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One-year surveillance of SARS-CoV-2 in wastewater from vulnerable urban communities in metropolitan São Paulo, Brazil

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ABSTRACT

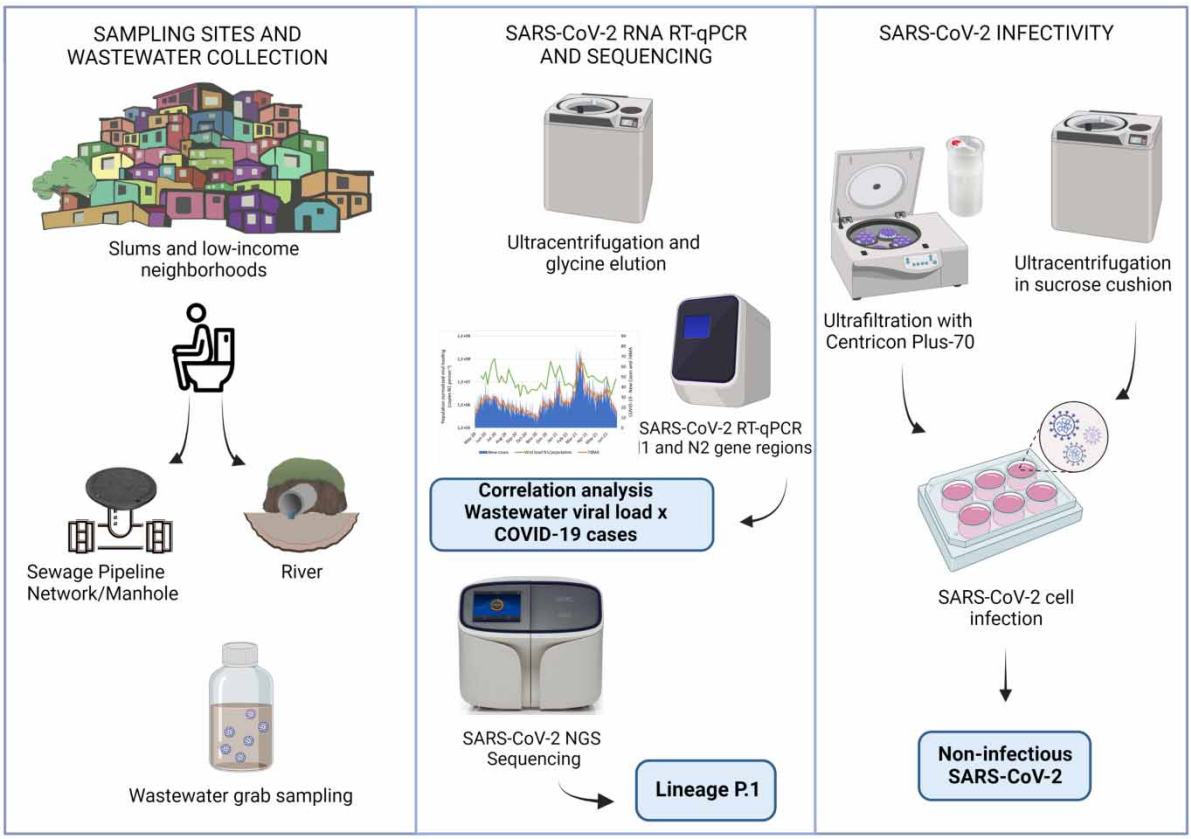
The current COVID-19 pandemic has emphasized the vulnerability of communities living in the urban outskirts and informal settlements. The lack of reliable COVID-19 case data highlights the importance and application of wastewater-based epidemiology. This study aimed to monitor the COVID-19 trends in four vulnerable urban communities (slums and low-income neighborhoods) in metropolitan São Paulo by assessing the SARS-CoV-2 RNA viral load in wastewater. We analyzed 160 samples from May 2020 to June 2021 with weekly or fortnightly samplings. The samples were ultracentrifuged with glycine elution and quantified by N1/N2 SARS-CoV-2 RT-qPCR. The results of positivity were 100% (Paraisópolis, Heliópolis and Cidade Tiradentes) and 76.9% (Vila Brasilândia). The new case numbers of COVID-19, counted from the onset of symptoms, positively correlated with SARS-CoV-2 N1 viral loads from the two largest communities ($p<0.001$). SARS-CoV-2 infectivity was tested in Vero E6 cells after concentration with the two techniques, ultrafiltration (Centricon® Plus-70 10 kDa) and sucrose cushion ultracentrifugation, but none of the evaluated samples presented positive results. Next-generation sequencing (NGS) analysis from samples collected in March and August 2021 revealed the presence of the clade 20 J (lineage P.1) belonging to the most prevalent circulating variant in the country. Our results showed that wastewater surveillance data can be used as complementary indicators to monitor the dynamics and temporal trends of COVID-19. The infectivity test results strengthened the evidence of low risk of infection associated with SARS-CoV-2 in wastewater.

Key words: environmental surveillance, infectivity, SARS-CoV-2, sequencing, vulnerable communities, wastewater

HIGHLIGHTS

- SARS-CoV-2 WBE can be a valuable tool to follow trends of COVID-19 cases in vulnerable communities (slums and low-income neighborhoods).
- Positive samples for SARS-CoV-2 RNA presented negative results in the infectivity assays performed with Vero E6 culture cell.
- Preliminary results of NGS sequencing analysis showed the circulation of lineage P.1, the predominant SARS-CoV-2 variant in clinical genomic surveillance

GRAPHICAL ABSTRACT



1. INTRODUCTION

The first occurrence of COVID-19 in Latin America was confirmed in São Paulo, Brazil, on February 26, 2020, two weeks before the World Health Organization declared COVID-19 a pandemic (Cucinotta & Vanelli 2020). Since then, the world has been under a global crisis with a severe impact on the public health system and economy (CRS 2021). The challenges the world has faced with the pandemic triggered an accelerated scientific and technological development in the areas of diagnosis, vaccine, logistics, modeling and epidemiological surveillance.

Environmental surveillance has been used for decades to evidence the circulation of waterborne pathogens in the population, particularly poliovirus, and more recently antimicrobial-resistant microorganisms, as a complementary approach to infectious diseases surveillance (Asghar *et al.* 2014; GPEI 2016; Sims & Kasprzyk-Hordern 2020; WHO 2020; Xagoraraki & O'Brien 2020). The first report of SARS-CoV-2 RNA detection in sewage in March 2020, in the Netherlands (Medema *et al.* 2020b), indicated that wastewater surveillance was also a sensitive tool to assess the circulation of SARS-Cov-2 in the community and the COVID infection trends. Since then, several research groups worldwide have been using this approach as an epidemiological device and as an early warning system (Haramoto *et al.* 2020; La Rosa *et al.* 2020; Wu *et al.* 2020; Ahmed *et al.* 2021; https://www.covid19wbec.org/collaborators). It has also been used as a valuable instrument to provide information about the emergence and abundance of SARS-CoV-2 strains (Nemudryi *et al.* 2020; Rimoldi *et al.* 2020; Crits-Christoph *et al.* 2021; Jahn *et al.* 2021). Many countries have already implemented a national wastewater surveillance system or program to support the government in formulating epidemic prevention policies for COVID-19, including the Netherlands (Rijksoverheid 2021), the USA (CDC 2021; Kirby *et al.* 2021), the UK (Wade *et al.* 2022), South Africa (NICD 2021), Japan (Takeda *et al.* 2021) and others.

Brazil has been severely affected by the COVID pandemic. By July 1, 2021, it was the second country in a total number of deaths (520,000), only ranking behind the USA (605,000). It was also ranked ninth in the number of deaths per million

inhabitants (Ritchie *et al.* 2020), with daily death rates ranging from 3,000 to 4,000 between March and April 2021. Given the testing limitations and the challenges faced in obtaining accurate clinical and epidemiological national data, several wastewater-based epidemiology (WBE) programs have been implemented by local governments and/or research projects mostly driven to assess spatial and temporal SARS-CoV-2 load fluctuations in the sewer system and wastewater treatment plants (WWTPs) of large metropolitan regions, supporting decision-makers on COVID-19 infection trends at the population level (ANA 2021; Claro *et al.* 2021; Fongaro *et al.* 2021; Mota *el al.* 2021; Prado *et al.* 2021).

Assessing the circulation of SARS-CoV-2 in the wastewater of a huge city as São Paulo has been a major challenge. With around 13 million people, the city is served by four WWTPs, responsible for the treatment of around 75% of the sewage generated. The remaining 25% are ultimately released into surface waters.

