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Detection of SARS-CoV-2 in wastewater as an earlier predictor of COVID-19 epidemic peaks in Venezuela

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Wastewater-based epidemiological surveillance has proven to be a useful and cost-effective tool for detecting COVID-19 outbreaks. Here, our objective was to evaluate its potential as an early warning system in Venezuela by detecting SARS-CoV-2 RNA in wastewater and its correlation with reported cases of COVID-19. Viral RNA was concentrated from wastewater collected at various sites in Caracas (northern Venezuela), from September 2021 to July 2023, using the polyethylene glycol (PEG) precipitation method. Viral quantification was performed by RT-qPCR targeting the N1 and ORF1ab genes. A significant association ($p < 0.05$) was found between viral load in wastewater and reported cases of COVID-19 up to six days after sampling. During the whole study, two populated areas of the city were persistent hotspots of viral infection. The L452R mutation, suggestive of the presence of the Delta variant, was identified in the only sample where a complete genomic sequence could be obtained. Significant differences ($p < 0.05$) between the physicochemical conditions of the wastewater samples positive and negative for the virus were found. Our results support proof of concept that wastewater surveillance can serve as an early warning system for SARS-CoV-2 outbreaks, complementing public health surveillance in those regions where COVID-19 is currently underreported.

Keywords Wastewater, SARS-CoV-2, COVID-19, Early warning, Epidemiology, Venezuela

Abbreviations

COVID-19	Coronavirus Disease 2019
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
RNA	Ribonucleic acid
PEG	Polyethylene glycol
VOC	Variant of Concern
WBE	Wastewater Based Epidemiology
WHO	World Health Organization
ACE-2	Angiotensin-converting enzyme 2
PCR	Polymerase Chain Reaction
RT-qPCR	Quantitative Reverse Transcription Polymerase Chain Reaction
Ct	Cycle threshold
NGS	Next Generation Sequencing
WS	Wastewater sample
EC	Electric conductivity
DO	Dissolved oxygen
RBD	Receptor Binding Domain
ORF1ab	Open reading frame 1a/1b gene
N1	Nucleocapsid protein N1 gene

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PRO	Propatria
COC	Coche
CAR	Caricuao
SB	San Bernardino
PET	Petare
CAT	Catia
CHAG	Chaguaramos
CHI	Chacaito
CHA	Chacao
EV	El Valle

Since its declaration by the World Health Organization (WHO) in March 2020[1], the COVID-19 pandemic, caused by the *betacoronavirus*SARS-CoV-2[2,3]has had a profound global impact on public health. According to WHO reports, by August 2024, the number of reported cases of COVID-19 exceeded 775 million, including 7 million deaths[4]. Ensuring early and accurate identification of the virus has been vital to controlling its transmission and spread, as well as implementing effective prevention.

SARS-CoV-2 is a positive-strand RNA enveloped virus with a genome size of approximately 30 kb[5] belonging to the order Nidovirales, family *Coronaviridae*, genus *betacoronavirus*[6]. The specific structural composition of this virus contains two large overlapping open reading frames, ORF1a and ORF1b, which are further processed to produce 16 nonstructural proteins (Nsp1 to 16)[7]. Additionally, it encodes 4 structural proteins, namely the spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins and 9 auxiliary proteins (ORF3a, ORF3b, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF9c, and ORF10)[8–12]. Several variants of SARS-CoV-2 emerged during the pandemic[13]. In particular, the Alpha, Beta, Gamma, Delta, and Omicron variants were classified as of concern by WHO (VOCs)[14]. These variants caused widespread concern and alarm between 2020 and 2022, leading to stricter prevention rules about staying home, complete country lockdown and limiting travel around the world[15,16].

SARS-CoV-2 viral entry into human cells is mediated by the S glycoprotein, a trimeric transmembrane protein. This is accomplished by interaction with the ACE2 (angiotensin-converting enzyme) receptor on the host cell's surface, which mediates viral entrance[6]. The ACE2 receptor is abundantly expressed in the small intestine[17]allowing virus replication. Genetic fragments of the SARS-CoV-2 virus, excreted by infected individuals[18–20], can be found in wastewater, and serve as biomarkers of infection. For this, wastewater-based epidemiology (WBE) has emerged as a sensitive and effective tool for monitoring the circulation of SARS-CoV-2 in a community through the detection of the viral RNA shed by infected individuals into wastewater. This method has shown great promise, as SARS-CoV-2 concentration trends in wastewater precede those of clinically reported COVID-19 cases[21–24]. Untreated wastewater can be considered a community excreta sample that, if monitored in a timely manner, can identify spikes in excreted viruses that may be related to outbreaks. WBE can be useful for the identification, early detection of outbreaks and forecasting of endemic viral diseases, resulting in a novel and alternative method of surveillance and monitoring of community health[25,26]. Indeed, this methodology has proven to be a highly effective approach for detecting and addressing public health problems, including viral disease outbreaks[27–29], bacterial diversity and antibiotic resistance[30,31], and substance abuse[32,33]. This approach was first theorized in 2001[33]and then implemented in 2005 to trace cocaine and other illicit drug use[34,35]as well as oseltamivir (Tamiflu) use during the 2009 influenza pandemic[36,37].

Several studies have shown that wastewater surveillance can provide early warning of community infection, with viral concentrations in wastewater increasing several days prior to identification through clinical testing[22,38–40]. WBE can complement the epidemiological surveillance by providing mass monitoring through a low-cost, efficient, and non-invasive approach[41]. It can also shed light on "hidden" prevalence rates for asymptomatic infections, poor health-seeking behavior, as well as enhance surveillance in settings with low diagnostic capacity[42]. WBE has been proven to be effective in monitoring the viral load in the wastewater catchment area, which may provide unambiguous predictions of future outbreaks. It may also aid in uncovering the ground reality of COVID-19 cases (including asymptomatic cases) by examining a larger population as opposed only clinically reported cases in the area[23,43–45]. The technique also lends itself to cost-effective, scalable implementation at the community-level. Collectively, these features position environmental surveillance as a powerful complement to traditional disease tracking methods, with transformative implications for pandemic preparedness and response.

Despite this, the detection method used in WBE has several uncertainties and lacks optimization and standardization, leading to varying results across different laboratories[46]. Biological differences in how individuals shed the virus, alongside the presence of other viruses in the wastewater itself, can influence the data collected[47–49]. Additionally, uncertainties arise during the sample collection and analysis process. Variations in virus concentration techniques, RNA extraction methods, PCR detection methods, susceptibility to PCR inhibition, and even the stability of viral RNA during transport through the wastewater system all contribute to result variability[46,50–53]. Additional factors include underreporting of cases due to reasons such as lack of testing capacity and individuals with mild symptoms not coming forward for testing[54,55]. As the WBE methodology continues to develop, the uncertainties associated with this method are likely to decrease.

