyethylene glycol 8000 (PEG) (Kocamemi *et al.* 2020; Wu *et al.* 2020a) seems to be a feasible alternative to ultracentrifugation. The modified version of this method used by La Rosa *et al.* (2020), however, applies PEG-dextran for overnight precipitation, which is more time-consuming and might not be suitable for a high number of samples.

The electronegative membrane is typically used for concentrating enteric viruses from wastewater (Vecchia *et al.* 2012; Symonds *et al.* 2014; Teixeira *et al.* 2017) with a modest recovery of 31–78% for human adenoviruses and polyomaviruses (Ahmed *et al.* 2015). Enveloped viruses, such as SARS-CoV-2, have high adsorption efficiency to the electronegative membrane (Haramoto *et al.* 2009) and the solid fraction of wastewater, compared with nonenveloped viruses (Ye *et al.* 2016).

Recently, Ahmed *et al.* (2020b) compared seven different virus concentration methods for RT-qPCR-based recovery of murine hepatitis virus (MHV), as a surrogate for SARS-CoV-2, from wastewater (Table 1). They reported that the two most efficient methods were the adsorption–extraction method without acidification at neutral pH (mean MHV recovery of 60.5%) and the one with the addition of 25 mM $MgCl_2$ (mean MHV recovery of 65.7%). The third most efficient method was the ultrafiltration using Amicon® Ultra-15 filter tubes (mean MHV recovery of 56.0%). However, this method's disadvantages were the co-concentration of polymerase chain reaction (PCR) inhibitors, the high price of the ultrafiltration centrifugal unit, and its lack of ability to process volumes larger than 15 mL at a time (Ahmed *et al.* 2020b). Therefore, among the methods tested, the adsorption using an electronegative membrane and direct RNA extraction can be successfully used for WBS as an efficient, rapid, and low-cost method for SARS-CoV-2 detection (as applied in Ahmed *et al.* (2020a) and Haramoto *et al.* (2020); Table 1).

Another way for virus concentration is the use of the aluminum hydroxide adsorption–precipitation method, as reported by Randazzo *et al.* (2020a, 2020b). This seems to be a good low-cost method, but it is not as straight forward as the adsorption–extraction method using electronegative membrane since it includes two agitation and centrifugation steps of 15 and 30 min each, respectively (as described in Symonds *et al.* (2014); Ahmed *et al.* (2015, 2020a, 2020b)).

After the virus concentration step, RNA can be extracted using commercial kits such as RNeasy PowerWater Kit and RNeasy PowerMicrobiome Kit, Qiagen (as reported by Ahmed *et al.* (2020a, 2020b)), or All Prep power-Viral DNA/RNA (Qiagen) (as reported by Symonds *et al.* (2014)).

Detection of SARS-CoV-2 by RT-qPCR

After RNA extraction, the detection of SARS-CoV-2 can be performed via available RT-qPCR assays (as summarized in Table 1). The choice of an adequate primer-probe set is crucial in this step because some primers are less sensitive than others. For instance, Medema *et al.* (2020) used different primer-probe sets recommended by the US Centers for Disease Control and Prevention (CDC), targeting the nucleocapsid protein (N) gene-regions N1, N2, and N3 (Lu *et al.* 2020) and Corman *et al.* (2020), in a European study, targeted the envelope protein (E) gene. Although Nalla *et al.* (2020) reported that N2 and E primer-probe sets are the most sensitive sets, Medema *et al.* (2020) indicated that the N1 was more sensitive to SARS-CoV-2 than the N2 and produced a signal in sewage samples even when the COVID-19 occurrence was as low as one known case in 100,000 people. The N3 and E primer-probe sets started to yield positive signals only when the prevalence was higher than 3.5 known cases per 100,000 people (Medema *et al.* 2020). On the other hand, Hata *et al.* (2020) reported positive signals using N2, N3, and NIID assays when the prevalence was around 1 case per 100,000 people and positive signal with only N3 when prevalence was lower than 1. Wu *et al.* (2020a) also used the N1, N2, and N3 primer-probe sets and reported that all

three produced RT-qPCR results with variable levels of SARS-CoV-2 in wastewater samples. Randazzo *et al.* (2020b) reported discrepancies among the results obtained from RT-qPCR with N1, N2, and N3 assays. The risk of false-positive samples detection by the N3 assay in low concentrated samples (Vogels *et al.* 2020) was eliminated by excluding the N3 primer-probe set from the US CDC protocol.

