Quantitative Reverse Transcription PCR Surveillance of SARS-CoV-2 Variants of Concern in Wastewater of Two Counties in Texas, United States

Laura M. Langan,* Megan O’Brien, Lea M. Lovin, Kendall R. Scarlett, Haley Davis, Abigail N. Henke, Sarah E. Seidel, Natalie Archer, Eric Lawrence, R. Sean Norman, Heidi K. Bojes, and Bryan W. Brooks

ABSTRACT: After its emergence in late November/December 2019, the severe acute respiratory syndrome coronavirus 2 virus (SARS-CoV-2) rapidly spread globally. Recognizing that this virus is shed in feces of individuals and that viral RNA is detectable in wastewater, testing for SARS-CoV-2 in sewage collections systems has allowed for the monitoring of a community’s viral burden. Over a 9 month period, the influents of two regional wastewater treatment facilities were concurrently examined for wild-type SARS-CoV-2 along with variants B.1.1.7 and B.1.617.2 incorporated as they emerged. Epidemiological data including new confirmed COVID-19 cases and associated hospitalizations and fatalities were tabulated within each location. RNA from SARS-CoV-2 was detectable in 100% of the wastewater samples, while variant detection was more variable. Quantitative reverse transcription PCR (RT-qPCR) results align with clinical trends for COVID-19 cases, and increases in COVID-19 cases were positively related with increases in SARS-CoV-2 RNA load in wastewater, although the strength of this relationship was location specific. Our observations demonstrate that clinical and wastewater surveillance of SARS-CoV-2 wild type and constantly emerging variants of concern can be combined using RT-qPCR to characterize population infection dynamics. This may provide an early warning for at-risk communities and increases in COVID-19 related hospitalizations.

KEYWORDS: Wastewater, WBE, COVID-19, VOCs, SARS-CoV-2, USA

1. INTRODUCTION

The use of wastewater-based epidemiology (WBE) for monitoring SARS-CoV-2 has been used globally as an invaluable tool to obtain information about trends in community spread at the population level in near real time, avoiding intrinsic delays and inconsistencies associated with individual clinical testing. As wastewater represents a composite of human wastewater from a specific catchment area, metagenomic sequencing of these samples may allow for the detection of viral variants and pathogen diversity across larger populations and regions without further burdening healthcare workers or facilities already at capacity. Although SARS-CoV-2 sequencing activities have expanded considerably as the pandemic has evolved at a global scale, sequencing capability varies markedly within and among countries. Such variable capacity in sequencing can impede surveillance of this constantly mutating virus. In the United States, this is particularly striking where SARS-CoV-2 sequencing for variant surveillance has been ranked 33rd (April 2021), and 35th (of 200 countries reporting sequencing data) as of December 2021 according to GISAID’s EpiCoV database (https://www.gisaid.org). In North America alone, the United States ranks fifth, sequencing approximately 4% of reported COVID-19 cases, in contrast to Canada where 10% of COVID-19 cases are sequenced; these differences have been attributed to multiple ongoing factors. To monitor the progression of the COVID-19 pandemic, modeling suggests that sequencing at least 5% of specimens that test positive for SARS-CoV-2 in a geographic region is necessary to reliably detect the emergence of novel strains or mutations at a prevalence level of 0.1−1%. In the United States, only 33% of states are hitting this threshold, with Texas currently sequencing an average of 3% of tested samples (accessed December 15, 2021). Though genomic surveillance remains a critically important tool for pandemic response, many laboratories do not have the resources (trained personnel, equipment, consumables, etc.) to support population level studies. However, it may be possible to...
supplement SARS-CoV-2 genomic surveillance with PCR-based variant detection, particularly in areas with limited sequencing capacity. Prior studies in the Netherlands have demonstrated that increases in the delta variant detected by reverse transcription (RT) PCR correlated well with data from genomic surveillance at the individual clinical level, with actionable data available approximately 2 weeks earlier.6

Given the substantial capacity building over the past two years for SARS-CoV-2 wastewater surveillance, we hypothesized that we could detect viral variants in wastewater samples, as has previously been accomplished with SARS-CoV-2 in other areas. In 2021, the list of variants of concern (VOCs) designated by the WHO included alpha (B.1.1.7), beta (B.1.351), gamma (P.1), and delta (B.1.617.2). In the present study, we examined the alpha and delta variants, and we identified that trends (increasing and decreasing) in wastewater data from treatment plant influents in two midsize Texas cities can be used to assess variant spread within the community. We further demonstrated the capacity of this technique at the community level to predict increases in case reports and/or hospitalizations. With the continuing emergence of variants, this approach can support public health decisions by enabling population-based triaging of samples to centers performing sequencing analysis, allowing for a more directed sequencing strategy.

2. MATERIALS AND METHODS

2.1. Sample Collection. One 24 h composite sample of wastewater influent was collected weekly from each of two publicly owned wastewater treatment plants (WWTPs) located in Waco and Denton, TX, USA. These regional WWTPs service multiple cities in McLennan and Denton counties, respectively. From both plants, influent was collected at the headworks by using 24 h time-weighted composite sampling (Teledyne ISCO, Lincoln, NE, USA) with a sampling interval of 15 min. The resulting solution was homogenized by hand and decanted into two (1 L) new high-density polyethylene (HDPE) bottles, or bottles washed in 10% hydrochloric acid (HCl) as per prior studies.8 The date assigned to the 24 h composite was the date when wastewater collection was completed. Samples from both locations were immediately stored at 4 °C and transported to the laboratory for processing. Weekly influent samples were collected at both locations (differing weekdays) over 37 weeks from March 11 to November 18, 2021, with some exceptions. Weekly samples were not collected three times for McLennan and seven times for Denton. Following transfer to the laboratory, 3.15 mL of water concentration buffer (Zymo Research, Irvine, CA, USA; R2042) was added to 45 mL of wastewater, incubated at room temperature for 15 min, and centrifuged at 5000 g for 15 min to concentrate viral particulates and solids. Thereafter, supernatant (~47 mL) was carefully removed and the remaining ~1.15 mL of concentrated stock was aliquoted (200 μL/vial) and immediately stored at ~80 °C.

2.2. Analytical Methods. Concentrated samples were processed and analyzed as described by Langan et al.7 [method 4 + Monarch Total RNA miniprep + Zymo OneStep PCR inhibitor removal columns]. One-step quantitative reverse transcription PCR (RT-qPCR) was performed with the Luna OneStep 4X protocol (New England Biolabs, Ipswich, MA, USA). Additional experimental details, including Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) reporting, are in the Supporting Information.

