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Wastewater surveillance of SARS-CoV-2 corroborates heightened community infection during the initial peak of COVID-19 in Bexar County, Texas

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ABSTRACT

The purpose of this study was to conduct a preliminary assessment of the levels of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA in wastewater at the Salitrillo Wastewater Treatment Plant in Texas during the initial peak of coronavirus disease 2019 (COVID-19) outbreak. Raw wastewater influent (24 h composite, time-based 1 L samples, $n = 13$) was collected weekly during June–August 2020. We measured SARS-CoV-2 RNA in wastewater by reverse transcription droplet digital PCR using the same N1 and N2 primer sets as employed in COVID-19 clinical testing. Virus RNA copies for positive samples (77%) ranged from 1.4×10^2 to 4.1×10^4 copies per liter of wastewater, and exhibited both increasing and decreasing trends, which corresponded well with the COVID-19 weekly infection rate (N1: $\rho = 0.558$, $P = 0.048$; N2: $\rho = 0.487$, $P = 0.092$). A sharp increase in virus RNA concentrations was observed during July sampling dates, consistent with the highest number of COVID-19 cases reported. This could be attributed to an increase in the spread of COVID-19 infection due to the Fourth of July holiday week gatherings (outdoor gatherings were limited to 100 people during that time). Our data show that wastewater surveillance is an effective tool to determine trends in infectious disease prevalence, and provide complementary information to clinical testing.

Keywords: wastewater-based epidemiology; SARS-CoV-2; COVID-19; droplet digital PCR; Bexar County; Texas

INTRODUCTION

At the initial stage of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) outbreak, several studies demonstrated the presence of viral genetic material in fecal samples of infected individuals (Wang *et al.* 2020; Xu *et al.* 2020). The genetic material of this enveloped RNA virus can travel in sewage pipelines and reach wastewater treatment plants (WWTPs) where wastewater can be sampled and tested for the viral genetic signals (Ahmed *et al.* 2020a; Gonzalez *et al.* 2020; Medema *et al.* 2020). Currently, the traditional approaches to community-wide health assessment are based primarily on clinical testing; however, wider access to testing for SARS-CoV-2 has so far been severely limited due to logistical difficulties and high costs (Leon *et al.* 2020; Lieberman-Cribbin *et al.* 2020). Therefore, aggregate information about disease prevalence within a certain community could be useful for monitoring its spread and the effectiveness of health risk assessments and mitigation

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measures. Together with clinical testing data, additional information could be extrapolated from the viral load in municipal wastewater. This approach is also known as wastewater-based epidemiology (WBE), which has been previously implemented for poliovirus monitoring in wastewater to support global efforts in eradication of poliomyelitis (Deshpande, Shetty and Siddiqui 2003; Asghar *et al.* 2014). Therefore, the rapidly evolving research area of wastewater surveillance has significant potential and high feasibility to serve as an early, cost-effective, community-level indicator of the presence of SARS-CoV-2 (Bivins *et al.* 2020b; Polo *et al.* 2020).

National and international research groups have been collaboratively developing techniques for SARS-CoV-2 wastewater surveillance, from sample collection, analysis, data interpretation, to data communication. Such studies from Australia, the Netherlands, Spain and the United States suggested that WBE could help in rapid identification of an increasing or decreasing trend of SARS-CoV-2 spread (Ahmed *et al.* 2020a; Bivins *et al.* 2020b; Gonzalez *et al.* 2020; Medema *et al.* 2020; Miyani *et al.* 2020; Peccia *et al.* 2020; Randazzo *et al.* 2020). In addition to these pilot studies, continuous monitoring and reporting of SARS-CoV-2 RNA levels in wastewater is being done in various health agencies and wastewater municipalities in the United States and around the world (Gawlik *et al.* 2021; Keshaviah, Hu and Henry 2021; McClary-Gutierrez *et al.* 2021; Weidhaas *et al.* 2021). Wastewater surveillance has the potential to be a sensitive tool to monitor the circulation of the virus in the population since SARS-CoV-2 RNA concentration correlates well with coronavirus disease 2019 (COVID-19) cases reported in a sewershed (D'Aoust *et al.* 2021; Weidhaas *et al.* 2021). Although the use of WBE for tracking COVID-19 has rapidly expanded, the implementation at small communities such as residential areas, rural towns and smaller sewersheds is largely limited. In these communities, clinical testing may also be quite limited due to logistical difficulties and accessibility. Therefore, wastewater-based testing may provide valuable information regarding disease burden in these communities.