WBE has been used to monitor the presence of SARS-CoV-2 in wastewater in different countries, and it has been documented that it can serve as an early warning system for COVID-19 outbreaks[56–60]. In September 2021, the experimental field test on the potential application of WBE in northern Venezuela began. We selected the city of Caracas as the study area, since this was the region in the country where the COVID-19 epidemic began and was best documented. Specifically, the objectives of the present study were: (i) detect the presence and evaluate the prevalence of SARS-CoV-2 RNA in the wastewater of Caracas; (ii) quantify the levels of RNA

detected; (iii) analyze the correlation between RNA levels and reported cases of COVID-19; and (iv) evaluate the effect of the physicochemical characteristics of wastewater on the detection of the virus. We expect that detection of the virus in wastewater will reflect the levels of viral infection in the community. The final aim was to demonstrate the usefulness of this tool as an early warning system for future disease outbreaks, even more so, when clinical laboratory tests and official reports on COVID-19 have decreased in Venezuela.

Materials and methods

Sample collection

Wastewater samples (WS) were collected directly from domestic influent discharge sewers, at locations specified in Fig. 1. Sampling was carried out from September 6, 2021 in seven urban sectors of the metropolitan area of Caracas (Distrito Capital and Miranda states): San Bernardino (N10°30'539" W066°54'211"), Caricuao (N10°26'068 W66°58'597"), Catia (N10°30'790 W66°56'996), Propatria (N10°30'296 W66°57'287"), Coche (N10°26'748 W066°55'701"), Los Chaguaramos (N10°29'303" W066°53'169"), and Petare (N10°29°191 W066°48'259) neighborhoods. In January and February 2022, sampling points were added in Chacao (N10°29'501" W066°51'332") and El Valle (N10°27'936" W066°54'360") sectors, respectively. Chacaito (N10°29'361" W066°52'210") was the last site to be included, in November 2022. These sectors include localities with predominantly residential areas and high commercial activity (Caricuao, Catia, Propatria, Coche, Chacao and Chacaito), moderate commercial activity (El Valle) and the presence of clinics and hospitals (San Bernardino, Los Chaguaramos). On the other hand, Petare is a densely populated area with slums, limited access to drinking water, some industry, and formal and informal sewer networks. However, this area faces a major problem regarding basic sanitation, as wastewater is not treated in the city's sewage system but is discharged into nearby rivers. Figure 1 shows the location of the sampling sites and the population density of the parishes in which they are located.

Due to the lack of functional sewage treatment plants in Caracas, wastewater sampling relied on a manual collection device inserted directly into the sewage system at specific locations within the sewer sheds. Approximately 1 L of WS was collected, packed in a sterile glass bottle, transported and maintained at 4 °C until processing at the laboratory. Samples were taken in each sampling campaign in the morning hours, thus not having a standardized sampling schedule. Physicochemical characteristics such as water temperature (T), pH, electric conductivity (EC), dissolved oxygen (DO), and turbidity were measured in situ using a sensor of water quality sonde (WTW™ MultiLine 3430™ Portable Digital Multiparameter). A weekly sampling frequency was followed during 2021 to change to a twice a month frequency during 2022 and 2023 due to logistical issues.

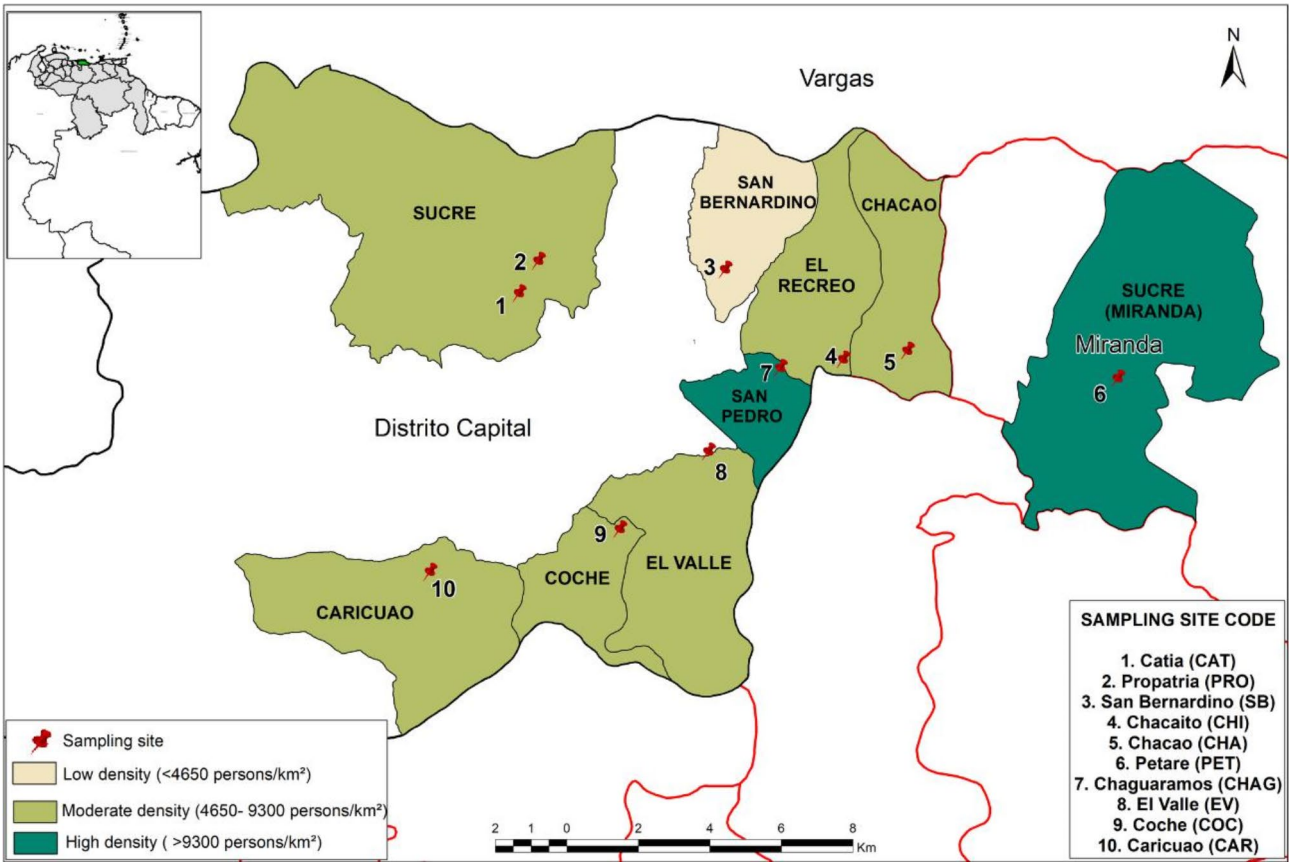

Fig. 1. Geographical distribution of sampling points. Sampling sites distributed across different parishes of Distrito Capital and Miranda states (known as Caracas city), including population density of the study area.