2.3. Case Count, Fatality, and ICU Hospitalization Data. Daily COVID-19 case count, fatality, and intensive care unit (ICU) hospitalization data were compiled by the Texas Department of State Health Services (DSHS) for both McLennan and Denton counties. In order to compare the SARS-CoV-2 signal in wastewater to the location-specific clinical picture, we used these counties’ daily COVID-19 case, fatality, and ICU census counts reported by Texas DSHS for the dates March 11, 2021–November 18, 2021. “Cases” were defined as the daily number of new COVID-19 cases confirmed with PCR reported to Texas DSHS by the counties’ health departments, and “ICU hospitalizations” were the daily number of hospitalized individuals in each county currently in the ICU who were confirmed to have an active COVID-19 infection, regardless of their reason for hospitalization. “Fatalities” are deaths for which COVID-19 is listed as a direct cause of death on the death certificate and do not include deaths of people who had COVID-19 but died of an unrelated cause. Fatalities are reported by the county in which the person lived as listed on the death certificate.

2.4. Data Analysis. While wastewater data has typically been reported as normalized to pepper mild mottle virus (PMMoV), recent studies have reported reductions in relationships between SARS-CoV-2 concentrations and COVID-19 incidences.5,9 As such, this study examined COVID-19 incidence, hospitalizations, and associated fatalities with wastewater signal with [denoted by “/PMMoV”] and without normalization to PMMoV. Unless otherwise stated, values of SARS-CoV-2 viral load in wastewater (genomic copies per liter; gc/L) are graphed with a log-10 scale on the y-axis.

Trend analysis consists of individual variant targets for B.1.167 (E484Q and P681R) and B.1.1.7 (HV69-70, A570, and Y144) compared to hospitalization data, as well as a geometric mean for all variant-specific (B.1.1.7 and B.1.617.2) targets against new case counts, hospitalizations, and fatalities, as has been reported previously when numerous wild-type targets are quantified simultaneously.10 B.1.1.7 was determined to be present in wastewater samples if at least two of the three variant sequences were detected. Whereas studies have reported stronger correlations with cumulative confirmed COVID-19 cases in a sewershed ($R^2 = 0.81$),11 for consistency with epidemiological data reporting, correlation and regression analyses between wild type (normalized and not-normalized) and variant genomic copies per liter (gc/L) were performed to examine whether statistically significant relationships existed between hospitalization and reported fatalities for each location individually with newly reported and 7, 14, and 21 day “floating” or rolling averages. As rolling averages are a way to reduce noise and smooth time series data, this approach has been used throughout the pandemic to understand and visualize case counts and deaths, which is especially important because new cases are typically not reported over weekends. Due to limited detection of B.1.1.7 (sample size < 10), correlation between parameters were calculated using Kendall’s $r_k$ coefficients, which is recommended for small research studies with $n < 10$.12 Further, as sample observations for both wild type and the delta variant are greater than 30, the central limit theorem applies whereby the sample mean approximates a normal distribution.13,14

https://doi.org/10.1021/acsestwater.2c00103
ACS ES&T Water XXXX, XXX, XXX--XXX
Statistical analyses and graphing were carried out with R (version 4.1.2 “Bird Hippie”) via the RStudio interface. Relationships between positive new cases and wastewater data for each location individually were calculated by using Pearson correlation and linear regression analyses ($\alpha < 0.05$). Calculations of rolling averages were carried out by using the “zoo” package in R, with default settings, with figures produced by using “ggplot2” packages.

3. RESULTS AND DISCUSSION

3.1. Viral RNA Detection Frequency and Relationship with COVID-19 Case Counts. 3.1.1. Wild-Type Form of SARS-CoV-2. Globally, communities are now using wastewater surveillance of SARS-CoV-2 to monitor trends at the community level, which supplements data gaps caused by limited diagnostic testing capacity in addition to asymptomatic infections. The economic value, in addition to the public health benefit, of wastewater-based epidemiology for disease monitoring is significant. Applying this approach, SARS-CoV-2 RNA was detectable in influents of the two wastewater facilities for 100% of the 65 unique influent samples, including duplicates sampled randomly throughout the process over a 9 month study period. The amount of bovine coronavirus (BCoV) was calculated as previously outlined with a recovery range of 10−12% identified, which was in agreement with prior research, although this is also higher than for BCoV run in DI water (Table S5). This result may be attributed to three factors, including osmotic pressure differences between BCoV spiked with DI water and the more complex wastewater sample matrix, calculation of recovery, and challenges in measuring the recovery of SARS-CoV-2 from wastewater, with limited standardization in the literature. It should be noted that BCoV has not been identified as an optimal surrogate for SARS-CoV-2, with obvious differences in behavior observed. Further work is ongoing to use digital droplet PCR to quantify the absolute number of BCoV to validate recovery estimates as this method facilitates accurate and precise quantification of nucleic acid targets without the need for calibration curves and any external standard.

Viral loads in wastewater were calculated with the N1 target assay, which was chosen based on preliminary comparisons with four other targets (N2, E-sarbeco, ORF1ab, RdRP), demonstrating the best overall sensitivity for wastewater, in agreement with prior studies. When viral titers (expressed as viral genomic copies per liter, gc/L) quantified at the two wastewater treatment plants were compared to epidemiological data (clinical COVID-19 positive cases), visible agreement can be seen between the viral signal in wastewater and reported new case counts (rolling 7 day averages). Specifically, increases in wastewater viral load precede increases in clinical cases by approximately 10 days, although this is variable and appears contingent on the dominant variant at the time of sampling.

Figure 1. Temporal analysis of 7 day rolling average new reported COVID-19 cases against SARS-CoV-2 signal in wastewater treatment plants for Denton County (A) and McLennan County (B) using the N1 target. Wastewater data (viz., SARS-CoV-2 (gc/L)) is graphed on a log scale. Increases in viral loads in wastewater precede increases in clinical cases by approximately 10 days, although this is variable and appears contingent on the dominant variant at the time of sampling.
these lead indicator trends can be extended to individual newly identified variants. With the use of the results of viral detection in wastewater using the N1 target against the reported new positive cases, significant ($p < 0.05$) relationships were observed at all examined time lags with $R$ ranging from 0.64 to 0.79 for McLennan County and from 0.46 to 0.55 for Denton County. The strongest significant relationship was observed using the 21 day rolling average of reported new COVID-19 cases for both Denton County ($R = 0.55, R^2 = 0.3$) and McLennan County ($R = 0.79, R^2 = 0.62$) (Figure S1). These relationships are similar to those previously reported for other locations in the United States ($R^2$ ranging from 0.2 to 0.4), although variability among sampled communities is expected and requires rigorous validation in order to identify contributing factors. Normalization with PMMoV did not strengthen observed relationships ($R^2$ ranging from $-0.06$ to 0.06, $p > 0.05$) compared to the unnormalized signal. Indeed, studies have previously reported that data normalization by an endogenous control agrees with the raw concentration data, minimizing the utility of this normalization.