COVID-19 arrived in the San Antonio metropolitan area in Bexar County, Texas, in late March 2020; however, the infection started spreading exponentially only in early June, which resulted in extended waiting times from test date to reporting date due to limited testing capacity. In an effort to provide real-time and complementary information to clinical testing data, we documented the levels of SARS-CoV-2 RNA in wastewater, measured using reverse transcription droplet digital PCR (RT-ddPCR), at a local WWTP in Bexar County, Texas, over the course of a 13-week period. We compared the viral RNA concentrations in wastewater with reported testing and infectivity data of patients from clinical testing to better evaluate the efficacy of using the wastewater data for tracking community infection dynamics. Since the testing period included the initial peak, we were able to ascertain the increase in COVID-19 cases as suggested by public testing data through the results of wastewater-based testing. The results presented here may enable the advancement of wastewater-based technologies for public health agencies to initiate effective interventions in disease control, as well as the collection of quantitative epidemiological data for a variety of anthropological investigations.

MATERIALS AND METHODS

Sample collection

Raw wastewater influent samples (24 h composite, time-based 1 L samples) were collected from the Salitrillo WWTP operated by the San Antonio River Authority in Bexar County, Texas. The Salitrillo WWTP services ∼17 000 connections with an average daily flow of 4.859 millions of gallons per day (MGD) and a permitted flow of 5.83 MGD. Bexar County has a population of ∼2 million people and is served by eight WWTPs with total combined wastewater treatment capacity of ∼235 MGD. Thus, the wastewater that comes to Salitrillo WWTP is only ∼2% of the entire county. Samples ($n = 13$) were collected weekly on every Tuesday from 2 June 2020 to 25 August 2020 using an ISCO 3700 autosampler (Teledyne ISCO, Lincoln, NE) at the influent intake point to ensure homogeneous population representation. Samples were stored on ice and delivered to the laboratory at the University of Texas at San Antonio and immediately concentrated upon delivery and frozen at –80°C, followed by molecular analyses within 7 days. The water-quality parameters (pH, temperature [temp], dissolved oxygen [DO], ammonia [NH_3], carbonaceous biological oxygen demand [CBOD] and total suspended solids [TSS]) for the wastewater influent samples ($n = 13$) during the study period are given in Table S1 (Supporting Information). pH was measured on-site using an IntelliCAL PHC101 probe and HQ40d portable multimeter (HACH, Loveland, CO). DO and temperature were measured on-site using an IntelliCAL LDO101 field luminescent/optical probe and HQ40d portable multimeter (HACH, Loveland, CO). NH_3 was measured using Salicylate Method 10205 (HACH TNTplus 832 kit), CBOD was measured using 5-day BOD test (standard method 5210B) and TSS was measured using gravimetric analysis (standard method 2540D) (APHA 2005).

Sample concentration and RNA extraction

Viruses were concentrated from wastewater samples using adsorption-extraction with electronegative membrane as previously described (Ahmed *et al.* 2020a,c). Briefly, wastewater samples were acidified with 2 *N* hydrochloric acid (Sigma-Aldrich, St. Louis, MO) until pH 3–4 was reached, and filtered in duplicate (200 mL each for a total of 400 mL) on a vacuum manifold (Thermo Scientific, Wilmington, DE) through 0.45 μm pore size, 47 mm diameter mixed cellulose ester membranes (GN-6 Metricel Membrane Disc Filter, Pall Laboratory, New York, NY) and immediately stored at –80°C until RNA extraction. Sterile deionized water controls were filtered with each sample event to check for cross-contamination during sample processing. RNA extraction was performed directly from the membrane filters using the RNeasy Power Microbiome Kit (Qiagen, Hilden, Germany) and in combination with automated robot QIAcube Connect (Qiagen, Hilden, Germany) according to the manufacturer's protocol, and the resulting RNA was eluted in 100 μL RNase-free water provided with the kit. Extraction blanks were also processed to test for contamination during the RNA extraction process. A NanoDrop One Spectrophotometer (Thermo Scientific, Wilmington, DE) was used to determine RNA purity and concentration (ng μL^{-1}). All RNA extracts were stored at –80°C and subjected to RT-ddPCR analysis within 7 days of RNA extraction.