Detection and quantification of SARS-CoV-2 RNA

The untreated wastewater samples were pasteurized placing on a preheated 60 °C water bath for 60 min to minimize exposure to pathogens, without significantly affecting the recovery and detection of genetic material[61–63]. After completion of pasteurization and when the samples reached room temperature, the viral concentration process was initiated.

Viral concentration was performed as previously described[64] with modifications. Briefly, 40 mL aliquots of the WS were treated with 10% of PEG 8000 (4.0 g) and 2.25% of NaCl (0.9 g) according to protocol. Once the solids were dissolved, the samples were placed in a refrigerator at 4 °C overnight. After this time, 60 µL of a 2% safranin solution was added to each tube mixing by inversion, and the tubes were centrifuged at 12,000 g for 120 min at 4 °C. Most of the supernatant was carefully discarded by decanting without disturbing the pellet. The pellet was resuspended with approximately 2 mL of the remaining supernatant and stored at -20 °C until processed. Distilled water was used as a negative control, and a nuclease-free water sample inoculated with a known amount of laboratory-grown heat-inactivated SARS-CoV-2 virus was the experiment's positive control.

For viral RNA extraction, the Viral Nucleic Acid Extraction kit from IBI Scientific (IB47403) was used, and the extraction was carried out according to the manufacturer's instructions. To evaluate the efficacy and robustness of the viral RNA extraction methodology, a comparative study was conducted using distilled water, fish tank water (characterized by a high organic matter content), and untreated wastewater samples. The inclusion of fish tank water allowed us to assess the method's performance in a matrix like real wastewater. Following concentration and extraction procedures, viral RNA was quantified via RT-qPCR. The dual detection of the N1 and ORF1ab SARS-CoV-2 genes enhanced the method's sensitivity and specificity. Results demonstrated the method ability to detect viral RNA in complex aqueous matrices, such as wastewater, without the addition of an exogenous virus. The absence of detectable viral RNA in specific wastewater samples might be attributed to low viral load, inhibitory substances, or inefficient viral particle concentration during the pretreatment steps.

Viral RNA was quantified by RT-qPCR, targeting the ORF1ab and N1 regions of viral RNA using the Sansure Biotech diagnostic kit (S3102E SC2 – Novel Coronavirus (2019-nCoV) Nucleic Acid Diagnostic Kit)[65,66]. For every 20 µL sample, 30 µL of the reaction mixture which consisted of 26 µL of SARS-CoV-2 specific PCR mix and 4 µL of the Enzyme mix included in the kit, was added, yielding a final reaction volume of 50 µL. Negative and positive controls for RT-qPCR included in the kit was used. RT-qPCR was performed on a Bioer LineGene 9600 Plus. Cycling parameters started with one cycle for reverse transcription at 50 °C for 30 min, one cycle of denaturation at 95 °C for 2 min, 45 cycles of denaturation at 95 °C for 15 s, 45 cycles of hybridization and polymerization at 60 °C for 20 s and one cycle for the final step at 25 °C for 20 s.

For genome copy quantification, a standard curve was generated using a SARS-CoV-2 standard. The E-gene RNA single positive control (Tib-Molbiol, Germany, Product No. 30743771) contains 10^{10} copies of RNA in one vial. An aliquot was generously donated by the National Institute of Hygiene Rafael Rangel in Caracas, Venezuela. This primary standard was used to create a secondary standard using SARS-CoV-2 obtained from cell culture. Briefly, the primary standard allowed us to create a calibration curve. Then, an aliquot of the virus obtained from infected cells in culture (under BSL3 conditions) was heat-inactivated and serially diluted (1:10 dilution factor). This process resulted in a double curve for the N1 and ORF1ab genes, with R2 values of 0.99 and 0.987 (Supplementary figures S1 and S2), respectively, ranging from 10^8 to 10 copies/mL. A single standard curve was used for different qPCR plates.

Then, gene copy data were converted to gene copies per liter (gc/L) using the equation reported by Flood et al[67].. with modifications:

$$Virus\ GC\ per\ liter = \frac{\frac{GC\ per\ reaction}{Vr} \times Ve \times \frac{Vf}{Vc}}{Vi} \times 1000$$

where: V_i = Initial volume of sample concentration in mL, V_f = Final volume of sample after concentration in mL, V_r = Volume of RNA template used per PCR reaction in µL, V_e = Final volume of RNA eluted from RNA extraction in µL, V_c= Volume of concentrated sample used for RNA extraction in mL. Samples were considered positive for Ct (Cycle threshold) values below 40[68,69].

SARS-CoV-2 variant analysis

Viral RNA from positive samples, with Ct below 34, were selected for complete genome sequencing by Next Generation Sequencing (NGS), as described previously[48]. The library was prepared with EasySeq™ RC-PCR SARS-CoV-2 WGS kit (NimaGen BV, Nijmegen, The Netherlands). Amplification of the RBD (Receptor Binding Domain) containing gene fragment in the viral S protein was also performed[70].