Alternatively, Ahmed *et al.* (2020a) used the N_Saberco assay designed in a Japanese study (Shirato *et al.* 2020) to detect SARS-CoV-2 and suggested that N_Saberco assay might be more sensitive than NIID_2019-nCOV_N assay. In contrast, La Rosa *et al.* (2020) did not obtain positive results using RT-qPCR with RdRp assay (primers targeting the RNA-dependent RNA polymerase gene and probe specific for SARS-CoV-2). They reported that this assay has low sensitivity compared with others and that the limit of detection observed was above 500 genome copies per reaction, as also mentioned by Vogels *et al.* (2020). Rimoldi *et al.* (2020), on the other hand, tested different primer-probe sets and assays (as shown in Table 1) and reported that detection using primers for the ORF1ab gene showed the highest frequency of positivity. Products were amplified in all positive samples, while the other two genes (N and E) failed to be amplified in two out of five positive cases. This observation indicates that the targeted genes have different sensitivity thresholds (Jung *et al.* 2020), and therefore that viral concentration estimates will depend on (and vary) according to the sensitivity of the employed primer-probe set.

Quantification of SARS-CoV-2 by RT-qPCR

A small number of studies to date reported concentration values of SARS-CoV-2 in sewage samples ranging from 1.9×10^0 to 1×10^5 copies/100 mL (Table 1). These numbers are likely dependent on many factors, such as the prevalence of the virus in the community, the epidemic curve position, the virus concentration method applied, and primer-probe sets used. Among the studies that detected SARS-CoV-2 in wastewaters, only three assessed the concentration method recovery efficiency. Medema *et al.* (2020) reported recovery efficiencies of F-specific RNA phages of 73% for the ultrafiltration method used. Hata *et al.* (2020) reported recovery efficiency of indigenous F-phage of 57%, and Randazzo *et al.* (2020b) reported recovery efficiencies of 6.2–10.8% for mengovirus (MgV) and 3.3–10.9% for porcine epidemic diarrhea virus (PEDV) in influent and effluent samples from WWTPs.

Applying RT-qPCR with primers for viral (E) gene and using the ultracentrifugation method for virus concentration, Wurtzer *et al.* (2020a) observed a SARS-CoV-2 detection limit of 1,000 equivalent viral genomes/L of sewage. The detection limit of the method was not always mentioned, although Wu *et al.* (2020a) reported that the spike-in experiments with the purified virus are currently underway to establish limits of detection.

Medema *et al.* (2020) highlighted that a reliable quantification of SARS-CoV-2 with RT-qPCR is dependent on the development of proper controls to consistently monitor the virus recovery (e.g., virus concentration control and internal control), the measurement of viral RNA yield, and the examination of RT-PCR inhibition. The authors suggested that a human coronavirus, such as 229E, could be potentially used as a control to perform a more reliable quantification.

This plethora of approaches, including different concentration methods, nucleic acid extractions, target genes, and primer-probe sets, have shown different data. The lack of information about the efficacy of these methods to study SARS-CoV-2 in sewage indicates a need to adopt protocols with acceptable detection limits. There is also a need for reproducible protocols to be used at different laboratories, including those in low-income countries, that can provide good recovery, high sensitivity, and low cost per sample. Thus, we suggest that the adsorption method that applies filtration through electronegative membranes followed by direct nucleic acid extraction from the membrane (Symonds *et al.* (2014) and Ahmed *et al.* (2015) for enteric viruses; and Ahmed *et al.* (2020a, 2020b); Haramoto *et al.* (2020) for SARS-CoV-2) can be used for SARS-CoV-2 detection in low-income countries because it requires common equipment that is normally available in environmental engineering laboratories (as will be further discussed).