SARS-CoV-2 viral RNA quantification

RT-ddPCR was used to measure SARS-CoV-2 RNA copies using the US Centers for Disease Control and Prevention (CDC) N1 and N2 primer/probe sets (Table 1) (Lu *et al.* 2020). The sets of primers and probes (2019-nCoV RUO Kit) were purchased from Integrated DNA Technologies (IDT; Coralville, IA). The RNA extracts were thawed on ice, and then quickly used for RT-ddPCR assays. The RT-ddPCR experiments were conducted on a Bio-Rad QX200 Droplet Digital PCR System (Bio-Rad, Hercules, CA) using the one-step RT-ddPCR advanced kit for probes (Bio-Rad). For each ddPCR well, a final reaction volume of 20 μL was used

Table 1. Primers and probes used in this study.

Assay	Primer/probe	Reference
CDC N1	F: GACCCCAAAATCAGCGAAAT R: TCTGGTTACTGCCAGTTGAATCTG P: FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ-1	Lu *et al.* (2020)
CDC N2	F: TTACAAACATTGGCCGCAAA R: GCGCGACATTCCGAAGAA P: FAM-ACAATTTGCCCCCAGCGCTTCAG-BHQ-1	Lu *et al.* (2020)
Bovine coronavirus	F: CTGGAAGTTGGTGGAGTT R: ATTATCGGCCTAACATACATC P: FAM-CCTTCATAT-ZEN-CTATACACATCAAGTTGTT-IBFQ	Decaro *et al.* (2008)

that comprised 5 μL 1× one-step RT-ddPCR supermix (Bio-Rad), 2 μL reverse transcriptase (Bio-Rad), 1 μL 300 mM DTT, 2 μL primer/probe mix (final concentrations were 670 and 170 nM primers and probes, respectively), 5 μL RNase-free water and 5 μL RNA template. For each assay, three no-template controls (NTCs) were used to check for cross-contamination. To test for PCR inhibition, each RNA extract was run at both undiluted and 10-fold dilution for both N1 and N2 assays. For droplet generation, 20 μL of reaction mixture and 70 μL of QX200 droplet generation oil for probes were transferred to DG8 gaskets and run on QX200 Droplet Generator (Bio-Rad). The resulting oil emulsion was then carefully pipetted to a new ddPCR 96-well plate and PX1 PCR Plate Sealer (Bio-Rad) was then used to foil-seal the plate. ddPCR plate was transferred to the C1000 Touch thermocycler (Bio-Rad) and run using following conditions: 60 min at 50°C for reverse transcription, 10 min at 95°C for enzyme activation, 40 cycles each of 30 s at 94°C for denaturation and 1 min at 55°C for annealing/extension (ramp rate of ∼2°C s^{-1}), and finally 10 min at 98°C for enzyme deactivation (Gonzalez *et al.* 2020). For droplet stabilization purposes, a 15-min 4°C cooldown step was added following the end of thermal cycling. Droplets were read using a QX200 Droplet Reader (Bio-Rad).

The ddPCR data were analyzed using the QuantaSoft Analysis Pro 1.0.596 (Bio-Rad) software and concentrations were calculated by either allowing the program to auto select a threshold or by manual calling when the program was not able to auto threshold. All wells with greater than 8000 accepted droplets were used for absolute quantification, however, majority of the data belonged to wells with greater than 10 000 droplets (Graham *et al.* 2021; McCluskey *et al.* 2021). Average concentrations per reaction were converted to copies per liter of wastewater using dimensional analysis (Table S2, Supporting Information). For samples outside the dynamic range of ddPCR, 10-fold dilutions were made until we could obtain a result within the dynamic range. Each run included extraction controls, positive controls and no-template controls, and 10-fold dilutions of RNA extracts were used to test for PCR inhibition. The positive control was the 2019-nCoV_N_Positive Control containing the complete nucleocapsid gene from SARS-CoV-2 purchased from IDT (Coralville, IA). Limits of detection (LOD) were calculated by running serial dilutions of the positive control in eight replicates distributed over multiple days, and determined as the lowest concentration at which over 60% of the replicates were positive (Gonzalez *et al.* 2020).