### 3.1.2. Alpha [B.1.1.7] Variant.

The first COVID-19 wave in 2020 was readily recognized using many targets on a global scale, and specifically N1 and N2 targets in North America. The variant B.1.1.7 (alpha) was first identified in the United Kingdom (U.K.) in autumn 2020 and quickly spread to more than 110 countries. The first identified clinical case of B.1.1.7 circulating in Texas was identified in Houston in mid-January 2021, by using genomic sequencing. With the use of clinical sequencing, B.1.1.7 was identified for the first time on February 3, 2021, and March 25, 2021, in Denton and McLennan counties, respectively; however, it was not detected in wastewater until early March (Figure 2A,B). Currently, the genomic surveillance program of the Centers for Disease Control and Prevention (CDC) assists in tracking population variants throughout the United States via contracting with private and public laboratories to sequence randomly selected clinical samples. Variant proportions are calculated with a weighted design using reported testing data. B.1.1.7 became the predominant variant in Texas (>50% prevalence) in mid-March, with the proportional total number of sequences of B.1.1.7 in Texas peaking in mid-April 2021. As noted above, while genomic surveillance is an important tool for pandemic response, with the application of new portable sequencing technologies showing some promise for variant tracking, many laboratories around the world do not have the resources (trained personnel, equipment, consumables etc.) to support population level studies. However, it may be possible to supplement SARS-CoV-2 genomic surveillance with PCR-based variant detection, while sequencing capacity is

![Figure 2](https://doi.org/10.1021/acsestwater.2c00103)
increasing. Detection of SARS-CoV-2 in wastewater required novel primers that amplify this virus sensitively and specifically, given low viral titers present in wastewater. Developing these primers does take time; however, new tools are emerging which will facilitate the more rapid development of targets to monitor existing and new variants in wastewater, and companies have started speedily producing standards specific to variants.

In early January 2021, a protocol was shared online to differentiate the B.1.1.7 lineage from other forms using nasopharyngeal swab samples; however, new tools are emerging which will facilitate the more rapid development of targets to monitor existing and new variants in wastewater, and companies have started speedily producing standards specific to variants.

In early January 2021, a protocol was shared online to differentiate the B.1.1.7 lineage from other forms using nasopharyngeal swab samples; however, new tools are emerging which will facilitate the more rapid development of targets to monitor existing and new variants in wastewater, and companies have started speedily producing standards specific to variants.

In early January 2021, a protocol was shared online to differentiate the B.1.1.7 lineage from other forms using nasopharyngeal swab samples; however, new tools are emerging which will facilitate the more rapid development of targets to monitor existing and new variants in wastewater, and companies have started speedily producing standards specific to variants.

Figure 3. Temporal analysis of 7 day rolling average new reported COVID-19 cases against SARS-CoV-2 signal in wastewater for the B.1.617.2 variant treatment plants for Denton County (A) and McLennan County (B) using targets for the P681R and E484Q. B.1.617.2 was defined as present if one out of the two tested targets was detected. Wastewater data (viz., SARS-CoV-2 [B.1.617.2 variant] (gc/L)) is graphed on a log scale. The overlap in the detection of variant fragments, specifically alpha (B.1.1.7) and delta (B.1.617.2) in wastewater, is outlined for Denton County (C) and McLennan County (D). While viral load in wastewater is typically graphed using a log scale, the overlap in variants is based on the raw genomic copies per liter (gc/L) due to the large degree of difference between observed detectable fragment levels.

The presence of a strong oxidant in the form of chlorine bleach in samples has previously been reported as increasing the probability of degradation of SARS-CoV-2 in water, though the level of such degradation cannot be determined. Further, a recent meta-analysis identified nine explanatory variables that could explain up to 50% of the observed variation including water temperature, air temperature, population size, water usage pattern, precipitation/rainfall, and sampling technique. Temperature is the main driver of microbial decay and can significantly alter the persistence of viruses in wastewater, with the storage of wastewater at lower temperatures (<4 °C) increasing the persistence of coronavirus in wastewater, although 8% decay per day is still reported using a spike in approach, and similar results have been reported in wastewater generally. Further, high temperatures (24–55 °C) have been shown to rapidly inactivate or decrease the environmental stability of SARS-CoV-2, therefore, higher water temperatures could increase decay rates of SARS-CoV-2 in wastewater. A cursory examination shows a large distribution in water temperature in the sampled locations in 2021, whereby temperatures typically range from 64 to 81 °F and from 55 to 81 °F (April–July) for McLennan County and samples, in both sampled locations (68–99 days), and the characteristics of this variant in terms of increased transmissibility of 43–90% and increased mortality compared to the original SARS-CoV-2 strain, it seems likely that other factors may be contributing to the observed disconnect.
Denton County, respectively. And while temperatures are typically less variable prior to April, there was an exception in mid-February when winter storm Uri brought a significant and prolonged severe cold wave to the United States, causing large blackouts, with 4−6 in. of snow in both sampling locations. The absence of B.1.1.7 signal in wastewater samples from these two locations prior to May could be related to water temperatures, air temperatures, and snow that collectively minimized social interactions. Given the comparative newness of monitoring for SARS-CoV-2 in wastewater, knowledge concerning the influence of weather extremes on viral signal is still limited, with research ongoing. It is also possible that this variant decreased concurrently with an initial increase in vaccinations in both locations and public health interventions such as stay-at-home orders for infected individuals. Vaccinations have been reported as reducing transmissions of wild type but also the alpha variant and thereby reducing the number of new SARS-CoV-2 infections.40 Prior studies have assessed the age specificity of infection fatality rates of COVID-19 (wild type), noting that infection fatality rates increase progressively with age,41 although this study did not account for the impact of variants. Children are reported as less affected by SARS-CoV-2 infections even with the spread of the alpha variant,42 with alpha reported to increase fatality and account for the impact of variants.43–45 and it is possible that this is also the case in the wastewater assayed had insufficient sensitivity over a 2 month period,47–49 consistent with previous reports of the detection of SARS-CoV-2. Less is known about how these suggested modifications may influence assessment of variants in wastewater and, further, if differences exist between digital droplet RT-PCR and RT-qPCR.