Estimation of infection prevalence based on SARS-CoV-2 sewage surveillance

According to Ahmed *et al.* (2020a), the prevalence of SARS-CoV-2 infection in a community within a specific sewage

collection area can be estimated using mass balance. The essential measurements are the total number of RNA copies in the wastewater per day (as measured by RT-qPCR) and the virus concentration in an infected person's feces per day. Each person excretes from 200 g (Wu *et al.* 2020a) to 250 g (Rose *et al.* 2015) of fecal material daily. The literature reports that the virus concentration in fecal material ranges from 6.0×10^5 (Zhang *et al.* 2020) to 3.0×10^7 genome copies per g of feces in the first week of symptoms, decreasing to 1.0×10^2 genome copies per g in the third week of symptoms (Woelfel *et al.* 2020). Using these data allows an estimation of the number of infected people in a specific region, as previously described by Wu *et al.* (2020a) and Nemudryi *et al.* (2020). Nevertheless, additional information on viral shedding in feces throughout the disease duration as well as from asymptomatic infected individuals is necessary to better predict the SARS-CoV-2 infection prevalence in the community based on virus concentration detected in sewage samples.

Wurtzer *et al.* (2020a) showed that the SARS-CoV-2 genomes in raw sewage were correlated with the number of COVID-19 confirmed fatal cases. They demonstrated that the detection of the viral genome occurred before the beginning of the exponential growth of the epidemy, indicating that wastewater survey may be used as an early tool to detect pathogens in the population. In a second study (Wurtzer *et al.* 2020b), they demonstrated that the SARS-CoV-2 viral load in raw wastewater was in good agreement with the dynamics of pandemic (i.e., the number of COVID-19 cases) observed in the Paris region.

Another study (Ahmed *et al.* 2020a) estimated the number of infected individuals in a catchment basin (population of 600,000 inhabitants) in the Southeast Queensland area applying Monte Carlo simulation, based on the RNA copy numbers observed in the wastewater. They reported that the estimated number of infected people and prevalence were strongly correlated with the log10 of SARS-CoV-2 RNA copies in fecal samples, followed by the RNA copies detected in wastewater and log10 grams of feces per person per day. In the Netherlands, Medema *et al.* (2020) estimated COVID-19 prevalence based on the sewage surveillance of SARS-CoV-2 using the following information: (i) the number of COVID-19 cases reported on the sampling day for the cities served by each WWTP where sewage samples were collected from and (ii) the number of people served by each WWTP investigated.

In the Valencia region in Spain, Randazzo *et al.* (2020a) showed that SARS-CoV-2 was undergoing community transmission earlier than previously believed, thus confirming that wastewater analysis is a sensitive and cost-effective strategy for COVID-19 epidemiological surveillance. Similar observations were reported by Peccia *et al.* (2020) that investigated SARS-CoV-2 RNA concentrations in primary sewage sludge in New Haven, CT. They showed that the virus RNA concentrations were highly correlated with the COVID-19 epidemiological curve and local hospital admissions. The study also demonstrated the potential of sludge-based surveillance in evaluating the local SARS-CoV-2 testing rate and estimating the number of new cases. Hata *et al.* (2020) also showed that the detection frequency of SARS-CoV-2 in wastewater samples was in agreement with the numbers of confirmed cases in the region investigated, being higher when it becomes above 10 cases in 100,000 people.

Wurtzer *et al.* (2020a) mentioned that the next steps include evaluating the virus viability in wastewater and the potential of wastewater as a vehicle for human exposure, whereas Rimoldi *et al.* (2020) investigated the presence and infectivity of SARS-CoV-2 virus in wastewaters and rivers. They did not recover infectious virus from wastewaters and surface waters that yield positive results for SARS-CoV-2 RNA detection. Hence, they reported no significant public health risks. However, more information on the infectious potential of wastewater in different conditions and temperatures should be provided. Moreover, none of these studies have shown the percentage of SARS-CoV-2 recovery from wastewater samples due to the risk associated with handling the virus and the requirements for a Biosafety Level 3 (BSL-3) facility. To date, only one study has reported the percentage of SARS-CoV recovery from wastewater (Wang *et al.* 2005), which was 1.02%.

The CDC recommends that virus isolation and culturing should be performed in BSL-3 settings, whereas routine diagnostic testing would be conducted in Biosafety Level 2 (BSL-2) laboratories (Centers for Disease Control and Prevention 2020).

Although initial experiments indicate that fecal loads of SARS-CoV-2 are not infectious (Hennig & Drosten 2020), the proper care should be taken when sampling and

handling sewage samples, and the researchers should adopt the established safety procedures for sewage collection (Ahmed *et al.* 2020a; Medema *et al.* 2020).