Data analysis

To determine the potential effect of the physicochemical characteristics of wastewater samples on the detection of the SARS-CoV-2 virus, a multiple linear correlation was performed considering the physicochemical variables determined in situ as independent variables and the viral load determined in the wastewater as the dependent variable. Additionally, a Kruskal-Wallis test by ranks was used with the physicochemical parameters measured in situ as independent variables (and samples), and positive or negative virus detection as the grouping dependent variable. Both analyses were performed with significance levels fixed at 5%. Data on daily COVID-19 cases in the different localities sampled were obtained through the official reports from the national health public system. For the statistical analysis only the N1 gene was used, since with this gene the viral concentration was higher with respect to ORF1ab, in addition to exhibiting a high proportion of positive samples. The linear relationship of daily reported COVID-19 cases and detected viral loads was explored using lagged or cross-

	Coeff.	Std.err.	t	*P*	R^2
Constant	5.5915	3.2364	1.7277	0.085106	
T (°C)	0.022284	0.11607	0.19199	0.84788	0.003294
pH	-0.34545	0.13335	-2.5905	0.010066	0.028502
EC (mS/cm)	-0.090517	0.041933	-2.1586	0.031699	0.000669
Turbidity (NTU)	0.001852	0.000495	3.7425	0.000219	0.02553
DO (mg/L)	0.034887	0.025821	1.3511	0.17771	0.00801

Table 1. Summary of multiple linear regression of the physicochemical variables (independent variables) and the concentration of SARS-CoV-2 (dependent variable) in wastewater. Multiple $R = 0.28563$. Multiple $R^2 = 0.081586$. $N = 297$. ANOVA $p = 0.00014614$.

Viral detection in samples	T (°C)	pH	EC (mS/cm)	Turbidity (NTU)	DO (mg/L)
Negative	27.35 ± 1.02 a	7.35 ± 0.93 a	5.22 ± 2.8 a	138.07 ± 198.47 b	6.07 ± 5.52 a
Positive	27.15 ± 1.23 a	7.15 ± 0.87 b	5.59 ± 4.22 a	238.87 ± 317.89 a	7.45 ± 8.10 a

Table 2. Physicochemical parameters in wastewater samples positive and negative for SARS-CoV-2.

correlation functions. Spearman's rank correlation tests were performed using the 2021 weekly data. To ensure consistency in the analysis, spatial variability was minimized when averaging the viral concentrations measured in each parish for each sampling week. Also, the COVID-19 cases reported in those parishes at 2, 3, 4, 5 and 6 days after sampling were averaged. Dispersion graphics were performed to visualize the temporal association of viral concentrations and COVID-19 new cases. A heatmap was created to show the prevalence pattern of SARS-CoV-2 across the different localities and sampling dates. To construct the heatmap, six categories were used for concentration in the display color scale, with the purple category being negative with values less than 10 gene copies per liter (gc/L) (Ct values greater than or equal to 40 or no detection of the studied genes). Finally, the R free-software, version 4.1.2 (The R-Development Core Team, http://www.r-project.org) was used for all analyses and figures.

Results

From September 6, 2021 to July 11, 2023, a total of 310 samples were collected, of which 217 were positive for at least one of the genes analyzed, representing a total positivity rate of 70%. During the initial sampling period (September 2021 - January 2022), a greater number of positive samples were observed, resulting in a positivity rate of 88.4%. In 2022 this rate decreased to 61.5% and in 2023, to 60.3%, in accordance with lower concentrations of viruses detected.

Effect of the physicochemical characteristics of wastewater on the detection of the SARS-CoV-2 virus

Caracas sewerage system is mainly combined, which means that along with domestic waste, sewers collect runoff and, in some sites industrial waste. As a result, differences in the physicochemical characteristics of untreated wastewater were detected at the different sampling sites (Supplementary figures S3). Temperature varied in a range between 23.5 and 30 °C. The pH values were determined between 3.39 and 8.88. Likewise, electrical conductivity values were recorded between 0.349 and 20.1 mS/cm. Turbidity was highly variable between zones, with values ranging from 3 (very clear wastewater) to 999 NTU (very turbid). Dissolved oxygen also showed variability from 0 to 6.8 mg/L.

Table 1 shows the result of the multiple linear correlation between the physicochemical variables and the viral load determined in the sampled wastewater. It is observed that the model fit (R-squared) was very low, however, the significance level was $p < 0.05$.

Table 2 shows the differences between the values of physicochemical parameters measured in the SARS-CoV-2 positive and negative samples. Despite the extreme dispersion of the data for some variables, the non-parametric test indicated significant differences between the pH ($p = 0.008551$) and turbidity ($p = 0.03172$) values between positive and negative samples. Samples that were positive for SAR-CoV-2 had lower pH values compared to the negative samples. Likewise, the positive samples presented higher turbidity values compared to the negative samples. The variables DO, CE and T did not show significant differences between positive and negative samples.

Mean ± standard deviation followed by different letters in the same column indicate statistically significant differences (Kruskal-Wallis, $p \leq 0.05$).

WBE as an early warning

The scatterplots of Fig. 2 show the relationship between the average number of COVID-19 reported cases in all localities sampled and the SARS-CoV-2 concentration in wastewater in those sites for 6-, 5-, 4-, 3-, and 2-days

post-sampling. In all cases, a linear trend can be observed in the graphs which shows a significantly positive correlation (Fig. 2).

For 2- and 3-days post-sampling, a correlation coefficient of $\rho = 0.68$ was determined ($p = 0.0139$ and 0.0129 respectively). The highest correlation was obtained at 4 days ($\rho = 0.83$ $p = 0.006$) however, even with 6 days a good fit of the model is obtained ($\rho = 0.81$; $p = 0.0012$).

Prevalence of wastewater SARS-CoV-2 positives in Caracas

Figure 3A shows the viral concentration determined in the different sampling periods. The highest values were recorded in September 2021, during the pandemic peak in the country, with a decreasing trend towards the end of the year. Then an increase in viral load in January and February 2022, with a tendency to decrease until May of that year. An increase in viral load was observed in July and August 2022, with a tendency to decrease towards the end of that year. Finally, an increase in viral load was observed between December 2022 and February 2023, a decrease in viral circulation between April and May 2023, and a new increase in viral concentration in wastewater in June 2023. This temporal pattern seems to be repeating. The graph shows a possible temporal pattern with an increase in cases in December-January and July-August.

When correlating the viral load with the active COVID-19 cases (Fig. 3B), the trend of COVID-19 cases, whether decreasing or increasing, was also observed in the concentration of SARS-CoV-2 detected in the wastewater samples. A Spearman correlation test showed the existence of this correlation (ρ (rho) = 0.573; $p = 0.0215$). Particularly, the increase in COVID-19 cases reported in January 2022 coincided with the high viral concentration in wastewater on the same date (Fig. 3B). Cases decreased between March and April 2022, and the viral load in wastewater decreased accordingly. Figure 3B also demonstrates that SARS-CoV-2 RNA was detected in wastewater even when COVID-19 cases were low or unreported and shows the dominance of the Delta variant during September-October 2021 and the Omicron variant during January-February 2022, which aligns with the corresponding peaks in COVID-19 cases and viral load in wastewater.