RNA recovery efficiency

To determine virus recovery efficiency of our concentration and extraction methodology, an attenuated vaccine strain of bovine coronavirus (Calf-Guard, Zoetis, Parsippany, NJ) was used as a surrogate viral RNA target. Wastewater influent samples were spiked with 10^6 copies of bovine coronavirus (BCoV) per liter of wastewater, directly before sample acidification and filtration. RNA extraction was performed using the same RNeasy Power Microbiome Kit (Qiagen) and RT-ddPCR quantification assay was performed on the bovine coronavirus target gene (see Table 2 for primers and probes). The 20 μL reaction volume was constituted from 5 μL 1× one-step RT-ddPCR supermix (Bio-Rad), 2 μL reverse transcriptase (Bio-Rad), 1 μL 300 mM DTT, 1.2 μL forward and reverse primers and 0.4 μL probe (with final concentrations of 600 and 200 nM, respectively), 4.2 μL RNase-free water and 5 μL RNA. NTC and positive control were included in each run. Quantification of bovine coronavirus was performed in a similar manner to the SARS-CoV-2 targeted assay, with respective reaction mixture constituents and thermal cycling conditions (Decaro *et al.* 2008). The resulting recovery efficiency was calculated using the ratio of bovine coronavirus concentration recovered by concentration seeded. Recovery efficiency was quantified using the last three samples collected in August 2020 and processed in duplicate. Random samples ($n = 3$) without BCoV added were tested to confirm there is no natural BCoV in the wastewater samples.

Data analyses

All statistical analyses and graphs were completed in GraphPad Prism version 9.0.0 (121). The wastewater virus RNA concentrations were reported as copies per L of wastewater. The correlation between SARS-CoV-2 RNA copies per L results for replicated RNA extractions of each weekly sample was analyzed using linear regression. Clinical testing data were obtained from the City of San Antonio's COVID-19 Open Data repository (https://cosacovid-cosagis.hub.arcgis.com/). The daily positive cases include COVID-19 cases reported on each date and the weekly infection rate is the percentage of positive COVID-19 tests in the last 7 days. Correlations between wastewater data and COVID-19 weekly infection rate were assessed via the Spearman correlation coefficient (ρ). The raw RT-ddPCR data have been provided in Table S3 (Supporting Information), and a Minimum Information for Publication of Quantitative Digital PCR Experiments (dMIQE) (Huggett 2020) checklist has been completed in Table S4 (Supporting Information).

RESULTS AND DISCUSSION

Performance of RT-ddPCR assays

The minimum copy number concentration that can be detected reliably was defined as the LOD for each assay. For N1 and

N2 assays, the LOD was estimated to be 31.6, and 19.3 copies/reaction, respectively. The N2 assay was more sensitive than the N1 assay for our molecular workflow. This is consistent with other studies using RT-ddPCR for estimation of SARS-CoV-2 virus RNA copies (Deiana *et al.* 2020; Gonzalez *et al.* 2020). We measured the SARS-CoV-2 RNA concentrations for two technical replicates for each composite sample. Replicated samples exhibited similar SARS-CoV-2 RNA concentrations for both assays. Concentration comparisons between replicates produced correlation coefficient of 0.96 for N1 primers ($P < 0.001$) and 0.91 for N2 primers ($P < 0.001$). PCR inhibition tests resulted in target copies proportional to a 10-fold dilution relative to the undiluted RNA templates, suggesting that PCR inhibition did not interfere with the amplification efficiency. For samples near or below LOD, no detectable SARS-CoV-2 was observed for 10-fold dilutions. Examples of positive and negative results for each assay are listed in Table S5 (Supporting Information). Filtration blanks, RNA extraction controls and no template controls were run to check cross-contamination and the absence of contamination in the ddPCR experiments was confirmed.