Therefore, most of the microbiology or environmental engineering laboratories (at Sanitary Engineering Departments) classified as BSL-2 will be able to perform detection and quantification of the virus from wastewater or other environmental samples if, at least, the following equipment is available: pH meter, refrigerated centrifuge, filtration system (filter holders and vacuum pumps), freezer (−20 °C), ultrafreezer (−80 °C), microcentrifuge, electrophoresis equipment, and real-time PCR detection system. Virus cultivation should not be performed in BSL2 laboratories, as already mentioned, being achievable via collaboration with different groups, laboratories with proper safety levels, and institutions.

The role, approaches, and challenges to perform WBS in low-income countries: the example of Brazil

Sanitation coverage and the pattern of COVID-19 distribution in Brazil

In 2017, 39.4 million Brazilians (i.e., 18.9% of the total Brazilian population) had no access to safe drinking water, and more than 101 million (around 46.8% of the population) had no access to sewage collection (SNIS 2018). Only 46% of sewage in Brazil is treated (SNIS 2018), which generates economic, environmental, and public health consequences. Almost 5 million cases of acute diarrhoeal disease were registered in Brazil in 2018 (Ministério da Saúde 2018). Brazil is a vast country with uneven economic and geographic distribution. Table 2 shows the sanitation coverage in the country's different regions in terms of water supply and the percentage of sewage treatment and collection. Sanitation numbers become even worse when we compare the different Brazilian regions. Only 13.3 and 36.3% of the urban population in the North and Northeast of the country, respectively, are served by sewage collection. This situation has improved in the Center-West, South, and Southeast parts of the country.

In Brazil, the first confirmed case of COVID-19 was reported on 25 February 2020, in São Paulo. There have been more than 1,368.195 confirmed cases on June 29 (Coronavirus Brasil 2020). The highest confirmed number of cases on June 29 was observed in the Southeast (475.989), Northeast (469.602), and North (257.723) regions of Brazil (Coronavirus Brasil 2020). Figure 1(a) shows the pattern of disease distribution in the country, regarding the number of confirmed cases of COVID-19 in the different regions of Brazil, with expressive occurrence in the capitals and large urban centers, where the urban clusters are significant. The high number of COVID-19 cases in Manaus (Amazon state), in the north, represents an exception because the demographic density of this city (158 inhabitants/km^2 – IBGE (2010)) is much lower than that of Recife (7,039 inhabitants/km^2 – IBGE (2010)), for instance, but the number of confirmed cases is similar (13,979 – Figure 1(a)). The low level of sanitation in Manaus (Figure 1(a) and 1(b)) might be one of the many possible explanations.

Considering the coverage of water supply (Figure 1(b)), the contamination by COVID-19 occurs predominantly in municipalities with more than 51% of the population served by water distribution, suggesting that water distribution has not been a factor in minimizing the virus spread. Other factors, such as population density and

Table 2 | Coverage of water and sewage services in municipalities with service providers, according to the geographic macro-region of Brazil (adapted from SNIS (2018))

	Network coverage (%)				Sewage treatment coverage (%)	
	Water		Sewage collection		Sewage generated	Sewage collected
Macro-region	Total[a]	Urban[a]	Total	Urban	Total	Total
North	57.1	69.6	10.5	13.3	21.7	83.4
Northeast[b]	**74.2**[b]	**88.7**	**28.0**[b]	**36.3**[b]	**36.2**	**83.6**
Southeast	91.0	95.9	79.2	83.7	50.1	67.5
South	90.2	98.6	45.2	51.9	45.4	95.0
Center-West	89.0	96.0	52.9	58.2	53.9	93.8
Brazil	83.6	92.8	53.2	60.9	46.3	54.5

[a]SNIS calculates the total service indices with the water supply and sewage services adopting the population served, informed by the service providers, and the total resident population, estimated by the Brazilian Institute of Geography and Statistics – IBGE. The urban area of the cities is better covered by water and sanitation facilities. The total includes rural population not well served (or not served at all), which decreases the percentage of the macro-region coverage.

[b]Northeast region data are shown in bold because the city of Recife (mentioned in Figures 2 and 3) is located in this region.