3.1.3. Delta [B.1.617.2] Variant. The third COVID-19 wave, categorized by the dominance of the B.1.617.2 variant (delta), was first identified in India in late 2020 and then labeled a variant of interest (VOI) in April 2021 and a VOC on May 11, 2021.28 However, studies in India have suggested that this was the dominant strain in some cities prior to May 2021.47 The B.1.617.2 variant, and its sublineages, is of particular concern for public health and the health care system due to its increased transmissibility compared to B.1.1.748 and increased resistance to neutralization by antibodies from plasma and serum.49 Symptomatic and asymptomatic individuals with this variant are associated with more onward transmissions than similar individuals infected with the alpha variant50 and, further, have a higher risk of hospitalization, need for intensive care unit admission, and mortality,51 although other factors also contribute to this such as comorbidities, age, and vaccination status. In Scotland, the risk of hospital admission with delta doubled compared to the alpha variant, with admission risk increased particularly in those with five or more relevant comorbidities.52 Likewise in Canada increases in hospitalizations (80−138%), ICU admissions (16−331%), and death (47−230%) were associated with the delta variant compared to other variants.53 While the delta variant was first reported in McLennan County on July 16, 2021, there is limited information on when it was first detected in Denton County. Delta was defined for the current study as target signal from the P681R and E484Q targets, as suggested by prior studies.54 It was first detected in Denton County influent wastewater as early as July 1, with viral fragments also detected in McLennan County influent on July 9, consistent with previous reports of the detection of SARS-CoV-2 fragments in wastewater days before first clinical confirmation. With the use of these targets, it is likely that increases in viral loads in wastewater precede increases in clinical cases by approximately 10 days; this pattern was visible for both sampling locations (Figure 3A,B). For Denton County, there was overlap in the B.1.1.7 and B.1.617.2 viral detections in wastewater, which was also observed for McLennan County but to a lesser degree (Figure 3C,D). Relationships between new case counts (0 lag, 7, 14, and 21
day rolling averages) and viral load quantified specific to the B.1.617.2 targets (geometric mean of genomic copies per liter for each paired target) were significant ($p < 0.05$) with $R$ values ranging from 0.51 to 66 for Denton County and $R^2$ ranging from 0.26 to 0.43 (Figure S1). Similar relationships were observed for McLennan County (Figure S2), with the strongest and most significant relationship observed with the 7 day rolling average of reported COVID-19 positive cases ($R^2 = 0.42$) and little difference between sampling locations ($R = 0.66$ and 0.65 for Denton and McLennan, respectively). Similar ranges of correlations have been reported previously with wild-type targets for SARS-CoV-2 following mass vaccination at a college campus and also at wastewater treatment plants and hospitals. These observations contrasted sharply with the B.1.1.7 variant detections. Viral loads in wastewater peaked and decreased prior to clinical positive detects, possibly due to protection from vaccines, providing an extra layer of information on COVID-19 infection dynamics and subsequently providing an early warning for increasing hospitalizations. From late July 2021, B.1.617.2 represented over 92% of all samples tested, although at this time new case counts were decreasing markedly in both locations.

To date, there are limited studies that apply targeted assays for the detection of variants of interest/concern across different wastewater samples or phases of the pandemic, which currently limits the ability of this method to assist in identifying emerging patterns. Studies that are available primarily relied on genomic surveillance, making comparisons to the less sensitive RT-qPCR method difficult. Johnson et al. tracked the geographic distribution of VOCs alpha, beta, and delta in the Western Cape of South Africa, observing the decreasing prevalence of alpha as delta became the dominant circulating variant, which is in agreement with results observed in the current study. Yaniv et al. also developed RT-qPCR assays for VOCs including delta, noting that wastewater detection can precede clinical COVID-19 cases reported by 2–3 weeks, a finding we also observed here. Furthermore, a recent article has outlined a digital RT-qPCR assay for mutation characteristics of several VOCs for use with settled solid samples, applying them to 35–156 wastewater samples as proof of concept and observing delta to increase over the month of June until it was at concentrations similar to that of the N-gene, which was also observed in the current study.

It is important to highlight that the targets we have used for the detection of B.1.617.2 variant in wastewater are not ones used in the prior outlined studies. While the P681R mutation in the S protein of the B.1.617 lineage is unique and newly identified in this variant of concern, there are numerous lineages associated with this strain, with phylogenetic analysis confirming the spread of both B.1.617.1 (kappa) and B.1.617.2 (delta) in India in early 2021, before delta became the dominant strain worldwide. Numerous targets, in addition to the P681R mutation, have been used to separate out the two lineages, including G142D and T478K, which have also been used to screen patients with RT-qPCR. The mutations L452R and E484Q within the receptor binding domain were specific to lineage B.1.617.1 (kappa) and B.1.617.3, while L452R and T478K were specific to the lineage B.1.617.2. Although this mutation has also now been found in the B.1.617.1 (kappa) B.1.617.2 (delta), and B.1.427/B.1.429 (epsilon) variants. In the present study, we used both targets (E484Q and P681R) initially to verify the presence of the B.1.617 lineage and later to differentiate between the kappa and delta variants when both variants were spreading, though a recent review reported that B.1.617.2 does not have the E484Q mutation. Thus, the continued identification of E484Q fragments in wastewater samples during the sampling period for the B.1.617.2 (delta) was particularly surprising, especially considering that delta was represented as the dominant circulating strain in Texas from late June (>50%). While it is possible that the target sequence proposed amplifies something else, we hypothesize that the continuing presence of the E484Q target in wastewater samples reflects the presence of a small cluster or clade of B.1.617.2 with the E484Q mutation. This has been observed frequently during the pandemic with studies demonstrating the emergence of mutations that may be endemic to specific areas but with limited transmissibility, or the convergent evolution of certain lineages. This hypothesis is partially supported by the presence of several mutation sequences in GISAID (https://www.gisaid.org/) associated with delta, including the submission of a mutation denoted as denoted as "B.1.617.2 + E484Q" or “B.1.617.2 lineage with S:E484Q”, with the earliest identification of this strain noted in Denmark. Genomic surveillance of these samples would be necessary to examine if this particular strain does relate to a mutation of B.1.617.2 or if the presence of E484Q is representative of other variants.

The U.K. (39%) and the United States (23%) have accounted for the majority of the published genomic sequences currently available, yet these represent an extremely small proportion of samples in comparison to the total number of RT-qPCR tests undertaken, both clinically and specifically using wastewater. Despite limited coverage (although this is improving) in the United States, and more so elsewhere, the E484Q mutation has a cumulative prevalence of <0.5% in the United States (55 sequences submitted) compared to three confirmed submissions in Texas (out of 155,085 submissions) as of late 2021, although this could be a significant underestimation given current coverage levels. Even in areas with excellent clinical surveillance systems, socioeconomic barriers and hesitancy to test make it difficult for epidemiologists and local public health agencies to maintain a clear picture of infection within communities. Complementary approaches that avoid this bias are therefore highly desirable. While the current contribution represents the first report of this phenomenon by RT-qPCR, it will be interesting to determine whether this trend is supported by genomic sequencing of these samples, which is currently underway, and if it can be extended further to identify other mutations that may be present and more feasible to implement than sequencing due to various constraints.