The city of São Paulo is responsible for the highest number of cases and deaths by COVID-19 among Brazilian cities, since the beginning of the pandemic. The city has the largest number of slums (favelas), with more than 1,700 reported by the Municipal Housing Secretariat (SEHAB, http://www.habitasampa.inf.br/habitacao/). It is estimated that there are more than 390,000 homes with more than two million inhabitants living in slums in São Paulo, about 11% of the city's population. The mortality rate of the new coronavirus in São Paulo can be up to 10 times higher in neighborhoods with the worst social conditions that coincide with the areas with the highest slum concentrations (PMSP 2020; Figueiredo 2021). However, cases confirmed by laboratory examination were not predominant, which may show greater difficulty in obtaining confirmation of suspected cases in these areas (PMSP 2020).

Poor sanitation conditions, water scarcity, hyper-dense dwellings, crowded households and lack of health care access, among other factors, make urban slums and other vulnerable communities major COVID-19 hotspots and relevant areas for the application of WBE programs; however, few studies have been reported in these communities. Mota *et al.* (2021) and Prado *et al.* (2021) in decentralized monitoring of SARS-CoV-2 RNA in sewages from Belo Horizonte and Rio de Janeiro, two other large Brazilian cities, showed that wastewater monitoring data is more sensitive for identifying hotspots in vulnerable areas than clinical/epidemiological data, allowing early intervention actions by public health authorities. Razzolini *et al.* (2021) followed the evolution of SARS-CoV-2 RNA concentration for 7 months in a contaminated stream that receives raw sewage from an urban underprivileged settlement in the city of São Paulo and observed a statistically significant correlation between SARS-CoV-2 concentration in water and COVID-19 cases in the community. The authors concluded that virus concentration in the environment reflects the epidemiological status of the community. Iglesias *et al.* (2021) in a similar study conducted in a low-resource community in Buenos Aires noted that SARS-CoV-2 measurements in the lagoon that receives sewage from the community could be applied to estimate the changes in the COVID-19 prevalence. Another frequent concern in these highly vulnerable regions is the possibility of fecal–oral and fecal–respiratory transmissions, although the viability of SARS-CoV-2 in sewage samples has not been demonstrated to date (Rimoldi *et al.* 2020; Westhaus *et al.* 2021).

The present study is part of the São Paulo State wastewater surveillance program for COVID-19 initiated in April 2020 and focused on assessing trends in SARS-CoV-2 RNA concentrations in wastewater from the two main favelas of the city of São Paulo (Paraisópolis and Heliópolis) and two low-income neighborhoods (Vila Brasilândia and Cidade Tiradentes), areas of high vulnerability considered as a priority for the fight against COVID-19 by the city Health Service System. SARS-CoV-2 concentration methods, genetic diversity and viability of SARS-CoV-2 were also evaluated.

2. MATERIAL AND METHODS

2.1. Sampling

Four communities with different population sizes were selected for SARS-CoV-2 monitoring, including Paraisópolis and Heliópolis, the two largest favelas in the City of São Paulo and two communities located in low-income neighborhoods (Cidade Tiradentes and Vila Brasilândia), the most affected by COVID-19 at the start of the study (Figure 1). The selection of the sampling sites had the support of the São Paulo State Sanitation Company (SABESP), which provided information about the sub-sewershed localization, flow rate and the respective population that contributes to the sewage discharge at the collection point, enabling the definition of sampling sites of each community. Paraisópolis does not have a sewage network infrastructure and monitoring was carried out by sampling surface water at a point in the stream that receives sewage discharge from the community. The other communities were monitored at manhole points of the collection network in sewer systems. Except for Cidade Tiradentes, where the collection point represents the entire sanitary sewage basin of the neighborhood, in the other locations, due to the complexity of the sanitary sewage system, points of easier and safer access,

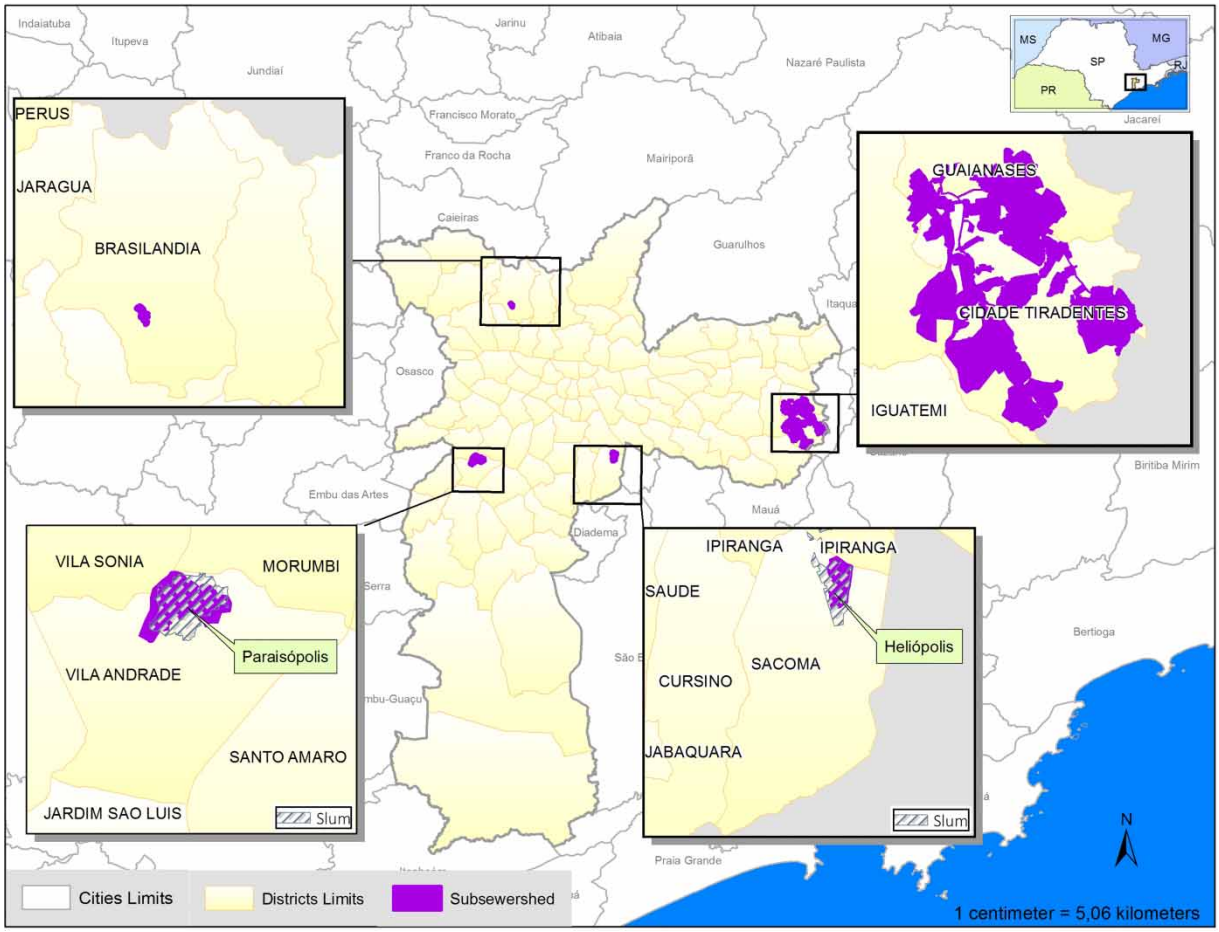


Figure 1 | Sampling locations and associated sub-sewersheds for the four selected vulnerable urban communities in São Paulo city.

representative of the community, were established. The geographic and population characteristics of each area are shown in Table 1. The average flow rates were calculated from the micromeasured flow data of treated water.

Grab samples were collected once a week, during the period from May to September 2020. From October 2020 onwards, the frequency of sampling was reduced to fortnightly.

For viruses' analyses, wastewater samples were manually collected in stainless steel buckets and transferred to sterile 1 L polypropylene bottles (APHA; AWWA; WEF 2017a). Temperature and pH were measured during sampling. Samples were transported to the laboratory on ice and kept refrigerated (4 °C) before analyses for a maximum of 24 h. Additionally, volumes of 500 mL were collected for chemical assay of total suspended solids (TSS) (APHA; AWWA; WEF 2017).