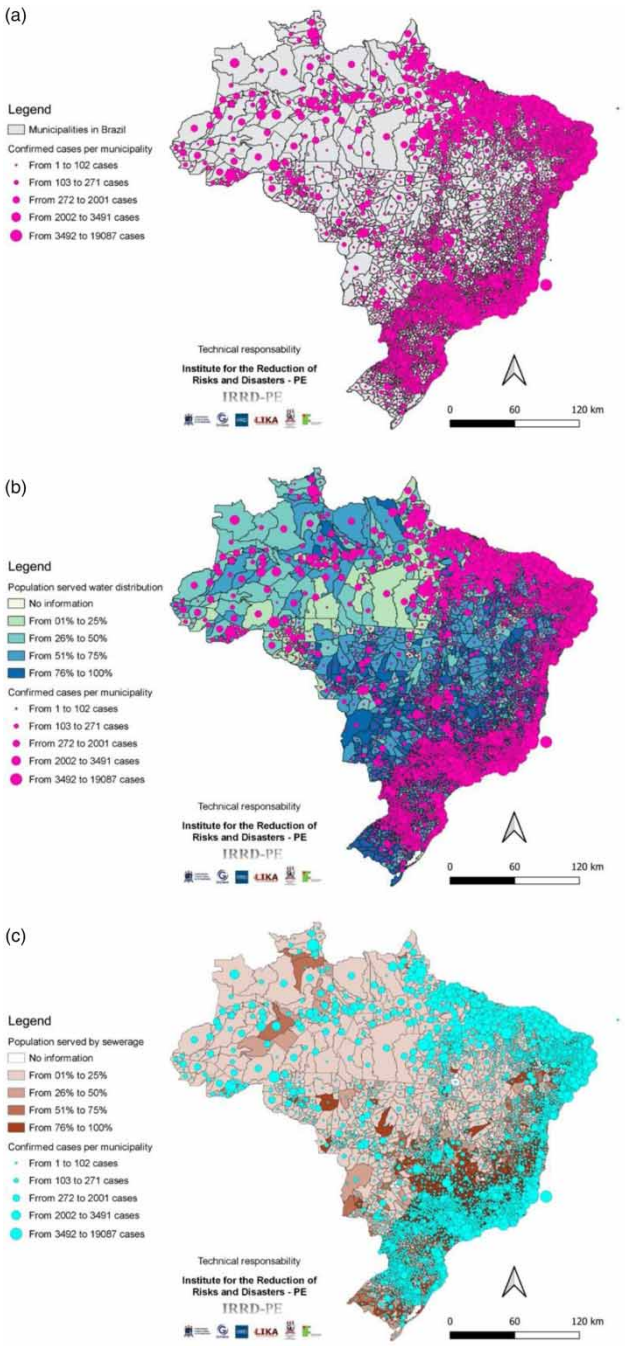


Figure 1 | Geospatial distribution map of confirmed cases of COVID-19 contamination in Brazil (a), in relation to water distribution in Brazilian municipalities (b), and in relation to sewage collection in Brazilian municipalities (c). Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/wh.2020.168.

intermittent water supply, probably have higher contributions. Although the contamination by COVID-19 occurs predominantly in municipalities where more than 76% of the population are sewer served (Figure 1(c)), this contamination is growing in municipalities with sewage collection lower than 50%, requiring attention to the potential transmission through sewage exposure.

About 13.6 millions Brazilians live in slums as in many other developing countries. These are sensitive places when we consider the potential of the COVID-19 rate of spread. These and other urban agglomerations are, in general, poorly served by water supply and sewage collection. That makes it challenging to apply the primary hygiene method of COVID-19 prevention, which is merely washing hands. Hand sanitizer is often not affordable. In urban agglomerations, all family members (sometimes 6–8 people) usually share a house of 40–50 m^2. Social distancing or self-isolation is not an option in these cases. Prevention is indeed more difficult when viruses are flowing at the front door within the open sewage. In these areas, WBS has three functions: to exhibit whether the virus is circulating, work as early warning, and help authorities to design appropriate public health interventions.