3.2. Relationships between Wastewater Concentrations and ICU Hospitalizations. The SARS-CoV-2 RNA load in raw wastewater has been reported as a leading indicator of COVID-19 positive cases, new hospitalizations, and ICU admissions by 5, 8, and 9 days, respectively. The strong and significant relationships between the wild-type form of SARS-CoV-2, detected using the N1 target, and reported new positive cases would support this observation (Figures S1 and S2), although the degree of difference between the two sampling locations is somewhat surprising. In contrast, the relationship between the two study locations in the present study for the delta variant was very similar irrespective of location. When wastewater data (wild type and variants) was extended to examine relationships with patients who have...
tested positive for COVID-19 and are being treated in ICUs, viral loads recorded for the raw N1 target demonstrated variable but significant \((p < 0.05)\) relationships depending on location, with a mean correlation coefficient \((R)\) range of 0.6−0.61 and \(R^2\) of 0.36 identified for Denton County (Figure S3A,D,G,J) compared to an \(R\) range of 0.74−0.76 and \(R^2\) of 0.58 for McLennan County (Figure S4A,D,G,J). Similar strong relationships between ICU patients and the N1 viral load in wastewater in Denton County were identified using the daily reported number and the 7 day rolling average of adult only ICU patients \((R = 0.61)\), compared to those in McLennan County which had the strongest relationship identified by using the 14 day rolling average of adult-only ICU patients \((R = 0.76, R^2 = 0.58)\). As with new case counts, no significant relationship was observed between these variables for the B.1.1.7 variant, although significant relationships varied depending on location (Figures S3B,E,H,K and S4B,E,H,K). As observed for the new case counts, the targets specifically for B.1.617.2 were also significantly \((p < 0.05)\) related to numbers of ICU patients with COVID-19 for both study locations. For Denton County (Figure S3C,F,I,L), the strongest relationship between the number of ICU patients and the B.1.617.2 viral load in wastewater was identified by using the 7 day rolling average number of adult-only ICU patients \((R = 0.49, R^2 = 0.19)\) compared to McLennan County (Figure S4C,F,I,L), which had the strongest relationship identified by using the 14 day rolling average of adult-only ICU patients \((R = 0.54, R^2 = 0.29)\). When wastewater data (wild type and variants) was extended to reported COVID-19 associated fatalities, the viral load recorded for the raw N1 target displayed significant \((p < 0.001)\) relationships with reported COVID-19 fatalities, but only for the McLennan County study location (Figure 4, for Denton County, see Figure S5), with the strongest relationship observed with the 21 day rolling average of fatalities \((R = 0.74, R^2 = 0.55)\).

SARS-CoV-2 follows a highly variable disease course, dependent on multiple factors, and it is becoming more evident that an individual’s immune system has a decisive influence on the progression of the disease, with detailed underlying molecular mechanisms of the SARS-CoV-2 mediated disease pathogenesis still largely unknown. Patients hospitalized with SARS-CoV-2 infections are major contributors not only to the rate of disease in a population but also to mortality in health care settings. Wastewater viral signal changes have been shown to predate changes in case burden in hospital settings as well as in other types of facilities and buildings. Notably, in the current study, vaccination rates vary between the two counties. As of November 18, 2021, Denton County had an overall higher vaccination rate with approximately 55% of the vaccine eligible population fully vaccinated compared with 46% in McLennan County. Wastewater samples collected from highly immunized communities have showed sharp increases in SARS-CoV-2 N-gene wastewater concentrations 2−3 weeks prior to an increase in active cases, which was also observed in Denton wastewater samples in this study (Figure 1A; 3 week lead) and

![Image](https://doi.org/10.1021/acs.estwater.2c00103)
generally similar to McLennan County samples (approximately 2 weeks). It is possible that differences in vaccination status may be one factor which may have contributed to the fewer significant relationships observed for Denton County (regardless of target) for new case counts, COVID-19 positive patients in ICU, and COVID-19 associated fatalities.

Other factors could have contributed to the reported observations (viz., viral loads in wastewater and COVID metrics such as new cases, ICU hospitalizations and mortality) in the present study. For example, we observed higher viral loads in McLennan County compared to Denton County. In recent research, Chan et al. investigated correlations with confounders such as state, population density, date of first documented COVID-19 case, social vulnerability, and percentage vaccinated, with the results suggesting linkages between extreme weather events, urbanization, and COVID-19. Differences in poverty rates, social vulnerability (which uses 15 social factors examined under socio-economic status, household composition and disability, minority status and language, and housing type and transportation access), vaccination rates, and access to hospitals may play a role in the observed differences in the relationship between the two sampled communities. Demographics for Denton County and McLennan County have reported poverty rates of 7 and 17%, respectively (https://www.census.gov). Social vulnerability for both sampling locations was examined using the CDC’s 2018 Social Vulnerability Index (SVI), as outlined in ref 71. The social vulnerability of each of the sampled locations was retrieved (https://svi.cdc.gov/map.html), with Denton County categorized as having a lower level of vulnerability (0.221) in contrast to McLennan County, which is categorized as having a higher level of vulnerability (0.844). Future wastewater based epidemiology investigations would benefit from an examination of demographics and social determinants of health within the contributing populations.

Estimates of infection based on COVID-19 case rates from testing individuals have been the standard for applying community interventions aimed at decreasing morbidity and mortality. However, studies are increasingly demonstrating the relevance of the incorporation (via modeling) of wastewater with epidemiological data to estimate COVID-19 case rates across communities, sometimes served by multiple different wastewater facilities. Yet uncertainties are still abundant. While it is possible to observe relationships between wastewater surveillance and clinical information, to estimate effective reproductive numbers, and even to analyze lag distributions, accounting for the variability in both the wastewater and population demographics requires further investigation.

For example, varying amounts of fecal RNA are shed depending on the person and the time since infection, both of which make it difficult to quantify individual numbers based off population data alone. Furthermore, while wastewater-based epidemiology has consistently fulfilled its initial promise as an approach for estimating population-wide COVID-19 prevalence, its relevance is somewhat limited due to the high degree of the global population not connected to the public sewerage network. Strong relationships between epidemiological data and wastewater viral loads could be expected in places where almost all of the population is connected to wastewater treatment, such as Austria, Denmark, Finland, Latvia, the Netherlands, or Norway as of 2019, compared to other regions where wastewater infrastructure is lacking or not present.