Table 1 | Wastewater sampling sites in low-income communities and estimated population residing in the area corresponding to each sub-sewershed

Sampling site	Collection point	Geographic coordinate	Average flow rate (L/day)	Estimated population
Paraisópolis (slum)	Stream	23° 36′28″ S 46° 43′11″ W	9,070,000	51,000
Heliópolis (slum)	Sewer pipeline (manhole)	23° 36′38″ S 46° 35′11″ W	2,060,000	9,800
Cidade Tiradentes	Sewer pipeline (manhole)	23° 33′36″ S 46° 25′06″ W	23,100,000	130,000
Vila Brasilândia	Sewer pipeline (manhole)	23° 27′54″ S 46° 41′30″ W	170,000	1,700

2.2. COVID-19 and SARI data

Correlation analyses were performed between confirmed cases of COVID-19 and SARI (Severe Acute Respiratory Infection) in the resident population of the area corresponding to the sub-sewersheds and SARS-CoV-2 RNA viral loads in wastewater samples. Confirmed cases of COVID-19 were defined according to the following criteria: laboratory tests, clinical (flu-like syndrome cases associated with anosmia or acute ageusia), clinical–epidemiological (flu-like syndrome cases and SARI that had close contact with a confirmed COVID-19 patient during the 14 days before the appearance of the symptoms) and clinical imaging (flu-like syndrome cases and SARI with tomography diagnose) results. A unified database with geocoded information of the residence addresses of all cases was provided by the Municipal Health Department of São Paulo, which gathered data from the SIVEP-Gripe and e-SUS Notifica databases (https://www.prefeitura.sp.gov.br/cidade/secretarias/saude/vigilancia_em_saude/doencas_e_agravos/coronavirus/index.php?p=313773). Confirmed cases of COVID-19 and SARI were counted considering the symptom onset date.

2.3. Temporal evaluation of SARS-CoV-2 RNA viral load

2.3.1. Virus concentration by ultracentrifugation and glycine elution

The ultracentrifugation and glycine elution method was used to concentrate the wastewater samples (Pina *et al.* 1998). Volumes of 40 mL of each concentrated sample were aliquoted and contaminated with 10^6 copies of bovine coronavirus (BCoV). After incubation of 1 h at 4 °C, samples were ultracentrifuged at 110,000 g for 1 h at 4 °C using a Sorvall RC 90 ultracentrifuge.

The supernatant was discarded, and the pellet was eluted with 4 mL of 0.25 N glycine buffer (pH 9.5) in an ice bath for 30 min, mixing by vortex each 5 min. After adding 4 mL of 2×phosphate-buffered saline (PBS, pH 7.2), the samples were centrifuged at 3,000 g for 20 min at 4 °C. Then, the supernatant was transferred to a new tube, previously weighed. After ultracentrifugation at 110,000 g for 1 h at 4 °C, the supernatant was carefully discarded, and the tube weight was re-recorded.

The pellet eluted in the remaining supernatant (approximately 300 μL) was transferred to 1.5 mL microtubes. The samples were kept under refrigeration (2–8 °C) until RNA extraction.

2.3.2. RNA extraction and RT-qPCR for SARS-CoV-2 and BCoV

The concentrated sample (140 μL) was extracted with the QIAamp® Viral RNA Mini kit (Qiagen, Germany) according to the manufacturer's instructions. Elution was performed in two steps with 40 μL of elution buffer (AVE), adding up to nearly 80 μL. Nucleic acid extracts were stored at −70 °C until viral quantification by RT-qPCR.

The RT-qPCR for SARS-CoV-2 was performed by quantifying the N1 and N2 gene regions according to primers, probes and reaction parameters described by the CDC protocol (CDC 2020). In addition, BCoV RNA concentrations were determined using primers and the probe previously described by Decaro *et al.* (2008). The BCoV probe was labeled with HEX at the 5′ and Black Hole Quencher 1 (BHQ1) at the 3′ end.

RT-qPCR assays for N1 and N2 were performed separately in 20 μL reactions containing 5 μL TaqPath™ 1-Step RT-qPCR Master Mix, CG (ThermoFisher, MA, USA), 1.5 μL of N1 or N2 primer and probe (2019-nCoV RUO kit, IDT, IA, USA) and 5 μL of extracted RNA. The thermal cycling conditions of one-step RT-qPCR were: 25 °C for 2 min, reverse transcription at 50 °C for 15 min, preheating at 95 °C for 2 min, 45 cycles of amplification at 95 °C for 3 s and 55 °C for 30 s. BCoV RNA was quantified using 900 nM of each primer and 200 nM of probe at the same conditions of N1 and N2.

The N1 assay was performed in a multiplex reaction with an RNA internal positive control (IPC), with the addition of 0.8 μL of VetMAX™ Xeno™ Internal Positive Control VIC Assay (Applied Biosystems, ThermoFisher) and 0.1 μL of Xeno RNA Control (Applied Biosystems, Thermo Fisher Scientific, MA, USA). If the *Cq* value of a wastewater sample increases more than 1−*Cq* compared with the reference *Cq* value, the sample is considered to have PCR inhibitors.

Standard quantification curves for N1 and N2 SARS-CoV-2 regions were constructed using serial dilutions of the 2019-nCoV_N_Positive Control kit (IDT, IA, USA), consisting of 2×10^4 to 2 copies/μL of a plasmid cloned with the N1 and N2 sequences of SARS-CoV-2. BCoV standard curves were constructed with serial dilutions of synthetic fragments (GeneArt, Thermo Fisher Scientific, MA, USA) containing BCoV sequences ranging from 2.5×10^5 to 2.5 copies/μL. Negative template control was included in all qPCR assays. All reactions were performed in duplicate in a StepOne Plus instrument (ABI, CA, USA), and data were extracted from ABI StepOne Software version 2.3.

The assay limits of detection (LODs) were defined as the minimum copy number/reaction with a 95% probability of detection. Samples that had at least one RT-qPCR replicate amplified with a *Cq* value of >40 were considered detected but not quantifiable.

The concentrations of N1 and N2 of SARS-CoV-2 and BCoV were obtained by the following equation:

$$\text{virus genome copies per L} = \text{genome copy number} * (\text{RNA}^{\text{total}}/\text{RNA}^{\text{PCR}}) * (\text{concentrate}^{\text{total}}/\text{concentrate}^{\text{extracted}}) * (1,000\ \text{mL/sample})$$

where $\text{RNA}^{\text{total}}$ is the total volume of RNA eluted (0.08 mL), RNA^{PCR} is the volume of purified RNA tested in RT-qPCR (0.005 mL), $\text{concentrate}^{\text{total}}$ is the total volume of water concentrate: Estimated volume of final concentrate=weight of tube with sample (g)−weight of tube (g), $\text{concentrate}^{\text{extracted}}$ is the volume of wastewater concentrate from which RNA was extracted (0.140 mL) and sample is the volume of original wastewater sample processed with ultracentrifugation procedure (40 mL).

To calculate the recovery efficiency of the total method, BCoV gene copies detected in the initial volume of 40 mL were divided by the BCoV gene copies spiked, and the result was expressed as a percentage.

Table 2 summarizes the RT-qPCR parameters obtained in the N1 and N2 SARS-CoV-2 and BCoV assays, for temporal evaluation of SARS-CoV-2 RNA concentrations in wastewater samples.

2.4. Infectivity assay of SARS-CoV-2

Wastewater samples with N1 and N2 concentrations greater than 10^4 GC/L in the RT-qPCR assays were submitted to the SARS-CoV-2 viability assay in cell culture.

The samples were processed using two different methods: ultrafiltration with Centricon® Plus-70 and ultracentrifugation in sucrose cushion (Summer & Smith 1987).

2.4.1. Ultrafiltration – Centricon® Plus-70

Approximately 200 mL of water or wastewater sample were filtered through a 0.22 μm PES filter (Stericup, Merck Millipore, MA, USA). Then, 60 mL of the filtered sample were centrifuged at 3,000 g for 20 min in a Centricon® Plus-70 filter (10 kDa) (Merck Millipore, MA, USA). The procedure was repeated until the volume of concentrate was reduced to approximately 1 mL. The filter was inverted and the concentrate was recovered after centrifugation (1,000 g for 2 min) and treated with 10 μl of 10 mg/mL gentamicin (Gibco, China).

2.4.2. Ultracentrifugation in sucrose cushion

The volume of 60 mL of 0.22 μm filtered sample was carefully added to the ultracentrifuge tube containing 6 mL of sucrose solution (25% w/w sucrose, 5 mM NaCl, 10 mM EDTA). After ultracentrifugation at 55,000 g for 75 min at 4 °C, the supernatant was discarded, and the pellet was resuspended in 1 mL of PBS (pH 7.2). The sample was treated with 10 μl of 10 mg/mL gentamicin (Gibco, China).

An aliquot of 140 μL of the concentrated samples was subjected to nucleic acid extraction and N1/N2 RT-qPCR, and the remaining samples were stored at −80 °C and posteriorly sent to the Laboratory of the Federal University of São Paulo.