Profile of the spread of COVID-19 in the metropolitan area of Recife

As a case study of a highly densely populated area, Figure 2 illustrates the profile of the COVID-19 spread for urban agglomerations in the metropolitan area of Recife, the capital of the Pernambuco state, Brazil. The disease first appeared on 05 March 2020, in high-income neighborhoods (Figure 3(a)) and started to spread to low-income neighborhoods (Figure 3(b) and 3(c)). After 10, 30, and 100 days, the number of cases in low-income urban agglomerates, such as in Olinda (red arrow – Figures 2 and 3), sharply increased, reaching 22, 398, and 5,760 confirmed cases, respectively. The real number would be much higher if the clinical testing index was also higher. Brazil has a testing coverage of about 14,445 people per million inhabitants, whereas in Spain, Belgium, and Israel, this number is higher than 100,000 people per million inhabitants (data from June 29) (Worldometers 2020). Massively testing the population of slums, urban agglomerates, and small remote municipalities for COVID-19 may be difficult, mainly due to economic and logistic issues. Despite the difficult access, sewage

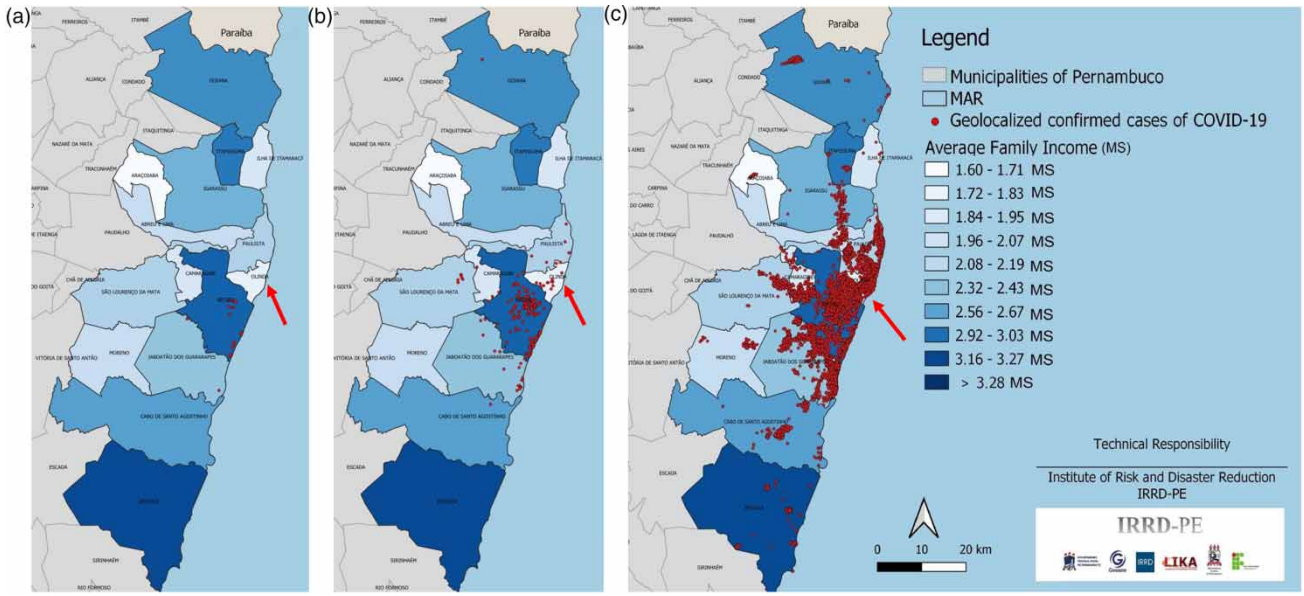


Figure 2 | Geolocalized confirmed cases of COVID-19 in the metropolitan area of Recife in relation to the average of family's income, expressed as minimum salary in Brazil: (a) 10 days after the first case (15 March 2020), (b) 30 days after the first case (04 April 2020), and (c) 80 days after the first case (25 May 2020). Red arrow highlights the city of Olinda. Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/wh.2020.168.