Observations in the present study present a relatively low cost, rapid, and independent approach to inform SARS-CoV-2 monitoring and public health interventions during the ongoing pandemic and potentially for other pathogens, which may be particularly important for communities that are vulnerable. The current study demonstrates that clinical and wastewater surveillance data can be combined to develop robust models to study COVID-19 infection dynamics of specific variants by RT-qPCR and provide an early warning for increased ICU utilization, with the potential to also provide early warning of potential fatalities. Echoing prior studies, the differences in the strength of the relationships to COVID-19 incidence and associated fatalities among these communities necessitate that rigorous validation should be performed at individual sites where wastewater surveillance programs are implemented.

4. CONCLUSIONS

Because clinical genomic sequencing capacity remains limited/low around the world, RT-qPCR approaches for COVID-19 variants were developed and applied to study two counties over a 9 month period. As the pandemic has progressed through the various variant dominant specific waves, rapid sharing of observations and the speed at which targets are developed, which can be added to ongoing wastewater surveillance projects, have dramatically increased. This was particularly demonstrated with the identification of the new dominant strain B.1.1.529 (omicron). On November 26, 2021, the WHO designated the newly detected B.1.1.529 as a VOC. Within days, companies had developed standards specific to this variant, and studies began to emerge outlining potential targets specific to the detection of this variant, with validation occurring in parallel, in contrast to the B.1.1.7 (alpha) variant, which had the first primers validated and available months after its emergence. For the B.1.1.529 variant, rapid monitoring was possible due to a single nucleotide variant, which was shared with a variant no longer prevalent (viz., HV69-70-Del). Thus, lead times in wastewater surveillance of an emerging variant were significantly reduced by redeploying previously validated assays, which was proposed in prior research. One of the major limitations of the targeted genomic surveillance or metagenomic sequencing, outside of the cost and specialized training required, is that rapid results cannot be obtained within the same time scale as RT-qPCR assays. Here, we demonstrate how RT-qPCR can be used to screen for wild type and putative VOCs, providing a flexible and effective approach to inform public health decisions.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestwater.2c00103.

Additional details on methods (viral RNA quantification and standard preparation), including sampling location characteristics and MIQE of experiment in addition to data (PDF).

AUTHOR INFORMATION

Corresponding Author
Laura M. Langan — Department of Environmental Science, Baylor University, Waco, Texas 76798, United States; Center for Reservoir and Aquatic Systems Research, Baylor
University, Waco, Texas 76798, United States; orcid.org/0000-0002-2095-7163; Email: laura.langan@baylor.edu

Authors

Megan O’Brien — Department of Environmental Science, Baylor University, Waco, Texas 76798, United States; Center for Reservoir and Aquatic Systems Research, Baylor University, Waco, Texas 76798, United States; Department of Public Health, Baylor University, Waco, Texas 76798, United States

Lea M. Lovin — Department of Environmental Science, Baylor University, Waco, Texas 76798, United States; Center for Reservoir and Aquatic Systems Research, Baylor University, Waco, Texas 76798, United States

Kendall R. Scarlett — Department of Environmental Science, Baylor University, Waco, Texas 76798, United States; Center for Reservoir and Aquatic Systems Research, Baylor University, Waco, Texas 76798, United States

Haley Davis — Department of Environmental Science, Baylor University, Waco, Texas 76798, United States; Center for Reservoir and Aquatic Systems Research, Baylor University, Waco, Texas 76798, United States

Abigail N. Henke — Department of Environmental Science, Baylor University, Waco, Texas 76798, United States; Center for Reservoir and Aquatic Systems Research, Baylor University, Waco, Texas 76798, United States

Sarah E. Seidel — Center for Health Statistics, Texas Department of State Health Services, Austin, Texas 78756, United States

Natalie Archer — Environmental Epidemiology and Disease Registries Section, Texas Department of State Health Services, Austin, Texas 78756, United States

Eric Lawrence — Environmental Epidemiology and Disease Registries Section, Texas Department of State Health Services, Austin, Texas 78756, United States

R. Sean Norman — Department of Environmental Health Sciences, Arnold School of Public Health, University of South Carolina, Columbia, South Carolina 29208, United States; orcid.org/0000-0003-4766-4376

Heidi K. Bojes — Environmental Epidemiology and Disease Registries Section, Texas Department of State Health Services, Austin, Texas 78756, United States

Bryan W. Brooks — Department of Environmental Science, Baylor University, Waco, Texas 76798, United States; Center for Reservoir and Aquatic Systems Research, Baylor University, Waco, Texas 76798, United States; Department of Public Health, Baylor University, Waco, Texas 76798, United States; orcid.org/0000-0002-6277-9852

Complete contact information is available at: https://pubs.acs.org/10.1021/acsestwater.2c00103

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This project was supported by the Centers for Disease Control and Prevention of the U.S. Department of Health and Human Services (HHS) as part of a financial assistance award (#HHS007972300001). The contents are those of the author(s) and do not necessarily represent the official views of, nor an endorsement, by CDC/HHS, or the U.S. Government. We are grateful for support of the City of Denton and the City of Waco, and Dr. Michelle Nemec with the Molecular Biosciences Center at Baylor University.