Figure 4 reveals a spatiotemporal pattern in the detection of the virus, highlighting Catia (CAT) and Caricuao (CAR) as critical points of viral infection (hotspots) during the study. By contrast, San Bernardino (SB) and Petare (PET) showed lower viral concentrations. The heatmap also reveals that during the period between

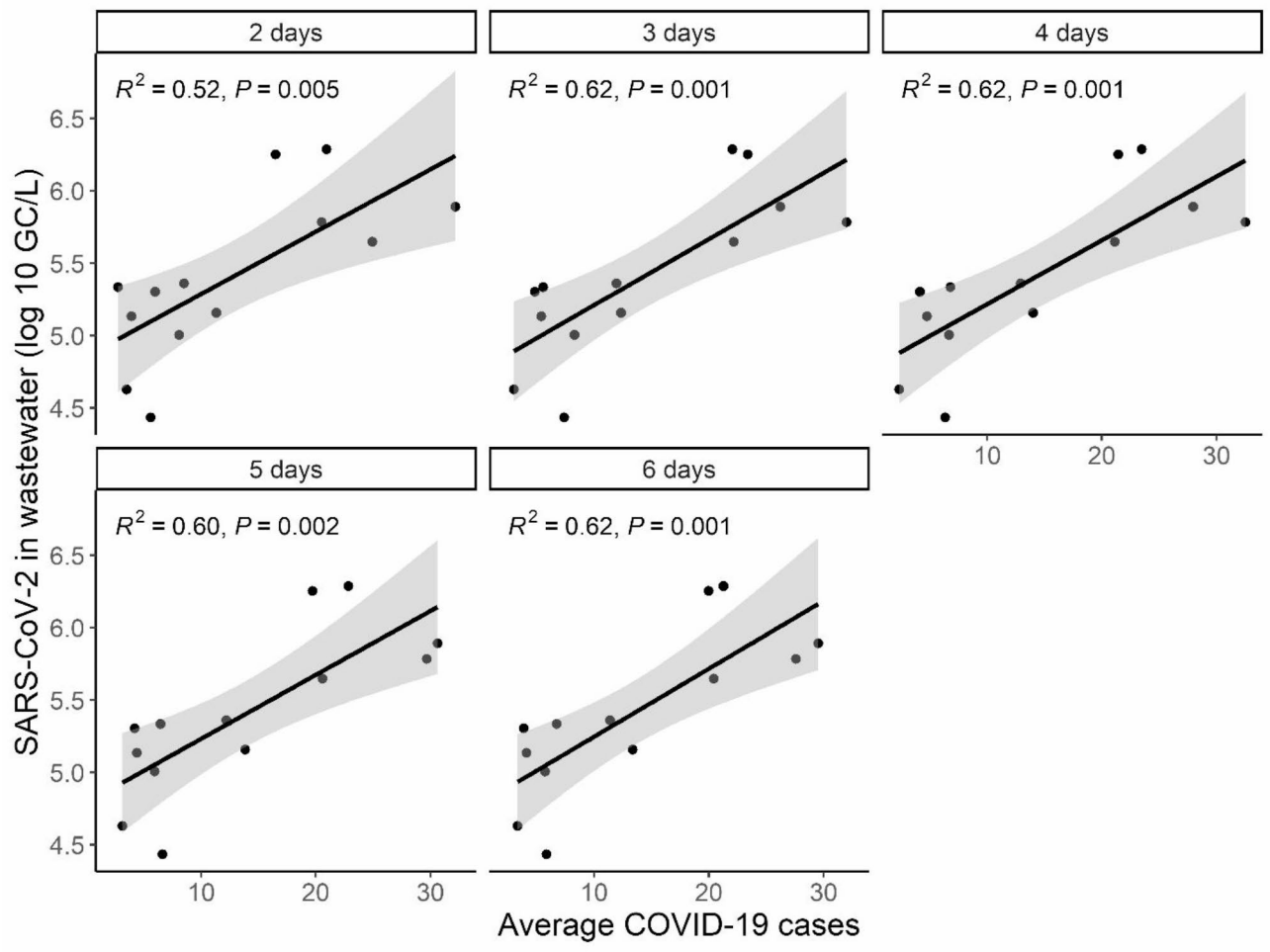

Fig. 2. Scatterplots for 6-, 5-, 4-, 3-, and 2-days post-sampling. Assessing the correlation between viral load in water and the average number of COVID-19 reported cases in all sectors sampled.