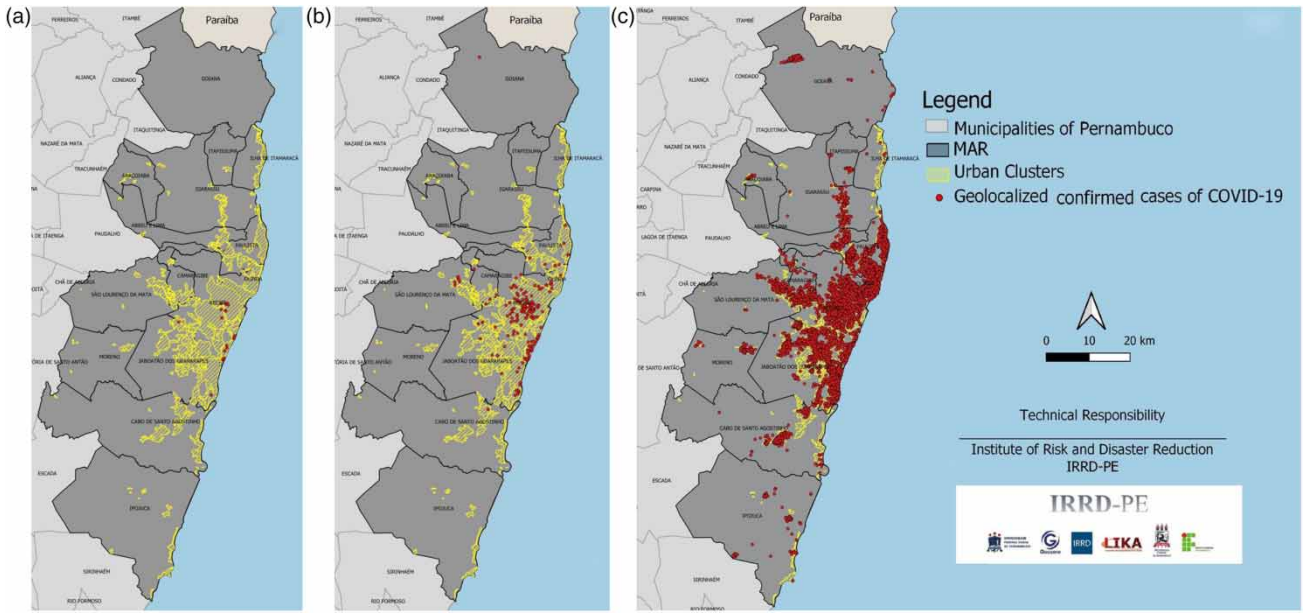


Figure 3 | Geolocalized confirmed cases of COVID-19 in the metropolitan area of Recife in relation to the urban clusters: (a) 10 days after the first case (15 March 2020), (b) 30 days after the first case (04 April 2020), and (c) 80 days after the first case (25 May 2020). Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/wh.2020.168.

surveillance in these areas has a higher contribution potential as a noninvasive community level detection tool.

DISCUSSION

Lessons from Brazil – recommendations for sewage surveillance in low-income settings

WBS has been shown as a complementary tool to monitor the occurrence of the virus in the community and reveal trends of disease prevalence in different countries (Table 1). The surveillance has been performed by sampling the influent of the WWTPs, which may not be representative in developing countries, where a significant portion of the sewage produced does not reach the WWTPs. This kind of surveillance makes it impossible to use the monitoring data to estimate disease prevalence in the whole population. Therefore, the monitoring and sampling plan for low-income countries should represent the local reality, as suggested in Table 3. These recommendations consider both sewer and non-sewer served areas, as well as the goals of describing trends for virus occurrence in a community to generate early warnings for possible upcoming waves.

Only 25% of the 5,570 Brazilian municipalities are covered by more than 50% of sewage collection (IBGE 2010). The monitoring plan in these municipalities should include collecting samples in the inlet of WWTP, strategic points of the sewer network of high- and low-income areas, including health vulnerability sites, which are usually well defined in the cities (Prado *et al.* 2020). At the sewer network, representative samples should be collected at the time of the day of higher water consumption, as described in Table 3. However, for WWTP, a 24 h composite sample can be more representative since it is more influenced by dilution over the day. Another alternative for WWTP would be to sample the primary sewage sludge because it has high solids content and might have higher virus concentration than the wastewater, enabling skipping the virus concentration step (Peccia *et al.* 2020). However, it is important to highlight that conventional activated sludge systems, with primary settlers, might not be common in low-income countries, where anaerobic reactors and stabilization ponds are the most used technologies (Von Sperling 2016).