Bovine coronavirus was used to assess recovery of our concentration and extraction methodology. The total recovery of surrogate virus for our workflow, which included sample acidification followed by electronegative filtration, was ∼2.4% (±1.3%). Our recovery estimates were consistent with another recent study using BCoV to assess viral recovery from wastewater influent samples; between 0.1% and 7% (Graham *et al.* 2021). However, they used a PEG precipitation method for concentration that is different from the current study using electronegative membrane adsorption. Additionally, Gonzalez *et al.* (2020) used the electronegative filtration workflow, similar to our study, and observed total recovery for BcoV as 4.8% (±2.8%). However, they added $MgCl_2$ to water samples prior to filtration, whereas we did not amend our samples with $MgCl_2$. This may have attributed to low recovery rate in our study.

Higher recoveries have been reported using electronegative filtration (Ahmed *et al.* 2020c), however, the authors used murine hepatitis virus (MHV) and quantitative reverse transcription polymerase chain reaction (RT-qPCR) for their workflow. Several studies improved the recovery efficiency using the electronegative filtration method by physical/chemical pretreatment of the samples. For instance, Juel *et al.* (2021) reported that with the same electronegative filtration method, the BCoV recovery significantly increased with the samples that have additional sonication treatment (from 3.85% to 23.7%). In an effort to compare different concentration methods by using human coronavirus OC43 as recovery target, it was reported that the mean recovery rate ranged from 0.038% to 9.05% across seven methods tested (Philo *et al.* 2021). It should be noted that recovery could be highly varied and depend on multiple factors such as concentration methods, characteristics of wastewater, and RNA extraction and quantification (Ahmed *et al.* 2020c; Jafferali *et al.* 2021; Kantor *et al.* 2021). Several studies have suggested to use both measured concentration data alongside with the recovery rate rather than attempting to correct the concentration for recovery efficiency (Graham *et al.* 2021; Jafferali *et al.* 2021; Kantor *et al.* 2021). Therefore, we did not correct concentrations reported in this study using measured recoveries. Concentration steps used in the current and other studies typically resulted in decreases in virus signal; however, concentration was eventually required to detect the low viral concentrations documented in wastewater samples at the initiation of the study.

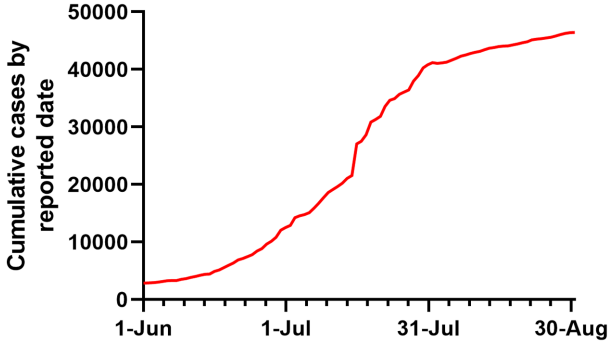

Figure 1. Documented cases of COVID-19 in Bexar County, Texas, for the study period.

COVID-19 cases in Bexar County, Texas

The first confirmed case of COVID-19 in Bexar County, Texas was reported on 12 March 2020, whereupon a Declaration of Public Health Emergency was issued, and by 1 June 2020, the total number of reported positive cases grew to 2839. After that, the cases started increasing rapidly, partly due to increased testing, reaching to around 46 000 cumulative cases by the end of August 2020 (Fig. 1). The peak of reported daily cases for Bexar County was documented in the weeks following the Fourth of July holiday, with highest case count of 5501 reported on 16 July. Our wastewater sampling for this study started on 2 June and ended on 25 August, thereby encompassing the 13-week period that witnessed the first peak of COVID-19 cases within the region.

Measurement of SARS-CoV-2 RNA in wastewater

We measured SARS-CoV-2 RNA in wastewater by RT-ddPCR using the same N1 and N2 primer sets as employed in COVID-19 clinical testing. Over the course of the 13-week period studied, virus RNA copies for positive samples (77%) ranged from 1.4×10^2 to 4.1×10^4 copies per liter of wastewater. These concentrations are consistent with other recent studies in the United States and worldwide (Gonzalez *et al.* 2020; Kumar *et al.* 2020; Peccia *et al.* 2020; Randazzo *et al.* 2020). It should be noted that the wastewater samples in this study were concentrated immediately on the same day of collection. We did not assess the effect of storage conditions such as freezing on the reliability of the SARS-CoV-2 RNA results as it was recently reported that SARS-CoV-2 RNA can remain detectable in wastewater samples stored at –20°C or –75°C (Medema *et al.* 2020; Hokajärvi *et al.* 2021).