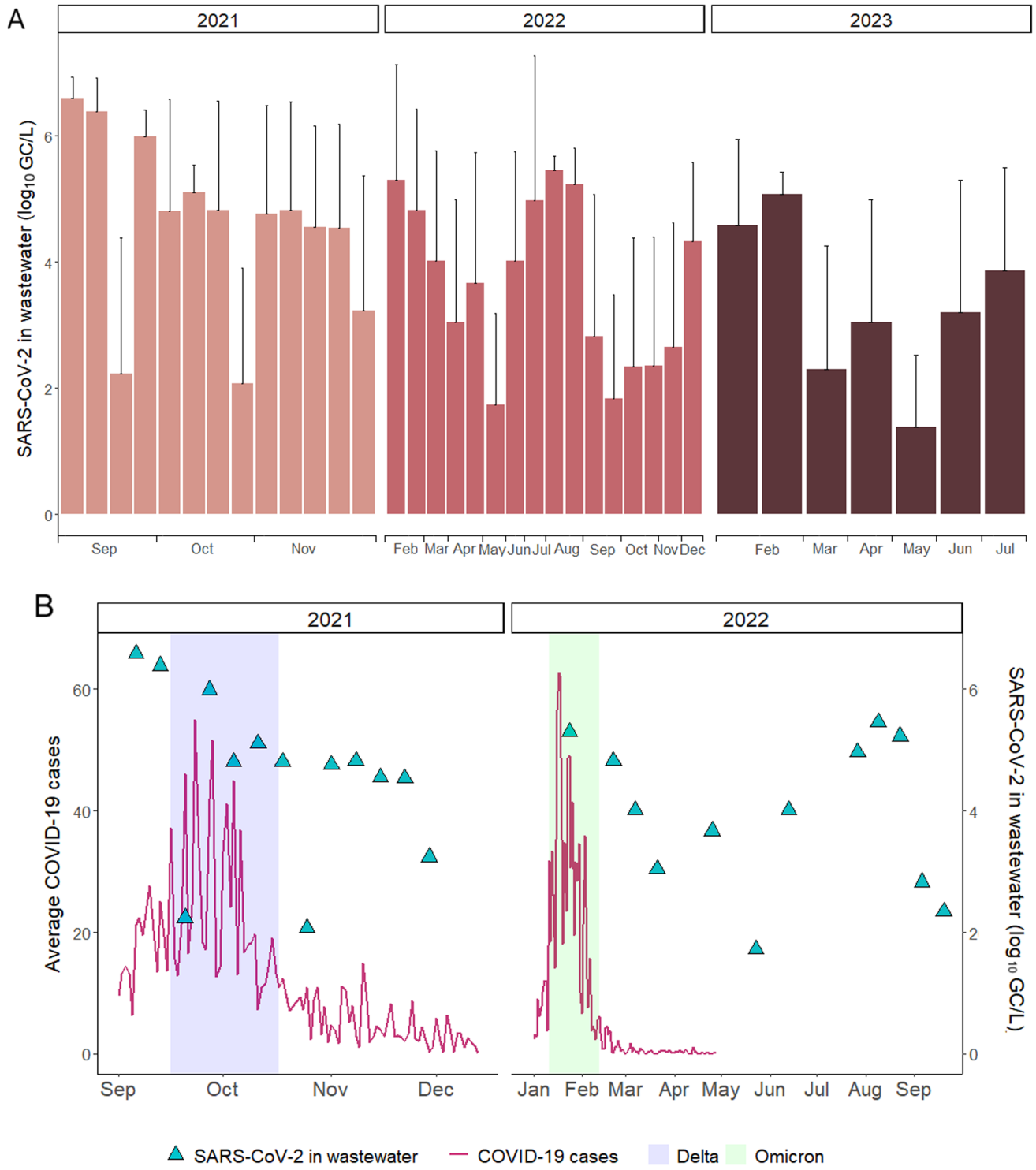

Fig. 3. Prevalence of SARS-CoV-2 RNA in wastewater from Caracas. (**A**) Average load of SARS-CoV-2 in wastewater during the different sampling periods. (**B**) Average COVID-19 cases and SARS-CoV-2 load in wastewater during the study. A similar trend between the two-time series is observed, corroborated by a significant correlation (ρ (rho) = 0.573; p = 0.0215). The shaded areas indicate the predominant variant (Delta, in lilac; Omicron, in green)[70] during our study. The official reports of COVID-19 cases were only available until April 2022.

September 2021 and January 2022, the viral concentration in wastewater was higher compared to the following months. Specifically, in July 2022 there was an increase in the concentration of SARS-CoV-2, suggesting a peak of infection that subsequently decreased starting in October 2022. A heatmap with the COVID-19 cases

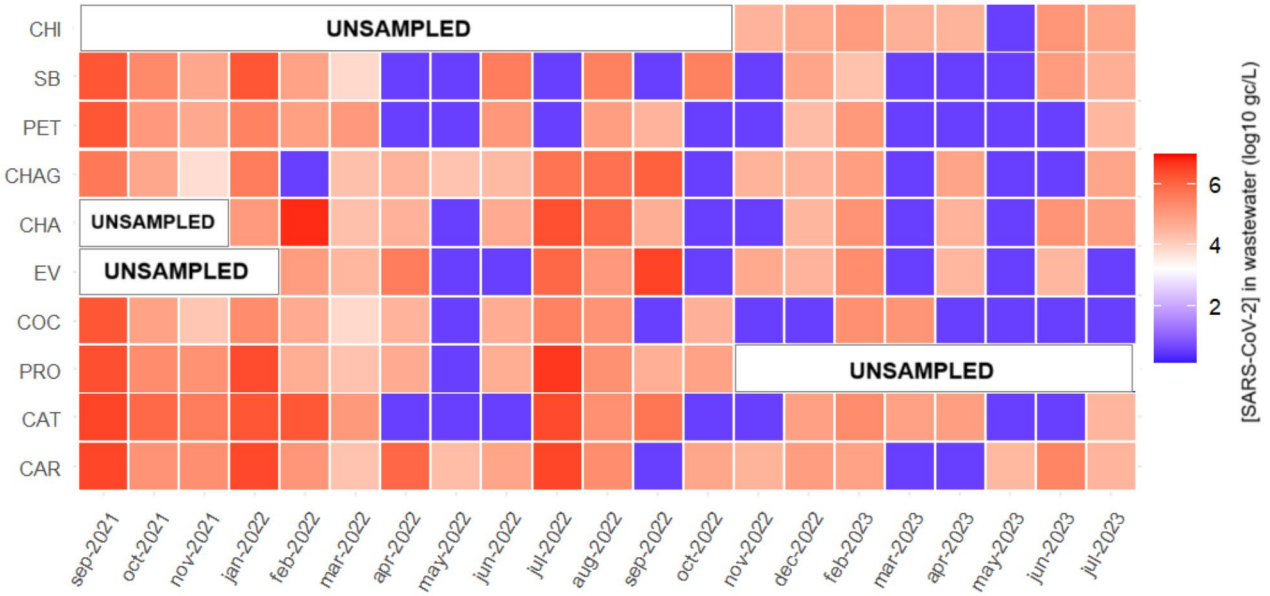

Fig. 4. Heatmap showing the change in SARS-CoV-2 concentration (expressed in $\log_{10}$gc/L) on the sampling dates, at each sampled site. CHI = Chacaito, SB = San Bernardino, PET = Petare, CHAG = Chaguaramos, CHA = Chacao, EV = El Valle, COC = Coche, PRO = Propatria, CAT = Catia, CAR = Caricuao. The warmer colors indicate higher viral concentration, while purple colors represent negative samples.

reported by parish until April 2022 can be observed in Supplementary figure S4. This figure shows that the trend of COVID-19 cases was consistent with the viral concentration observed in wastewater (Fig. 4).

Genome sequencing

A complete genome sequence could be obtained for one sample from Catia (CAT) October 4, 2021, GISAID accession number EPI_ISL_15104826. This isolate was classified as an unresolved lineage B, probably due to its low coverage (83.3%). It harbored however the L452R mutation, suggestive of a Delta VOC. The sequence was submitted to BLAST analysis to identify sequences with high similarity both in GenBank and GISAID, and no hit retrieved a Delta VOC. Phylogenetic analysis showed that the sequence from virus in water got grouped near but outside the Delta lineages in the tree (Fig. 5). Two sequences from water samples collected during the same year in Austria and USA grouped correctly in the Delta VOC clade, as expected (Fig. 5). As stated before, the presence of the L452R mutation in this sequence suggests that the sample belonged to the B.1.617.2 (Delta VOC lineage), in agreement with the circulation of this variant, at the time of sampling, in the capital city of the country. Thus, the analysis of the viral sequence from the Venezuelan water sample strongly suggests the presence of a Delta VOC circulating in water, as expected for the time of sampling.