For municipalities not well served by sewer systems, the hotspot samples (Table 3) have an important role because these wastewaters are usually representative of a high number of people. Since in some common hotspots, such as airports, shopping malls, and universities, it is difficult to predict the source of infected people, public schools and community toilets, when available, could represent an easier track to the community health, in areas not served by sewage collection. The urban slums are reported as key hotspots for WBS in low-income countries (Pandey *et al.* 2021). Rainwater drainage systems should also be included

Table 3 | Sampling strategies for WBS in low-income countries

Condition	Sampling points	Sampling approach
Municipalities (and communities) with sewer network	• Different points along the sewerage network • Outlet of sewage collection basins • Inlet of WWTP • Surface water (rivers and streams) before the discharge point of the treated effluent from WWTP[a]	Collect 500 mL of sewage for 3 h, sampling every 20 min. Build a 4.5 L of composite sample. When WWTP is available: collect 1 L of composite 24 h raw sewage sample and/or collect 1 L of primary sewage sludge.
Municipalities (and communities) without sewer network	Channels, downstream of slum's hills, ditches, inlet of septic tanks	
Hotspots: hospitals, bus stations, schools, universities, shopping malls, community centers, small and middle size factories.	Outlet of sewage collection	

[a]To consider those neighborhoods where the population is not yet served by sewers and thus raw sewage is discharged into the river.

for WBS when it drains sewage, as it is possible to consider the drainage basin as the influent community.

Considering the viral concentration of SARS-CoV-2 reported in the literature (Table 1) that ranged from 1.0×10^2 to 1.0×10^6 genome copies/L of sewage, and considering that 48% of the Brazilian population (i.e., 100 million inhabitants) are not covered by sewage collection and treatment and that one person generates 160 L of sewage per day (SNIS 2018), it is estimated that the viral load discharged into Brazilian rivers and water bodies could be in the range of 1.0×10^{12} to 1.0×10^{16} genome copies per day. Therefore, these numbers indicate an urgent need to increase sewage collection and treatment in Brazil to improve the population health, not only during the COVID-19 pandemics but in the medium and long term due to the other viruses that have the fecal–oral route confirmed (e.g., adenoviruses, noroviruses, and rotaviruses). Even considering that the transmission route by sewage is unlikely or has not been proved yet for SARS-CoV-2, the potential transmission should not be neglected in places with poor sanitation coverage because infectious SARS-CoV-2 has been documented in feces of one COVID-19 patient (Xiao *et al.* 2020).

In summary, based on the information available to date, the answers to the questions asked in the introduction are as follows:

1. There is no single and standard protocol for SARS-CoV-2 concentration from wastewaters. Thus, based on the available information reported to date and reviewed in this study, the adsorption-direct RNA extraction method using electronegative membrane can be a suitable option for SARS-CoV-2 concentration and detection if proper controls of the process and RT-qPCR are employed. This method is rapid, efficient, and requires only standard laboratory equipment, being feasible for low-income countries. Another possibility, when appropriate, would be to perform virus detection from primary sewage sludge, avoiding the concentration step.
2. The most sensitive primer-probe set combination for SARS-CoV-2 detection in sewage samples by RT-qPCR described until now are N1, N2, and the N_Sarbeco assay, all targeting the nucleocapsid protein gene.
3. The SARS-CoV-2 RNA concentration measured in the sewage ranges from 1.0×10^2 to 1.0×10^6 genome copies/L.
4. It is possible to estimate the number of infected people in a specific sewage catchment area if the virus concentration in the wastewater per day and the virus concentration in the feces of an infected person per day are known.
5. In low-income countries, such as Brazil, where the clinical testing is deficient, WBS can be used as a complementary and possibly early tool to detect pathogens in a community, considering that specific and representative sampling points in areas with and without sewerage systems are chosen.

CONCLUSIONS

One of the biggest challenges regarding SARS-CoV-2 is its unpredictability. In the case of low-income countries, such as Brazil, due to its continental size and economic diversity, WBS seems to be an excellent and urgent need to track the virus and assess the real COVID-19 prevalence. This tool can help health authorities on planning protection measures and mitigate virus spread. In urban agglomerations, such as slums and confined communities where the sanitation coverage is low, WBS has enormous potential to be used as a complementary and sometimes the main tool to understand SARS-CoV-2 circulation in a community. Wastewater surveys may provide an alternative and possibly early tool to detect pathogens in the population when clinical tests are difficult for economic or logistic reasons. Despite the challenges, the detection of SARS-CoV-2 in sewage can be performed by the adsorption–extraction method using negatively charged membranes because it requires common laboratory equipment available in most laboratories in low-income countries.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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