We quantitatively compared SARS-CoV-2 RNA concentrations in wastewater with publicly reported data on daily positive cases and positive cases in hospitals (Fig. 2). The virus RNA levels for both the N1 and N2 assays exhibited both decreasing and increasing trends over the course of the study period (Fig. 2A), which corresponded well with the publicly reported data for daily positive cases (Fig. 2B) and weekly infection rate (Fig. 2C). The daily positive cases include COVID-19 cases reported on each date and the weekly infection rate is the percentage of positive COVID-19 tests in the last 7 days. The weekly SARS-CoV-2 wastewater concentrations were positively correlated with the weekly infection rate when wastewater data lagged 1 day, for both N1 ($\rho = 0.558$; $P = 0.048$) and N2 assays ($\rho = 0.487$; $P = 0.092$). In general, the changes in SARS-CoV-2 RNA concentrations in wastewater corroborated with the daily cases by reported date; however, we did not attempt to measure the lag between wastewater data and public testing data since the

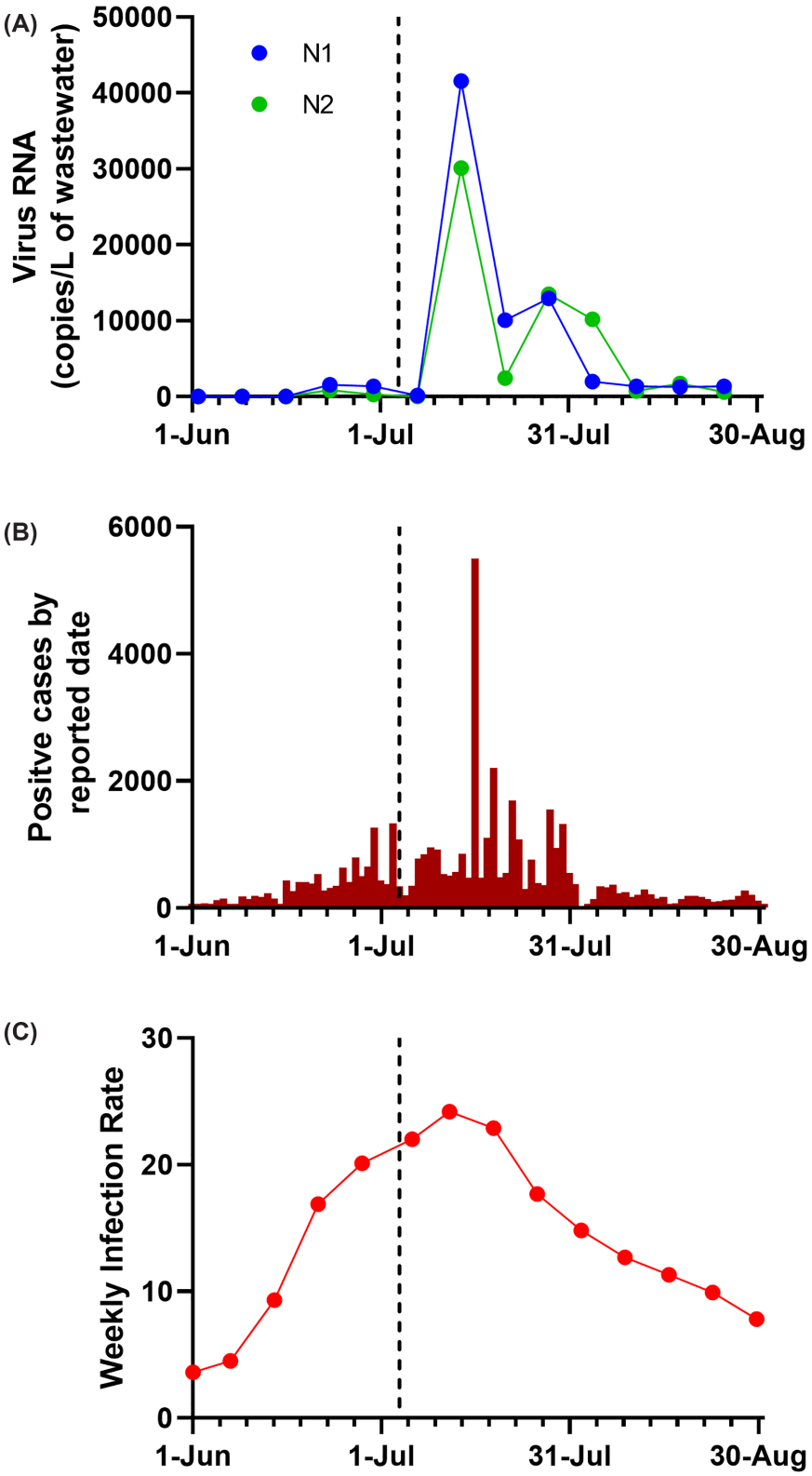

Figure 2. Wastewater SARS-CoV-2 RNA concentration time course and clinical COVID-19 outbreak indicators. **(A)** Wastewater SARS-CoV-2 RNA concentrations (copies L^{-1} of wastewater) for the Salitrillo WWTP in Bexar County, Texas. **(B)** Number of positive COVID-19 daily cases reported for Bexar County, Texas. **(C)** Weekly infection rate of COVID-19 in Bexar County, Texas. The black vertical dashed line indicates 4 July 2020.

wastewater results were documented for weekly samples, while the public test results were reported for daily cases. Wastewater samples collected during the initial dates (June 2020) showed either no detections or low concentrations (<2000 copies L^{-1}). A sharp rise in concentration was observed on 14 July (>30 000 copies L^{-1} for both N1 and N2), after which all samples showed positive detections over the course of the following weeks. Following the peak on 14 July, there was a gradual decrease in the virus RNA concentration for the later sampling dates.