REFERENCES

(1) World Health Organisation. Guidance for Surveillance of SARS-CoV-2 Variants: Interim Guidance, 9 August 2021; World Health Organisation; 2021.
(2) Crawford, D. C.; Williams, S. M. Global Variation in Sequencing Impedes SARS-CoV-2 Surveillance. PLOS Genetics 2021, 17 (7), No. e1009620.
(3) Maxmen, A. Why US Coronavirus Tracking Can’t Keep up with Concerning Variants. Nature 2021, 592 (7854), 336–337.
(4) Vavrak, D.; Speroni, L.; Curnow, K. J.; Oberholzer, M.; Moeder, V.; Febo, P. G. Genomic Surveillance at Scale Is Required to Detect Newly Emerging Strains at an Early Timepoint. medRxiv (Infectious Diseases (except HIV/AIDS)), January 15, 2021, 2021.01.12.21249613. DOI: 10.1101/2021.01.12.21249613.
(5) Molenkamp, R.; Fanoy, E.; Derickx, L.; de Groot, T.; Jonges, M.; Leenstra, T.; Nijhuis, R.; Pas, S.; Vahidnia, A.; von Wintersdorff, C.; Mulder, B.; Koopmans, M. Supplementing SARS-CoV-2 Genomic Surveillance with PCR-Based Variant Detection for Real-Time Actionable Information, the Netherlands, June to July 2021. Euro Surveill 2021, 26 (40), 2100921.
(6) Graham, K. E.; Loeb, S. K.; Wolfe, M. K.; Catoe, D.; Sinnott-Armstrong, N.; Kim, S.; Yamahara, K. M.; Sassoubre, L. M.; Mendoza Grijalva, L. M.; Roldan-Hernandez, L.; Langenfeld, K.; Wigginton, K. R.; Boehm, A. B. SARS-CoV-2 RNA in Wastewater Settled Solids Is Associated with COVID-19 Cases in a Large Urban Sewershed. Environ. Sci. Technol. 2021, 55 (1), 488–498.
(7) Langan, L. M.; O’Brien, M.; Rundell, Z. C.; Back, J. A.; Ryan, B. J.; Chambliss, C. K.; Norman, R. S.; Brooks, B. W. Comparative Analysis of RNA-Extraction Approaches and Associated Influences on RT-QPCR of the SARS-CoV-2 RNA in a University Residence Hall and Quarantine Location. ACS EST Water 2022, DOI: 10.1021/acsestwater.1c00476.
(8) Wolfe, M. K.; Archana, A.; Catoe, D.; Coffman, M. M.; Dorevich, S.; Graham, K. E.; Kim, S.; Grijalva, L. M.; Roldan-Hernandez, L.; Silverman, A. I.; Sinnott-Armstrong, N.; Vugia, D. J.; Yu, A. T.; Zambrana, W.; Wigginton, K. R.; Boehm, A. B. Scaling of SARS-CoV-2 RNA in Settled Solids from Multiple Wastewater Treatment Plants to Compare Incidence Rates of Laboratory-Confirmed COVID-19 in Their Sewersheds. Environ. Sci. Technol. 2021, 8 (5), 398–404.
(9) Corchis-Scott, R.; Geng, Q.; Seth, R.; Ray, R.; Beg, M.; Biwas, N.; Charron, L.; Drouillard, K. D.; D’Souza, R.; Heath, D. D.; Houser, C.; Lawal, F.; McGinlay, J.; Menard, S. L.; Porter, L. A.; Rawlings, D.; Tong, Y.; Scholl, M. L.; Siu, K. W. M.; Weisener, C. G.; Wilhelm, S. W.; McKay, R. M. L. Averting an Outbreak of SARS-CoV-2 in a University Residence Hall through Wastewater Surveillance. Microbiol. Spectrum 2021, 9, e0079221.
(10) Hinz, A.; Xing, L.; Doukhanine, E.; Hug, L. A.; Kassen, R.; Ormeci, B.; Kibbee, R. J.; Wong, A.; MacFadden, D.; Nott, C. SARS-CoV-2 Detection from the Built Environment and Wastewater and Its Use for Hospital Surveillance. FACETS 2022, 7, 82.
(11) Weidhaas, J.; Anderud, Z. T.; Roper, D. K.; VanDerslice, J.; Gaddis, E. B.; Ostermiller, J.; Hoffman, K.; Jamal, R.; Heck, P.; Zhang, Y.; Torgersen, K.; Laan, J. V.; LaCross, N. Correlation of SARS-CoV-2 RNA in Wastewater with COVID-19 Disease Burden in Sewersheds. Sci. Total Environ. 2021, 775, 145790.
(12) Bonett, D. G.; Wright, T. A. Sample Size Requirements for Estimating Pearson, Kendall and Spearman Correlations. Psychometrika 2000, 65 (1), 23–28.
(13) DeRiggi, D. A Central Limit Theorem for Correlated Variables with Limited Normal or Gamma Distributions. Communications in Statistics - Theory and Methods 2019, 48 (21), S213–S222.
(14) Maddox, A. B. Introduction to Statistical Methods, 3rd ed.; Kendall Hunt Publishing: 2017.
Maximize Contributions in the Fight Against COVID-19.

Bibby, K.; Wastewater-Based Epidemiology: Global Collaborative to
R. J.; Nilsson, D.; Noble, R. T.; van Nuijs, A.; Peccia, J.; Perkins, T.
Islam, Md. T.; Jones, D. L.; Kasprzyk-Hordern, B.; Kitajima, M.;
Vela, J.; Farkas, K.; Fernandez-Casi, X.; Gerba, C.; Gerrity, D.;
Girones, R.; Gonzalez, R.; Haramoto, E.; Harris, A.; Holden, P. A.;
Islam, Md. T.; Jones, D. L.; Kasprzyk-Hordern, B.; Kitajima, M.;
Kotlarz, N.; Kumar, M.; Kuroda, K.; La Rosa, G.; Malpei, F.; Mautus,
M.; McLellan, S. L.; Medema, G.; Meschke, J. S.; Mueller, J.; Newton,
R. J.; Nilsson, D.; Noble, R. T.; van Nuijs, A.; Peccia, J.; Perkins, T.
A.; Picking, A. J.; Rose, J.; Sanchez, G.; Smith, A.; Stadler, L.;
Stauber, C.; Thomas, K.; van der Voorn, T.; Wigginton, K.; Zku, K.;
Bibby, K. Wastewater-Based Epidemiology: Global Collaborative to
Maximize Contributions in the Fight Against COVID-19. Environ. Sci.
Technol. 2021, 54 (13), 7754−7757.

LaTurner, Z. W.; Zong, D. M.; Kalvapalle, P.; Gamas, K. R.;
Tervilliger, A.; Crosby, T.; Ali, P.; Avadhana, V.; Santos, H. H.;
Weesner, K.; Hopkins, L.; Piedra, P. A.; Mareso, A. W.; Stadler, L. B.
Evaluating Recovery, Cost, and Throughput of Different Concentration
Methods for SARS-CoV-2 Wastewater-Based Epidemiology. Water
Res. 2021, 197, 117043.

Kantor, R. S.; Nelson, K. L.; Greenwald, H. D.; Kennedy, L. C.
Challenges in Measuring the Recovery of SARS-CoV-2 from Wastewater.
Environ. Sci. Technol. 2021, 55 (6), 3514−3519.

Acosta, N.; Bautista, M. A.; Holman, J.; McCaldie, J.; Beaudet,
A. B.; Man, L.; Waddell, B. J.; Chen, J.; Li, C.; Kuzma, D.; Bhattachar
S.; Leal, J.; Meddings, J.; Hu, J.; Cabaj, J. L.; Ruecker, N. J.; Naugler,
C.; Pillai, D. R.; Achari, G.; Ryan, M. C.; Conly, J. M.; Frankowski,
K.; Hubert, C. R.; Parkins, M. D. A Multicenter Study Investigating
SARS-CoV-2 in Tertiary-Care Hospital Wastewater. Viral Burden
Correlates with Increasing Hospitalized Cases as Well as Hospital-
Associated Transmissions and Outbreaks. Water Res. 2021, 201,
117369.