As an alternative strategy for variant analysis, the amplification in 30 samples with a viral load estimated to be above $6{,}4\times10^5$ copies of the N1 gene per liter (based on a Ct cutoff of 34) of a small gene fragment corresponding to the Receptor Binding Domain (RBD) of the Spike protein was attempted. However, this strategy was unsuccessful, probably due to the low viral load or damaged RNA in the samples.

Discussion

Wastewater-based epidemiology enables early detection of pathogens in wastewater, even before epidemiological outbreaks arise in the community[73]. It also complements clinical methods by identifying asymptomatic cases and serving as a reliable early warning system. This capability assists health authorities in making informed decisions to control virus spread. During the COVID-19 pandemic, wastewater surveillance emerged as a cost-effective alternative to frequent diagnostic testing[74]. Our study in Caracas, Venezuela, confirmed the presence of SARS-CoV-2 RNA in wastewater samples collected from various sampling localities. Systematic analysis of wastewater at sentinel points can provide an estimate of COVID-19 cases at the level of the entire population[75–77]. We validated the correlation between reported COVID-19 cases and SARS-CoV-2 concentration in wastewater, supporting the hypothesis that the level of virus in wastewater reflects community infection levels. Notably, the virus could be detected in wastewater 6 – 4 days before official case reporting, emphasizing its potential for early tracking[78]. The findings of this study suggest the possibility of implementing an early warning system, with a time window of 4 to 6 days before an increase in clinical data is observed in the areas analyzed. These results are consistent with previous work conducted in Italy[79], where SARS-CoV-2 RNA was detected in wastewater before cases were documented. Studies conducted in Paris[21], Spain[39]and England[80]also demonstrated the early detection capabilities of this approach, emphasizing the importance of environmental monitoring. The persistence of SARS-CoV-2 RNA in wastewater varies but remains detectable for days to weeks[81]. Overall,

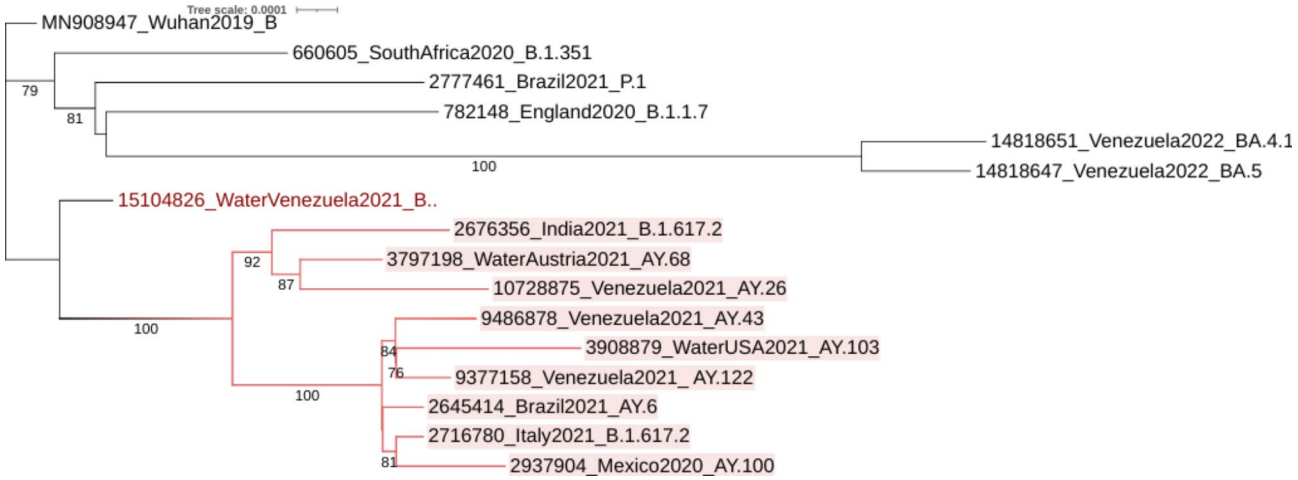

Fig. 5. Phylogenetic analysis of the viral isolate from water in Venezuela. Maximum likelihood tree with model of substitution: TIM + FO + I was constructed with IQ-tree, with 1000 bootstrap replicas[71,72]. Sequences shaded in pink belong to different sub-lineages of the Delta VOC. The sequence from Venezuelan wastewater (October 4, 2021) is shown in red, lineage B. (undefined, although more closely related to the Delta VOC sequences). Other lineages and sub-lineages of different variants were also included: B (ancestral virus, Wuhan), B.1.351, P.1, B.1.1.7, BA.4 and BA.5.1 (Beta, Gamma, Alpha, and two Omicron VOC sub-lineages).

wastewater surveillance can play a crucial role in responding to pandemics, outbreaks, and risk mitigation in general.

In our study, we observed an expected correlation between the concentration of SARS-CoV-2 in wastewater samples and reported COVID-19 cases. This finding aligns with similar studies, such as Maida et al[82]. in Italy, who also detected a correlation between SARS-CoV-2 presence in wastewater and georeferenced cases in areas served by treatment plants during the study period. Additionally, Medema et al[68]. and De Freitas et al[83]. reported a positive and significant correlation between viral load in wastewater and clinical cases.

Even when reported COVID-19 cases were low, we still detected SARS-CoV-2 RNA in wastewater. This detection may be attributed to the positive impact of vaccination campaigns and reduced symptoms (asymptomatic cases) in the population. Wastewater surveillance complements traditional epidemiological methods by identifying asymptomatic or mildly symptomatic cases that might otherwise go unreported but still pose a risk of contagion[69].

Over time (from September 2021 to July 2023), we observed a decrease in positive samples. Vaccination campaigns in Venezuela likely influenced the decline in infected individuals and, consequently, the SARS-CoV-2 concentration in wastewater. In early 2022, the high concentrations found in the samples could be due to the epidemic peak caused by the Omicron variant in the city[70]. Epidemic peaks coincided with reported local outbreaks, possibly influenced by climatic conditions (lower temperatures and frequent rainfall)[84]. Regarding this, the study of Morris et al[85]. presents a model that determines the effect of temperature and relative humidity on the persistence in the environment of enveloped viruses, including SARS-CoV-2, and their results suggest that low temperatures and increased humidity relative, characteristic of mild winters, favor the persistence of viruses in the environment.