The higher viral loads during the July sampling dates could be attributed to an increase in the spread of COVID-19 infection due to the 4 July holiday week gatherings. The spike in wastewater virus RNA concentrations was consistent with the rising numbers of COVID-19 cases within the community (Selcraig 2020). According to public health experts (Gonzalez 2020; Hayden 2020), large gatherings of families and friends, and travel over the 4th July week helped the spread of coronavirus, although other factors may also have contributed to a higher number of new cases. It should be noted that we observed low virus RNA concentrations on 7 July, right after the 4 July weekend. Since the incubation period of SARS-CoV-2 ranges from 2 to 14 days (Lauer *et al.* 2020), it is highly unlikely that the individuals infected during the 4 July weekend could have shed the virus on or before 7 July. Consequently, the sharp increase documented on 14 July may have resulted from the viral shedding from individuals infected during the 4 July weekend. For clinical data, the highest cases were reported on 16 July (5501 cases; Fig. 2B), while our WBE data showed highest viral RNA levels 3 days earlier. It is noted that we were only sampling once per week, thus the actual peak of WBE data may have occurred before or after 14 July. A weekly monitoring frequency was insufficient to determine the relative changes in SARS-CoV-2 RNA levels in wastewater during peak infection; therefore, future studies might benefit from having a more frequent sampling campaign. Our observation was consistent with previous studies (Medema *et al.* 2020; Peccia *et al.* 2020; D'Aoust *et al.* 2021), where monitoring wastewater influent for SARS-CoV-2 RNA has shown the potential to detect changes in COVID-19 spread in communities, leading individual test results by 2–7 days and hospitalization rates by 1–4 days. After public holidays such as Fourth of July and Thanksgiving where large gatherings may result in spread of infection, immediate wastewater results can provide complementary information on infection dynamics. There are multiple factors that could impact the early warning capability of WBE and the correlation of wastewater viral RNA signal with reported/active case numbers of COVID-19, such as clinical testing rate, characteristics of the sewage system and wastewater sampling frequency. In addition, most studies that have indicated WBE as a leading indicator of COVID-19 spread have reported for a specific region/sewershed; therefore, care should be taken when comparing WBE results among different areas. In our study, we have demonstrated the applicability of WBE for a small sewershed region.