Table 2 | Performance of N1 and N2 SARS-CoV-2 and M gene BCoV RT-qPCR assays from a composite of seven standard curves

	Range				
Assay target gene	Efficiency (*E*) (%)	Linearity (R^2)	Slope	*Y*-intercept	Genome copies/reaction LOD
SARS-CoV-2 N1	91.3–101.8	0.994–0.998	−3.290 to −3.572	36.213–38.089	10
SARS-CoV-2 N2	91.2–101.1	0.996–1.000	−3.296 to −3.552	37.608–39.304	10
BcoV M gene	91.6–102.5	0.991–0.998	−3.262 to −3.511	45.593–48.782	12

2.4.3. Cell culture for viral isolation

All experiments were conducted using two biological replicates and two technical duplicates. The experiments for viral isolation and initial passages were performed in a biosafety level 3 laboratory (BLS3), in accordance with WHO recommendations and under the laboratory biosafety guidance required for the SARS-CoV-2 at the BLS3 facilities at the Federal University of São Paulo.

Concentrated samples which previously tested positive for SARS-CoV-2 RNA were managed for viral isolation and cytopathic effect (CPE) observation in cell culture (Araujo *et al.* 2020). We used for the experiments the Vero E6 cell line (ATCC® CRL-1586™) maintained in Minimum Essential Medium (MEM; Gibco), supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Gibco). The Vero E6 cells were kept in a humidified 37 °C incubator in an atmosphere of 5% CO_2. After reaching 80% confluent monolayer, we seeded cells in 24-well plates within a concentration of 5×10^5 cells for each well and in 6-well plates within a concentration of 7.5×10^5 cells for each well. After 24 h, for the 24-well plates, we removed the culture medium, washed the wells three times with PBS 1× and inoculated them with aliquots of 150 µL of MEM medium supplemented with 150 µL of concentrated wastewater sample. For the 6-well plates, 200 µL of the concentrated wastewater sample were added to 800 µL of supplemented MEM medium. For both experiments, after 3 h of incubation, the supernatants were removed to perform the N RT-qPCR assay (Corman *et al.* 2020) to evaluate eventually remaining virus in the supernatants after incubation. Then, the medium volumes in each well in 24 or 6-well plates were completed to 500 µL and 3 mL of MEM supplemented with 2.5% FBS and 1% penicillin–streptomycin, respectively. The Vero E6 cells were incubated under humidified 37 °C with a 5% CO_2 atmosphere and were observed daily for CPE up to 6 days. The supernatant was collected, and virus replication was confirmed through CPE with an optical microscope and RT-qPCR for SARS-CoV-2. We used a positive control of SARS-CoV-2 for all experiments, kindly given by Prof. José Luiz Proença-Módena (University of Campinas – UNICAMP, SP, Brazil).

2.5. Statistical analysis

For statistical analysis, concentrations below the LODs were assigned a value equal to the LOD divided by the square root of 2 (Croghan & Egeghy 2003). The population-normalized viral loads (gene copies/capita/day) were estimate by multiplying (gene copies/L)×(L wastewater/day)×(1/sub-sewershed population).

The values of N1 and N2 RNA concentrations were transformed using the log10 function. The Wilcoxon test for paired data (Bauer 1972) was used to compare mean N1 concentrations for the two methods of concentration of SARS-CoV-2. The significance level adopted for the statistical tests was 5%.

Scatterplot diagrams were created to assess correlations between quantitative variables (recovery percentage and TSS). Linear models were fitted to assess the significance of correlations and trends. Confidence intervals of 95 and 99% were fitted for linear models. Multiple comparisons were tested using the statistics proposed by Dunn (1961).

Spearman correlations of population-normalized viral loads with new cases of COVID-19 (7-day cumulative) were calculated using IBM® SPSS® Statistics version 27.0, with a p-value statistically significant of <0.05. A cross-correlation analysis (Haugh 1976; Polanco-Martinez *et al.* 2019) was used to assess whether a time displacement of the COVID-19/SARI case dataset (7-day cumulative) impacts the strength of the correlation between N1 SARS-CoV-2 viral loads dataset quantified in wastewater samples. For viral loads, the frequency was fortnightly and when more than one data was available in the fortnight, the mean values were used. Analyses were performed using the R language with RStudio.

2.6. SARS-CoV-2 sequencing

Whole viral genome sequencing was carried out by a Brazilian reference laboratory located at the Instituto Adolfo Lutz (IAL, Center for Interdisciplinary Procedures, Strategic Laboratory). The partnership for sample sequencing was only made possible from March 2021, and therefore, only samples with concentrations above 10^5 GC N1 L^{-1}, collected from that date onwards, were sequenced. An aliquot of extracted RNA underwent a retrotranscription (RT) step where cDNA was synthesized using the SuperScript IV VILO Master Mix according to the manufacturer's instructions (Thermo Fisher Scientific, MA, USA). Fifteen microliters of cDNA were used to amplify SARS-CoV-2 full-length genome using an AmpliSeq SARS-CoV-2 panel (Thermo Fisher Scientific, MA, USA) (Alessandrini *et al.* 2020). The library was adjusted to 30 pM and then loaded onto an Ion Chef instrument (Thermo Fisher Scientific, MA, USA) for emulsion PCR, enrichment and loading onto an Ion 530 chip on the Ion GeneStudio S5 Prime Series system (Thermo Fisher Scientific, MA, USA). Reads from the library were aligned with the Wuhan-Hu-1 NCBI Reference Genome (Accession No. MN908947.3) in Torrent Suite v. 5.10.1.

For mutation calling, the following plugins were used: Coverage Analysis (v. 5.10.0.3), Variant Caller (v.5.10.1.19) and COVID19AnnotateSnpEff (v.1.0.0), a plugin specifically developed for SARS-CoV-2 that can predict the effect of base substitution (Alessandrini *et al.* 2020). The software Geneious R9 was used to visualize each sample's torrent variant caller (TVC) bam files to check the consistency of nucleotide calls and analyze sequencing data. Raw sequence reads were aligned to the complete genome of the SARS-CoV-2 Wuhan-Hu-1 isolate (Genbank Accession No. NC_045512.2).

The resulting contigs were subjected to a blast search using Ugene software (Okonechnikov *et al.* 2012) to identify members of the betacoronavirus genera. To classify and determine the mutational pattern of our sequences, NextClade v1.5.0 clade assigner (https://clades.nextstrain.org/) was used.

3. RESULTS

3.1. SARS-CoV-2 RNA quantification

We detected N1 or N2 of SARS-CoV-2 RNA in 153 (95.6%) of the 160 wastewater samples analyzed over the study period. Of these 153 samples, 150 (93.8%) had quantifiable concentrations of SARS-CoV-2 RNA. The frequency of positive samples in each community and the SARS-CoV-2 RNA concentration ranges are summarized in Table 3.

The N2 RT-qPCR detected SARS-CoV-2 RNA in 152 (95%) samples, while for the N1 RT-qPCR assay, 149 (93%) samples were positive. No significant differences were observed between the concentrations (genome copies/L) of N1 and N2 in wastewater samples, although N1 values were systematically higher than N2. The linear model (GAMLSS) relating N1 to N2 concentrations presented an R^2 value of 99.85%. Therefore, only results related to N1 data were presented for statistical analyses.

3.1.1. Concentration methods

Preliminary tests were performed to select the most effective concentration method for recovering SARS-CoV-2 RNA from wastewater samples (n=13) naturally contaminated. The ultracentrifugation and glycine elution method was compared with the ultrafiltration method using Centricon® Plus-70 (10 kDa). Statistical analysis (Wilcoxon test) showed that the ultracentrifugation results were statistically superior to ultrafiltration (W=85.0; p=0.003418; Figure 2).

3.1.2. Recovery efficiency

The recovery efficiency using BCoV control was evaluated only in samples ultracentrifuged with glycine elution. Considering all the results obtained in the laboratory including other sampling locations, with the analysis of wastewater samples (n=267), the average recovery percentage was 8.3% (s.d. 11.7%). The characteristics of the different sample matrices significantly influenced the results. Statistical analysis showed that these differences in recovery efficiencies might be associated with variations in TSS. Samples with higher amounts of TSS showed lower recovery rates of BCoV (t=−4,933 with g.l.=335; p<0.001; 95% confidence interval (−0.3570 to −0.1580); Figure 3).