Hotspots with high population density and commercial activity in Caracas such as Catia (CAT) and Caricuao (CAR) contributed to a greater number of infections. Genomic characterization of positive samples was limited, with only one almost complete genome likely from the Delta variant. The difficulties in obtaining full complete genome sequences from wastewater have been previously reported by many authors, and alternative strategies have been developed, based for example on the detection of variant specific mutations[86]. The integrity of the genome which may affect small fragment could be also affected by environmental factors such as temperature[87,88].

Our results showed that pH and turbidity could influence the viral concentration detected. Wastewater samples with pH values around 7.1–7.4 had more positive results than samples with higher pH. This is likely due to the impact of pH on viral particles, as prior research found[89]SARS-CoV-2 is inactivated at alkaline pH. This sensitivity to pH could be because most viruses have an isoelectric point (pI) ranging from 3.5 to 7[90]. Then, RNA is not stable in high alkaline conditions[91]. The findings align with Amoah et al[92]., who observed higher viral loads in wastewater with pH 7.1–7.4. These results suggest that moderately acidic conditions favor greater stability of SARS-CoV-2 in wastewater. However, since wastewater is a complex matrix, it contains other elements that could influence the stability of the viral particles in the water, such as sulfates, ammonium, phenols, among others. Consequently, this could affect the detection of SARS-CoV-2 RNA in wastewater. Although the solid fraction of the wastewater analyzed was not quantified, the turbidity value was used as an indicator of the concentration of solids in the wastewater. It was determined that greater turbidity was associated with positive samples. A similar result was reported by Amoah et al[92]., who observed that an increase in total solids corresponds to an increase in SARS-CoV-2 RNA. About it, Forés et al[93]. reported that approximately 23% of SARS-CoV-2 in wastewater was attached to the solid fraction. This could explain how higher turbidity values were associated with a higher

positivity rate. An increasing total solids concentration could be associated with an increasing recovery from SARS-CoV-2.

Study limitations

The application of wastewater-based epidemiology for regular SARS-CoV-2 surveillance still has several methodological challenges that may affect prevalence estimation. Globally, there is a lack of centralized infrastructure for the collection and treatment of wastewater, which makes it difficult to collect and process representative samples of the entire population and represents a limitation for the WBE due to the lack of access to sufficient human excreta for analysis[94]. Additional factors can complicate cross-sample comparisons are the wide ranges in sewer sizes, which affect the amount of time wastewater spends in the sewage system and thus the degradation of the SARS-CoV-2 signal[95].

Indeed, wastewater contains numerous inhibitors whose concentration and diversity are influenced by factors such as population size, surrounding industry and agriculture, and climate[96]. Accuracy is affected by the uncertainty of SARS-CoV-2 RNA persistence in sewers as well as many other variables, and the impact of each step on prevalence estimation is largely unknown[97]. One of the possible causes of variability in our results may have been the use of a single standard curve for several RT-qPCR assays. Further optimization and standardization of experimental protocols is needed to reduce these sources of variation.

Particularly, the present study has several limitations. One of the possible causes of variability in our results may have been the use of a single standard curve for several RT-qPCR assays. Added to this, due to the lack of operational wastewater treatment plants in the city, samples were collected directly from the sewer system, which is combined. This prevents a more in-depth analysis of the viral load in wastewater, such as determining the number of people who deposit their waste at each sampling point. Furthermore, the water supply is not the same at all sampling sites and influences the composition and concentration of the virus in the wastewater. On the other hand, social and economic differences between sampling sites, including the coverage and quality of drinking water service, hygiene practices, and final waste disposal, may contribute to the variability in the detected viral load. In many vulnerable communities, waste is not channeled into the sewage system but is dumped directly into rivers or streams, making it challenging to accurately assess the prevalence of the virus in the population.

Despite methodological limitations, this study suggests that environmental surveillance of SARS-CoV-2 in wastewater in Caracas can be an effective early warning tool for Venezuela. By monitoring the concentration of SARS-CoV-2 in wastewater, public health officials can receive early warnings of potential outbreaks. This information can then be used to implement measures such as contact tracing, isolation, and quarantine to prevent the spread of the virus. Indeed, the experience with wastewater surveillance during COVID-19 has demonstrated that these data are useful for informing public health action and that wastewater surveillance is worthy of further development and continued investment[95].

Challenges and future directions

The pandemic inspired us to quickly implement a novel method of environmental monitoring, therefore, as a future perspective, it is expected that this methodology will continue to be applied not only for COVID-19 but for new emerging and re-emerging infectious diseases. Indeed, a large set of microorganisms of pathogenic nature, including viruses, bacteria and protozoa, have been identified in aqueous matrices, especially in wastewater. Therefore, there is an urgent global need for these pathogens to be tracked, as could be illustrated by the reemerging Monkeypox virus. WBE has proven to be a valuable component of the response to the COVID-19 pandemic and will continue to be a critical data source for public health actions in response to the disease. As at-home testing increases and clinical laboratory tests or official reports decrease, as is currently the case in Venezuela, monitoring wastewater to detect new variants and their spread becomes increasingly important[95]. Continued optimization of protocols will improve the reliability of future studies and mitigate biases and limitations.

Conclusion

The correlation between wastewater SARS-CoV-2 levels and reported COVID-19 cases underscores the immense potential of environmental surveillance as a complementary public health tool, especially in regions with substantial underreporting. This study further exemplifies the valuable insights that can be gleaned from this approach in Venezuela, directly linking wastewater viral concentrations to local epidemiological trends. By applying WBE, health authorities could proactively respond to possible outbreaks, thus implementing timely preventive measures, which contributes to providing effective public health. Continued research and improved methodologies will further enhance the value of wastewater epidemiology in public health surveillance efforts.

Data availability

Sequence data supporting this study's findings have been deposited in the GISAID repository, accession number EPI_ISL_15104826. The dataset used and analyzed during the current study are available in FIGSHARE dataset repository https://doi.org/10.6084/m9.figshare.27130536.v1.

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Author contributions

MBM: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. HRR: Conceptualization, Methodology, Validation, Formal analysis, Data curation, Writing – review & editing, Supervision. FHP: Conceptualization, Methodology, Validation, Formal analysis, Writing – review & editing. MEG: Conceptualization, Data curation, Visualization, Writing – review & editing. RCJ: Investigation, Writing – review & editing. NM: Investigation. MR: Investigation. AZF: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – review & editing, Funding acquisition, Project administration, Supervision.

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