In this study, the wastewater surveillance data for the prevalence of SARS-CoV-2 was documented for a small sewershed region within Bexar County, while the clinical COVID-19 testing data are reported for the entire Bexar County. It was not possible to obtain the clinical testing data at the resolution of the sewershed region due to logistical and ethical issues, and the cases reported could have been related to disease incidence in other parts of the Bexar County. Thus, wastewater-based testing is particularly useful for tracking disease trends in small communities, where public testing cannot currently provide real-time and comprehensive information about the health of a community due to logistical difficulties and high cost. The limitations of public testing are compounded in low-resource settings and disadvantaged populations where testing every individual is impractical, slow and cost prohibitive. Therefore, wastewater-based testing may provide more comprehensive data sets covering whole population and accounting for spatial and temporal variability. Overall, the results of this study suggest that wastewater surveillance of disease biomarkers may provide an alternative and straightforward means of deducing the spread of the disease within the community. We suggest that in epidemiological studies, the use of these methods could provide complementary information.

The advantages of wastewater analysis are that the sample collected is more representative of the population (as the whole population continuously contributes to its wastewater in real time), is collected noninvasively, has low collection costs and is effectively anonymous due to contribution of material from many individuals. Our approach allows 'remote' community-wide monitoring of COVID-19 as well as other infectious diseases by providing a means to assess the levels of specific genetic biomarkers in wastewater (Deshpande, Shetty and Siddiqui 2003; Sims and Kasprzyk-Hordern 2020). Conducting viral wastewater surveillance, however, can be limited by several difficulties such as the stability of the genetic material in wastewater (Ahmed *et al.* 2020b; Bivins *et al.* 2020a), sampling variability in the case of grab against composite sampling methods (Ahmed *et al.* 2020d), variability in concentration methods (Ahmed *et al.* 2020c) and assays with high detection limits that disable sensitive detection in samples with low virus concentrations (Farkas *et al.* 2020; Sims and Kasprzyk-Hordern 2020). Therefore, efforts are needed to develop robust methods that enhance the applicability to concentrate SARS-CoV-2 genetic material. In this study, several SARS-CoV-2 wastewater surveillance limitations were addressed. The methodology included 24-h composite sampling that is more representative of the service area population than the grab method. The viral recovery effectiveness from the concentration method used was assessed using a model bovine coronavirus during the method development. In addition, the quantitative assays used were based on digital PCR (RT-ddPCR) that are shown to provide higher sensitivity and accuracy than RT-qPCR assays when low viral loads are expected (Deiana *et al.* 2020; Liu *et al.* 2020; Suo *et al.* 2020). This facilitated the generation of quantitative data on SARS-CoV-2 prevalence reported out in viral RNA copies per volume of wastewater for the specific treatment service area. Thereby, direct comparison of SARS-CoV-2 genetic trace levels in different time periods was conducted where high measurements are anticipated, as well as a comparison between WBE virus levels and number of reported clinical cases in Bexar County, Texas.

In this study, the wastewater sample collection was conducted on a weekly basis; however, other studies have suggested at least two to three samples per week for a timely intervention (Feng *et al.* 2021). The recovery efficiency of the concentration method used is around 3%, and may vary from sample to sample and requires further evaluation. Overall, further methodological improvements will be required if the intended use of SARS-CoV-2 monitoring in wastewater is to provide early warning system on the presence of COVID-19 in the community.

CONCLUSIONS

Overall, the findings from this study suggest that WBE is an effective tool to determine trends in infectious disease prevalence, and provide complementary data to clinical testing. This

information can be an invaluable resource for authorities to map hot spots of disease spread, assess trends over time/seasons, prompt intervention and correlate the wastewater results with public testing. Moreover, an increase in COVID-19 cases and wastewater SARS-CoV-2 RNA levels after holiday weekends (as demonstrated in this study) may warrant special public health interventions during these occasions.

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SUPPLEMENTARY DATA

Supplementary data are available at *FEMSMC* online.

Conflict of interest. None declared.

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