Table 3 | Frequency, average and range concentrations of SARS-CoV-2 RNA (N1 and N2) in wastewater samples at four vulnerable communities of São Paulo city, Brazil

		Frequency of positive samples (%)		SARS-CoV-2 RNA Average (range) genome copies/L	
Sampling site	**Number of analyzed samples**	**N1**	**N2**	**N1**	**N2**
Paraisópolis	40	97.5	100	9.7×10^4 (<LOD – 7.2×10^5)	5.5×10^4 (DNQ – 5.8×10^5)
Heliópolis	41	97.6	100	1.5×10^5 (<LOD – 1.7×10^6)	6.3×10^4 (2.1×10^3–8.4×10^5)
Cidade Tiradentes	40	100	100	1.2×10^5 (1.3×10^4–5.8×10^5)	6.0×10^4 (4.9×10^3–5.8×10^5)
Brasilândia	39	76.9	76.9	4.1×10^4 (<LOD – 2.7×10^5)	2.6×10^4 (<LOD – 2.0×10^5)

DNQ, detected but not quantifiable; LOD, limit of detection.

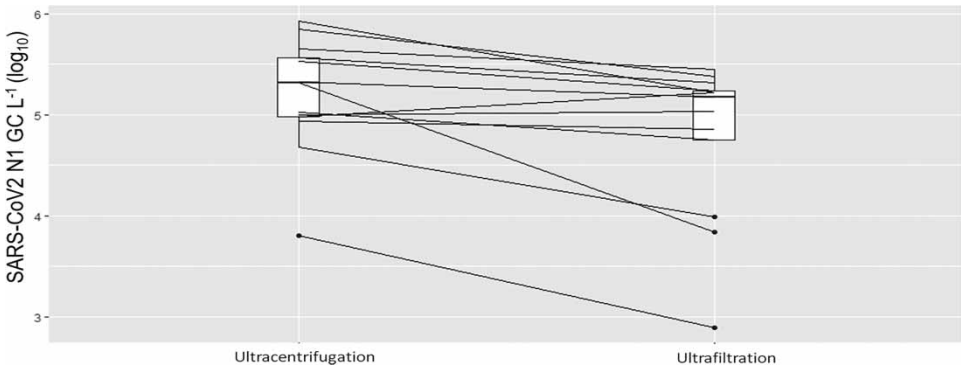


Figure 2 | Comparison between log-transformed N1 concentration (copies/L) results in wastewater samples concentrated by ultracentrifugation (with glycine elution) and ultrafiltration with Centricon® Plus-70 methods.

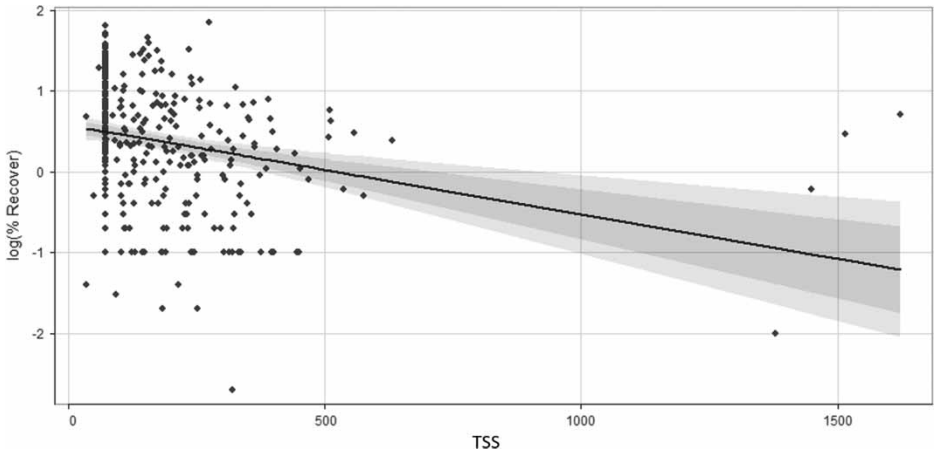


Figure 3 | Influence of the variable total suspended solids amount (TSS) on the recovery efficiencies of SARS-CoV-2 RNA by ultracentrifugation and the glycine elution method.

3.1.3. RT-qPCR inhibition

All the N1 SARS-CoV-2 assays were performed in multiplex RT-qPCR with an internal RNA positive control to ascertain inhibitors' interference present in the RNA samples. The presence of inhibitors in the samples was not observed since the IPC amplification results did not show differences greater than 1 *Cq* compared to the reference control IPC. RNA extracts (n=9) were also analyzed in different volumes (5, 1 and 0.5 μL) by N1 and N2 RT-qPCR, and no significant differences were observed between the values of copies/L concentrations.

3.1.4. SARS-CoV-2 RNA in wastewater samples and COVID-19/SARI cases

The evolution of population-normalized SARS-CoV-2 loads observed in wastewater from May 2020 to June 2021 in the four communities is shown in Figure 4(a). The periods of highest concentrations (May to June 2020, December 2020 to January 2021 and March 2021) coincided with records of an increase in new cases of COVID-19 in the city of São Paulo (Figure 4(b)). The alert levels of the São Paulo Coronavirus Control Plan that were in force during the study period are also plotted in Figure 4(b) (see Supplementary material for classifying criteria).

Considering the population-normalized viral load data in the wastewater of each community (Figure 5), we observed a greater similarity to the new cases of temporal variations only in the Cidade Tiradentes and Paraisópolis communities, which correspond to the sub-sewersheds of the largest contributing population. This observation was confirmed by the

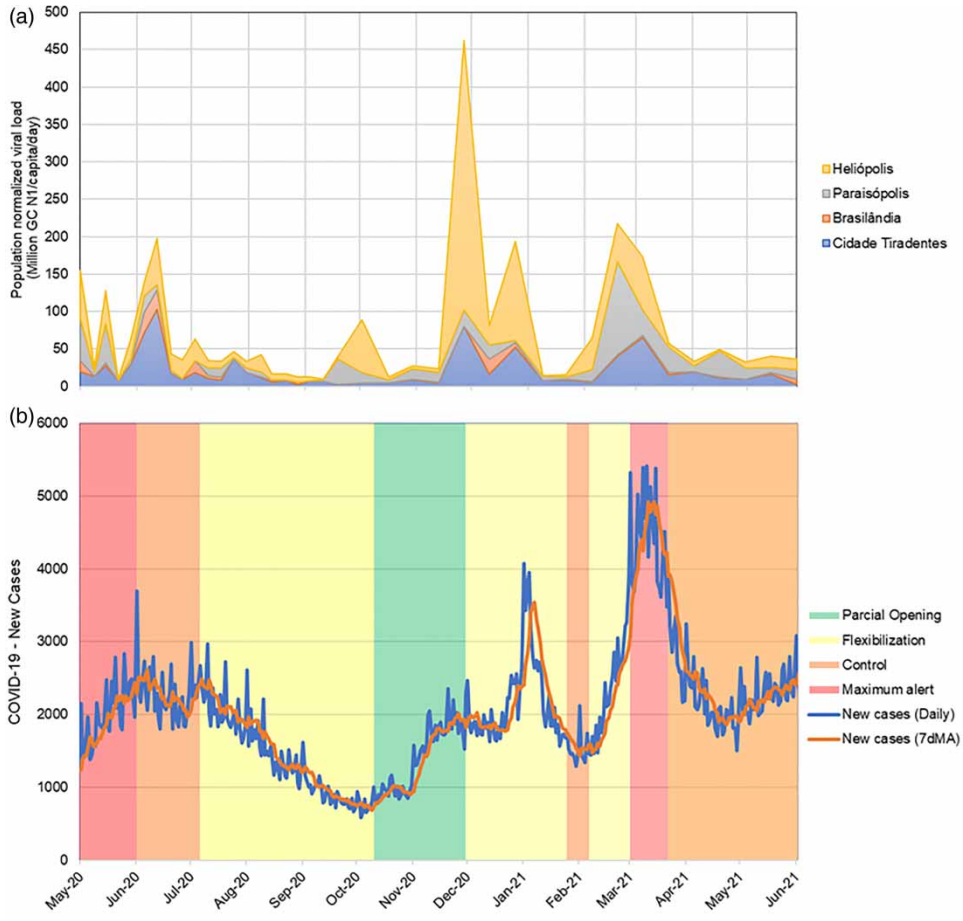


Figure 4 | Population-normalized SARS-CoV-2 loads for the four communities (a) and new cases and 7-day moving average new cases (7dMA) of COVID-19 (b).

statistical analysis of the Spearman correlation, in which significant positive correlations were observed in both communities ($p<0.001$) (Figure 6).

To assess the differences in the behavior of the temporal curves, the viral loads of SARS-CoV-2 RNA were correlated to the curves of the number of 7-day cumulative cases for each community using a cross-correlation analysis. Although the viral load curves in wastewater and reported cases of COVID-19/SARI presented similar trends in different periods, no significant correlation was observed considering the entire study period (Figure 7). Only time series of Cidade Tiradentes, for lag 0, the correlation coefficient was close to the limit of 95%; however, the representativeness was not guaranteed, since it did not reach robust CI.

3.1.5. Virus isolation

The positive concentrated samples for N1 and N2 SAR-CoV-2 RT-qPCR were inoculated in the previously prepared plates of Vero E6 cells. The samples were frozen and then used for virus isolation with daily observation for CPE by optical microscopy. Twenty-four hours post-infection (24 h.p.i.), all samples were compared with the positive control, and no morphological changes were observed. Three days post-infection (3 d.p.i), the cultures continued without any sign of CPE. After 6 d.p.i, no virus propagation was observed by CPE in the cultures, but all supernatants were collected. Samples of wastewater tested in the Vero E6 cell line had no sign of virus infectivity (Figure 8(a) and 8(d)). For all independent experiments performed in cell culture, CPE was observed in positive controls for the SARS-CoV-2, and negative controls had no signs of morphological changes (Figure 8(e) and 8(f), respectively). RT-qPCR further confirmed these results, corroborating the lack of

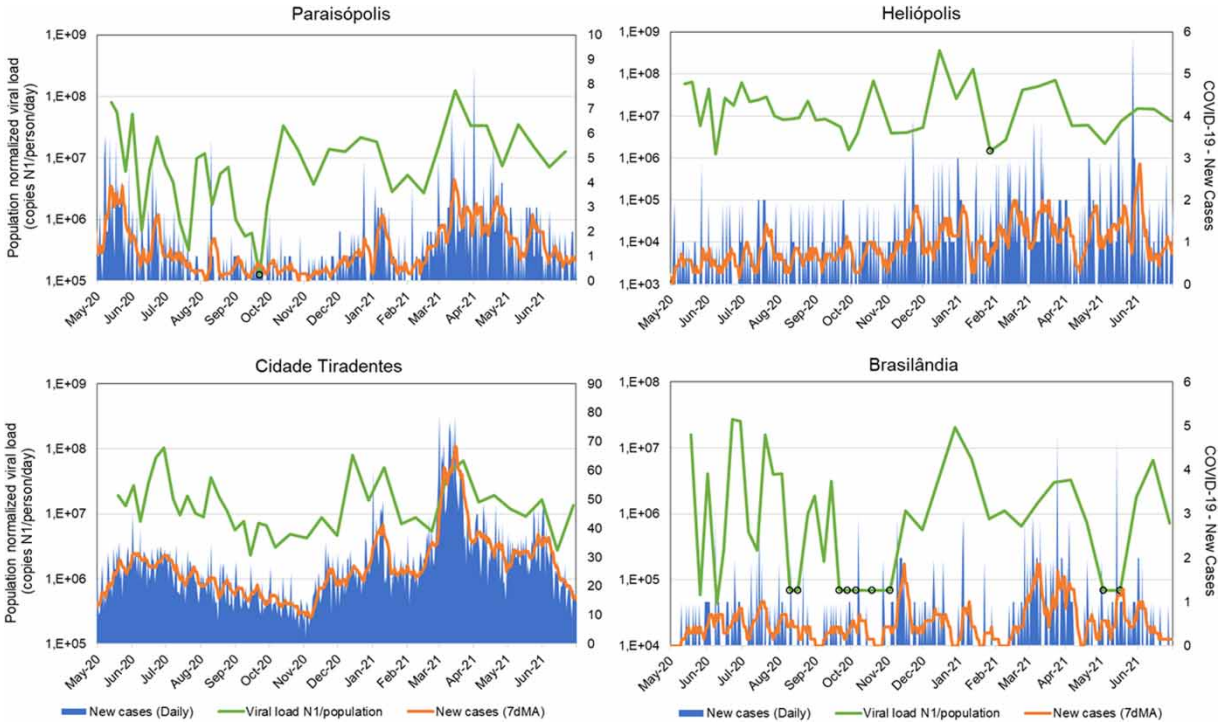


Figure 5 | Population-normalized SARS-CoV-2 RNA viral loads in wastewater and number of COVID-19/SARI cases in vulnerable communities from São Paulo city, sampled between May 2020 and June 2021. Hollow dots indicate samples below the limit of detection.

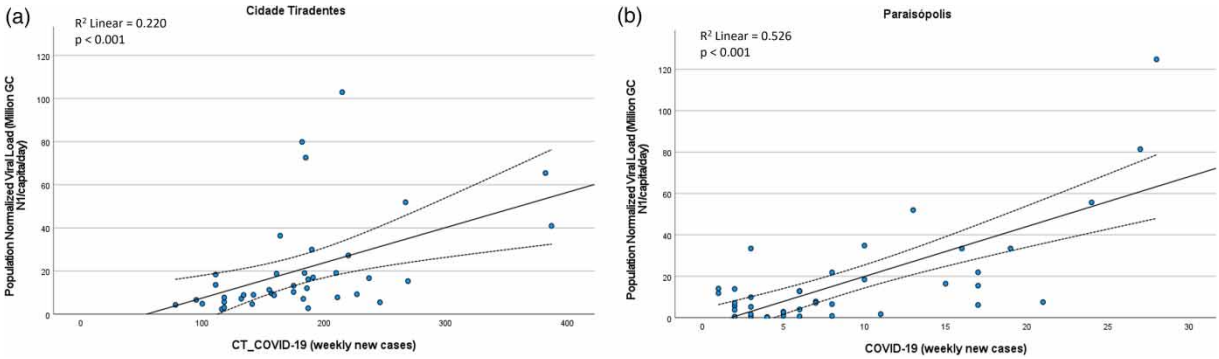


Figure 6 | Spearman's correlation between population-normalized SARS-CoV-2 viral load and weekly new cases of COVID-19 from (a) Cidade Tiradentes and (b) Paraisópolis data.

SARS-CoV-2 detection in the 6 d.p.i culture supernatants (Table 4). Similar results were observed for negative control samples. Positive controls used in the Vero E6 cells presented clear CPE and SARS-CoV-2 RNA detection in the supernatant by RT-qPCR using gene N as target.

3.1.6. SARS-CoV-2 genomes in wastewater samples

We performed NGS on three ultracentrifuged (with glycine elution) wastewater samples, and we were able to recover only one nearly complete genome of SARS-CoV-2. The results of the BlastN search using these genomes indicated that our sequences presented similarities ranging from 96.0 to 99.9% with SARS-CoV-2 reference sequences. Based on the SARS-CoV-2 Spike region (3,822 bp), we performed genotyping of the sequences identified in this study (Table 5). According to

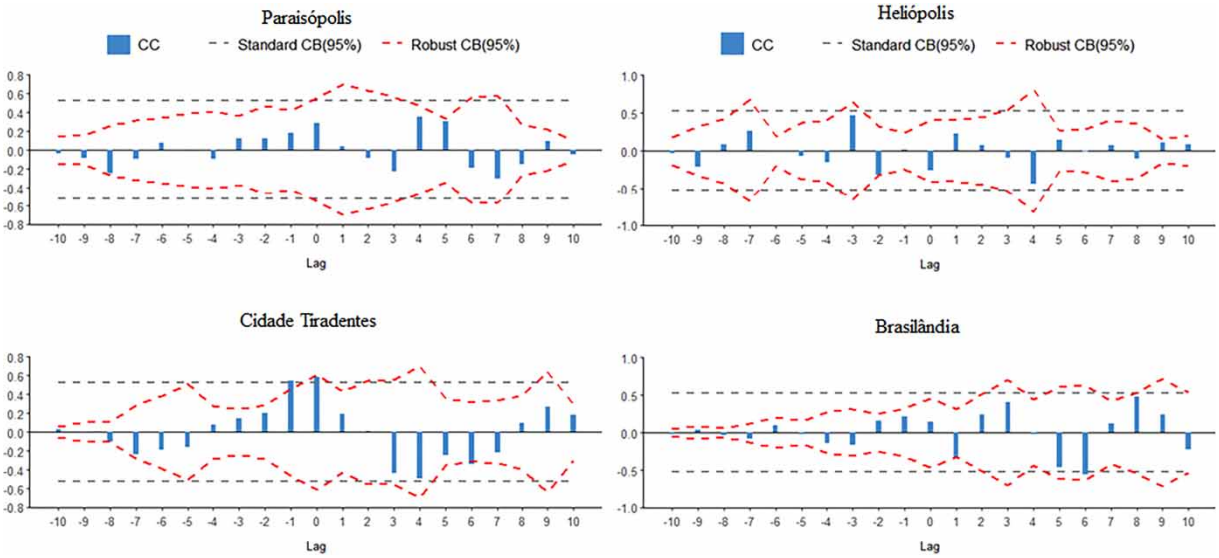


Figure 7 | Cross-correlation analysis between SARS-CoV-2 RNA viral loads and 7-day cumulative cases of COVID-19/SARI time series, in four vulnerable communities of São Paulo city, from May 2020 to June 2021.

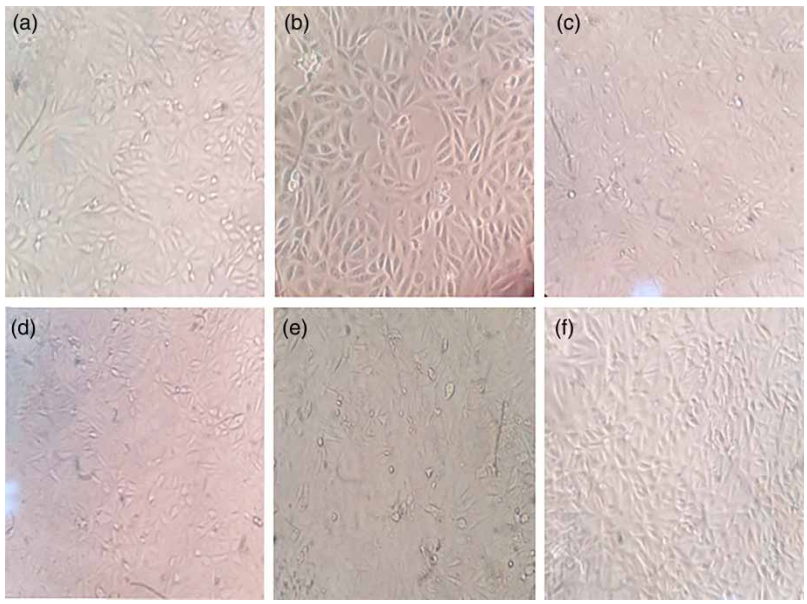


Figure 8 | Wastewater samples tested in Vero E6 cells, observed under optical microscopy. (a,b) Cells infected with the wastewater samples concentrated by ultrafiltration with Centricon® Plus-70 and (c,d) ultracentrifugation in sucrose cushion. The samples of wastewater tested in the cell culture had no sign of virus infectivity. (e,f) Cells infected with the SARS-CoV-2 virus and uninfected cells, respectively.

the clade assignment of the NextClade application, sequences 915 and 920 belong to the clade 20 J (also known as lineage P.1 or variant gamma).

4. DISCUSSION

This study presents data from SARS-CoV-2 monitoring in wastewater samples from vulnerable communities (slums and low-income neighborhoods) located in São Paulo city. During the 1-year study from May 2020 to June 2021, SARS-CoV-2 RNA (N1 or N2) was detectable in all samples from Cidade Tiradentes (n=40, ~130,000 individuals), Paraisópolis (n=40, ~51,000

Table 4 | SARS-CoV-2 RNA concentrations in wastewater samples submitted to infectivity assay in Vero E6 cells, and the corresponding results of evaluating the presence of CPE and the detection of SARS-CoV-2 replication by RT-qPCR

Sample ID	Sampling site	Sampling date	Concentration method	SARS-CoV-2, N1 copies/L (*Cq*)[a]	Cell culture (CPE-SARS-CoV-2)	SARS-CoV-2 N gene detection post-infection
2006032	Paraisópolis	26/05/2020	Ultrafiltration	2.1×10^4 (35.4)	Negative	Negative
2006073	Cidade Tiradentes	02/06/2020	Ultrafiltration	4.6×10^4 (32.9)	Negative	Negative
2006075	Heliópolis	02/06/2020	Ultrafiltration	8.9×10^4 (34.4)	Negative	Negative
2006199	Heliópolis	16/06/2020	Ultrafiltration	1.1×10^6 (30.4)	Negative	Negative
2006201	Cidade Tiradentes	15/06/2020	Ultrafiltration	1.6×10^6 (30.0)	Negative	Negative
2006250	Cidade Tiradentes	22/06/2020	Ultrafiltration	1.5×10^5 (33.9)	Negative	Negative
2006248	Heliópolis	23/06/2020	Ultrafiltration	7.1×10^4 (35.8)	Negative	Negative
2006251	Paraisópolis	23/06/2020	Ultrafiltration	1.1×10^5 (33.2)	Negative	Negative
2011128	Cidade Tiradentes	14/12/2020	Ultrafiltration	2.8×10^5 (31.7)	Negative	Negative
			Sucrose cushion ultracentrifugation	6.2×10^4 (34.1)	Negative	Negative
2011132	Heliópolis	15/12/2020	Ultrafiltration	8.8×10^5 (30.1)	Negative	Negative
			Sucrose cushion ultracentrifugation	1.1×10^5 (33.2)	Negative	Negative
2103497	Paraisópolis	15/03/2021	Ultrafiltration	2.4×10^5 (32.0)	Negative	Negative
			Sucrose cushion ultracentrifugation	2.6×10^5 (31.8)	Negative	Negative

[a]Quantification prior to inoculation in cell culture.

Table 5 | Genotyping of SARS-CoV-2 detected in wastewater samples by illumina sequencing

					NextClade[a]		
Sample ID	Sampling site	Sampling date	N1 (copies/L) *Cq*	Genome coverage (%)	Complete genome	Orf1ab	Spike
915	Paraisópolis	10/05/2021	2.0×10^5 32.4	99.6	20 J (Gamma, V3)	20B	20 J (Gamma, V3)
918	Heliópolis	09/03/2021	2.4×10^5 32.1	97.1	NA	NA	NA
920	Paraisópolis	29/03/2021	1.9×10^5 32.4	80	NA	NA	20 J (Gamma, V3)

NA, genotype not assigned due to poor quality of the sequence.
[a]NextClade v1.5.0 clade assigner (https://clades.nextstrain.org/).

individuals) and Heliópolis (n=41, ~10,000 individuals), and in 30 of a total of 39 from Vila Brasilândia, with an estimated population of 1,700 individuals contributing to sub-sewershed. The temporal variations of SARS-CoV-2 RNA were evaluated in ultracentrifuged wastewater samples, and the N1 and N2 concentrations ranged from 1×10^3 to 1×10^6 gene copies/L (Table 3). These values are within the range of SARS-CoV-2 RNA concentrations reported in wastewater around the world (https://www.covid19wbec.org/covidpoops19; https://sphere.waterpathogens.org/map).

Ultracentrifugation and glycine elution was the method of choice to determine the time series of SARS-CoV-2 RNA concentrations as it resulted in significantly higher SARS-CoV-2 N1 concentrations than the Centricon® Plus-70 ultrafiltration method (p<0.001) (Figure 2). Some authors, when comparing the recovery results of SARS-CoV-2 or enveloped viruses (MHV, BCoV, PMMoV) obtained by different concentration methods, observed that ultrafiltration using Centricon® Plus-70 had better performance than other methods such as CP-Select™ ultrafiltration (Forés *et al.* 2021), PEG 8000 precipitation (Gerrity *et al.* 2021), Amicon, Macrosep, Vivaspin ultrafiltration, PEG 8000 precipitation (Boogaerts *et al.* 2021), PEG 6000 precipitation (Bertrand *et al.* 2021) and the adsorption–elution method using electronegative membranes (Sherchan *et al.*

2020). Our comparative study was based on a few samples (n=13) previously filtered on 0.22 μm membranes (not centrifuged) before the Centricon concentration step, a difference that may have reduced the effectiveness of the method but was necessary for the viability test in cell culture.

Few studies have comparatively evaluated the ultracentrifugation method. Ahmed *et al.* (2020) compared seven different concentration methods and had better recovery efficiency of MHV in ultracentrifugation (33.5 ± 12.1%) than Centricon® Plus-70 ultrafiltration (28.0 ± 9.10%), although the most efficient was the adsorption–extraction method with $MgCl_2$ pretreatment (65.7 ± 23.8%). Prado *et al.* (2021) reported mean recoveries of 27.4 ± 8.64% of BRSV, using the same ultracentrifugation method in the wastewater sample concentration. The viral recoveries reported within this study (8.3 ± 11.7%) are in line with those reported by other comparative studies with recoveries of BCoV of no greater than 20% for the majority of samples (Jafferali *et al.* 2021; Philo *et al.* 2021; Wilder *et al.* 2021).

The ultracentrifugation technique employed in this work was originally developed to concentrate non-enveloped enteric viruses that exhibit lower partitioning to solids present in wastewater compared to enveloped viruses (Ye *et al.* 2016). Due to the strong interaction of enteric viruses with solid particles, the considerable viral loss probably occurs during the glycine elution step followed by centrifugation. This could explain the lower recovery rates in samples with higher amounts of TSS.

SARS-CoV-2 (N1 and N2 gene regions) was detected consistently throughout the monitoring period, except in Vila Brasilândia, where the virus was not found in 23% of the samples, probably as a consequence of false-negative results due to the low flow rate (Weidhaas *et al.* 2021).

The SARS-CoV-2 densities were greater than 4 $\log_{10}$ GC/L for about 80% of the samples, with 36% of these showing values greater than 5 log GC/L. Heliópolis was the place that showed the highest concentrations of viral RNA, 39% of the values were above 5 log GC/L, with a peak in December (1.7×10^6 GC/L), followed by Cidade Tiradentes (32.5%), Paraisópolis (30%) and Vila Brasilândia (12.8%) (Supplementary Fig. S1). These data are in line with those reported in other Brazilian cities (Claro *et al.* 2021; Mota *et al.* 2021; Prado *et al.* 2021).

Comparing the population-normalized viral load in the four monitored sites, both slums, Heliópolis and Paraisópolis, presented higher values of N1/capita/day over the course of the 1-year study (Figure 4(a)). Trends in viral load showed an increase in three different periods, at the beginning of the monitoring (May/June 2020), at Christmas time (December 2020/January 201) and after the carnival holiday (March 2021), which coincide with the peaks of the curve of COVID-19 new cases (daily and 7-day moving average (dMA)) reported by the São Paulo city (Figure 4(b)). After July, new COVID-19 cases and deaths decreased, but the facilitation of preventive measures in mid-October 2020 (partial opening) increased the number of daily deaths cases, exceeding 1,000 per day in mid-December (Ministério da Saúde 2020). The emergency of the new SARS-CoV-2 variant lineage P.1 in January 2021 in the city of Manaus, with more significant transmission potential, added to a greater people circulation in Carnival (mid-February) led to a new peak of cases and deaths in March.

However, when we look at the sub-sewershed level, trends in SARS-CoV-2 wastewater loads versus COVID-19 cases are distinct (Figure 5). At Cidade Tiradentes and Paraisópolis, the wastewater viral loads followed a trend similar to new cases of COVID-19 (daily and 7 dMA) reported for the population of these watersheds over the monitoring time period (p<0.001) (Figure 6); the same was not observed for the other two communities. Also, the cross-correlation analysis, considering the viral loads of SARS-CoV-2 RNA and the 7-day cumulative COVID-19 cases for each community, showed no significant temporal dependence among the COVID-19 cases and the respective virus load for the four communities. Although it was not possible to establish a model for predicting cases of COVID-19 in these communities based on the SARS-CoV-2 data in the respective sewersheds, following the trends of SARS-CoV-2 wastewater loads in the four vulnerable communities allows the Public Health Service to have a more refined picture of infected individuals circulating within these sewersheds.

It is important to mention some limitations in our study. The monitoring was conducted with weekly or fortnightly sampling with weekly or biweekly frequency, which may have reduced the representativeness of SARS-CoV-2 RNA concentrations, especially in the Brasilândia and Heliópolis communities. The sampling frequency is one important aspect to identifying the COVID-19 incidence dynamics. Some studies indicated that the least resource-intensive sampling scheme that maintained a high degree of confidence in capturing case trends was two nonconsecutive days per week (Feng *et al.* 2021; Graham *et al.* 2021). Another important aspect is that the lack of reliable data on COVID-19 cases does not allow a more accurate comparison with SARS-CoV-2 wastewater surveillance results. This issue is mainly due to the high rate of underreported cases, resulting from the limitation in the number of tests for COVID-19, with rates much lower than in

developed countries (https://www.worldometers.info/coronavirus/). Veiga Silva *et al.* (2020) estimated an underreporting rate of deaths of 35.28% in the city of São Paulo, considering data up to May 2020. Kupek (2021) estimated an underreporting rate of deaths by COVID-19 of 22.62% in 2020 in Brazil. Furthermore, the underreporting rate is more accentuated in low-income communities due to the limited availability of tests in the public health system (de Souza *et al.* 2020). Another limiting factor for comparing data is the considerable percentage of missing data (date of initial symptoms, ZIP code), especially in slum residents.

A few studies have evaluated the infectivity in cell cultures of SARS-CoV-2 isolated from water and sewage samples (Rimoldi *et al.* 2020; Razzolini *et al.* 2021; Westhaus *et al.* 2021), and as reported in the present study, the presence of infectious SARS-CoV-2 was also not evidenced. Factors like the propagation process, susceptibility of cells and incubation time may affect the sensitivity of viral culture. Furthermore, pretreatment processes by filtration and concentration of samples (Giacobbo *et al.* 2021), as well as storage and freezing–thawing procedures, can also contribute to the inactivation of SARS-CoV-2 particles. Therefore, regarding viral culture, there is a lack of standardized methods for the recovery of viable SARS-CoV-2.

Although studies have shown that viral particles of SARS-CoV-2 remain viable at 20–24 °C in river water samples (1.9–2.3 days, T90) and sewage (1.2–1.6 days, T90) experimentally contaminated (Bivins *et al.* 2020; de Oliveira *et al.* 2021; Sala-Comorera *et al.* 2021), many factors contributed to the inactivation of SARS-CoV-2 in environmental waters such as temperature, pH, exposure to UV, antagonistic microorganisms and presence of disinfectants (La Rosa *et al.* 2020; Giacobbo *et al.* 2021; Paul *et al.* 2021; Tran *et al.* 2021). However, due to the high viral load released into the environment, especially in communities without access to a sewage collection network, the low rate of sewage treatment in Brazil (SNIS 2020) and the low rate of virus removal in conventional sewage treatments from activated sludge processes, there is a clear need for further investigation to clarify the real microbiological risk associated with waterborne transmission of COVID-19 (Kumar *et al.* 2021).

The preliminary sequencing analyses detected the circulating variants, identified as the 20 J (P.1) variant, in two of the three samples evaluated. However, the complete virus genome was achieved in only one sample. Additional tests are needed to assess the sequencing approach's feasibility, including the RNA concentration and RNA purification steps. The results agreed with the SARS-CoV-2 genomic surveillance data in clinical samples conducted during the same period of collection of wastewater samples. In São Paulo city, the P.1 variant was identified in 64.4% of the 73 samples analyzed in the first week of March 2021 (http://www.prefeitura.sp.gov.br/cidade/secretarias/upload/saude/situacao_-covid19_03_26_03_2021.pdf). Considering the variants circulating in the metropolitan São Paulo in 2021, P.1 was predominant in March (84.6%), April (94.7%) and May (96.3%) (https://butantan.gov.br/assets/files/Covid/Boletim_epidemiologico/relatorio.pdf).

SARS-CoV-2 wastewater surveillance proved to be an efficient tool to detect and monitor trends and the prevalence of COVID-19 cases in communities, as demonstrated in several studies worldwide carried out from wastewater analysis (WWTP, sub-sewershed and facilities) and surface water (Medema *et al.* 2020a). In low-income communities with high rates of transmission and underreporting cases, SARS-CoV-2 WBE has an even more critical role because it can provide information on infection trends without being influenced by the availability and access to clinical testing resources or data on healthcare-seeking behavior.

5. CONCLUSIONS

This study demonstrated that in vulnerable communities such as low-income neighborhoods and slums, where underreporting rates are pronounced, wastewater surveillance could be an essential tool to provide reliable indicators of COVID-19 prevalence, despite the difficulty of validating the strategy and demonstrating its value to clinical surveillance.

The ultracentrifugation with the glycine method showed greater sensitivity in quantifying SARS-CoV-2 compared to the Centricon® Plus-70 ultrafiltration method. We observed that the different characteristics of wastewater matrices influenced the concentration method recovery. The total solids amount negatively influenced the recovery efficiency.

Wastewater-based genomic epidemiology can provide complementary and more comprehensive data on the circulation of variants in a community. Preliminary results of NGS sequencing evidenced the circulation of the P.1 lineage, the predominant variant in the area and periods analyzed. SARS-CoV-2 RNA positive sewage samples showed no replication of

SARS-CoV-2 in Vero E6 cell culture, which reinforces the evidence suggesting a low risk of transmission of SARS-CoV-2 by wastewater contamination.

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CONFLICT OF INTERESTS

The authors declare that there are no competing interests in this article.

DATA AVAILABILITY STATEMENT

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

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