Lazuka, A.; Arenal, C.; Soyeux, E.; Sampson, M.; Lepeule, A.-S.;
Deleuze, Y.; Pouradier Duteil, S.; Lacroix, S. COVID-19 Wastewater
Based Epidemiology: Long-Term Monitoring of 10 WWTP in France
Reveals the Importance of the Sampling Context. Water Sci. Technol.
2021, 84 (8), 1997−2013.

Peccia, J.; Zulli, A.; Brackney, D. E.; Grubaugh, N. D.; Kaplan,
E. H.; Casanovas-Massana, A.; Ko, A. I.; Malik, A. A.; Wang, D.;
Wang, M.; Warren, J. L.; Weinberger, D. M.; Arnold, W.; Omer, S. B.
Measurement of SARS-CoV-2 RNA in Wastewater Tracks Community
Infection Dynamics. Nat. Biotechnol. 2020, 38 (10), 1164−1167.

Feng, S.; Roguet, A.; McClary-Gutierrez, J. S.; Newton, R. J.;
Kloezko, N.; Meiman, J. G.; McLellan, S. L. Evaluation of Sampling,
Analysis, and Normalization of SARS-CoV-2 Concentrations in
Wastewater Access COVID-19 Burdens in Wisconsin Communities.
ACS EST Water 2021, 1, 2055.

Greenwald, H. D.; Kennedy, L. C.; Hinkle, A.; Whitney, O. N.;
Fan, V. B.; Crits-Christoph, A.; Harris-Lovett, S.; Flamholz, A. I.; Al-
Shayeb, B.; Liao, L. D.; Beyers, M.; Brown, D.; Chakrabarti, A. R.;
Dow, J.; Frost, D.; Koekemoer, M.; Lynch, C.; Sarkar, P.; White, E.;
Kantor, R.; Nelson, K. L. Tools for Interpretation of Wastewater
Surrogate Murine Hepatitis Virus RNA in Untreated Wastewater to
Estimate Transmissibility and Impact of SARS-CoV-2 Lineage
K.; Keogh, R.; Eggo, R. M.; Funk, S.; Jit, M.; Atkins, K. E.; Edmunds,
W. J. Estimated Transmissibility and Impact of SARS-CoV-2 Lineage
K. 1.1.7 variants in England. Science 2021, 372, 846−850.

Li, X.; Kulandaivelu, J.; Zhang, S.; Shi, J.; Sivakumar, M.;
Mueller, J.; Luby, S.; Ahmed, W.; Cohn, L.; Jiang, G. Data-Driven
Estimation of COVID-19 Community Prevalence through Wastewater-
Based Epidemiology. Science of The Total Environment
2021, 819, 137497.

Ahmed, W.; Bertsch, P. M.; Bibby, K.; Haramoto, E.; Hewitt, J.;
Huyngh, F.; Gyiwalai, P.; Korajkic, A.; Riddell, S.; Cherian, S. P.;
Simpson, S. L.; Sririkanchana, K.; Symonds, E. M.; Verhagen, R.;
Vasan, S. S.; Kitaima, M.; Bivins, A. Decay of SARS-CoV-2 and
Surrogate Murine Hepatitis Virus RNA in Untreated Wastewater to
Inform Application in Wastewater-Based Epidemiology. Environ.
mental Res. 2020, 191, 110992.

Markt, R.; Mayr, M.; Peer, E.; Wagner, A. O.; Lackner, N.;
Insam, H. Detection and Stability of SARS-CoV-2 RNA Quantification in Wastewater Solids. PeerJ.
2021, 9, e11933.
Analysis, and Public Policy Implications.

B.; Walsh, S. P.; Meyerowitz-Katz, G. Assessing the Age Specificity of Impact of Vaccination on New SARS-CoV-2 Infections in the United Kingdom. Nat. Med. 2021, 27 (8), 1370–1378.

(41) Levin, A. T.; Hanage, W. P.; Owusu-Boateng, N.; Cochran, K. B.; Walsh, S. P.; Meyerowitz-Katz, G. Assessing the Age Specificity of Infection Fatality Rates for Patients under Age of 70 Years and Hospitalization Risk Overall. Acta Microbiol. Immunol. Hung. 2021, 68 (3), 153–161.

(42) Wolfe, M.; Hughes, B.; Duong, D.; Chan-Herur, V.; Wiginton, K. R.; White, B. J.; Boehm, A. B. Detection of SARS-CoV-2 Variant Mu, Beta, Gamma, Lambda, Delta, Alpha, and Omicron in Wastewater Settled Solids Using Mutation-Specific Assays Is Associated with Regional Detection of Variants in Clinical Samples. Appl. Environ. Microbiol. 2022, 88, e00045-22.

(43) Graber, T. E.; Mercier, É.; Bhatnagar, K.; Fuzzen, M.; D’Aoust, P. M.; Hoang, H.-D.; Tian, X.; Towhid, S. T.; Plaza-Diaz, J.; Eid, W.; Alain, T.; Butler, A.; Goodridge, L.; Servos, M.; Delatolla, R. Near Real-Time Determination of B.1.1.7 in Proportion to Total SARS-CoV-2 Viral Load in Wastewater Using an Allele-Specific Primer Extension PCR Strategy. Water Res. 2021, 205, 117681.

(44) Li, X.; Zhang, S.; Shi, J.; Luby, S. P.; Jiang, G. Uncertainties in Estimating SARS-CoV-2 Prevalence by Wastewater-Based Epidemiology. Chem. Eng. J. 2021, 415, 129039.

(45) Ghosh, A. K.; Kaiser, M.; Molla, Md. M. A.; Nafisa, T.; Yeasmin, M.; Ratul, R. H.; Sharif, Md. M.; Akram, A.; Hosen, N.; MacDonald, R.; Amin, Md. R.; Islam, A.; Hoseque, Md. E.; Hamid, O.; Latnd, O.; Lytton, S. D. Molecular and Serological Characterization of the SARS-CoV-2 Delta Variant in Bangladesh in 2021. Viruses 2021, 13 (11), 2310.

(46) Earnest, R.; Uddin, R.; Matluk, N.; Renzette, N.; Siddle, K. J.; Loretth, C.; Adams, G.; Tomkins-Tinch, C. H.; Petrone, M. E.; Rothman, J. E.; Breban, M. I.; Koch, R. T.; Billig, K.; Fauer, J. R.; Vogels, C. B. F.; Turbett, S.; Bilguvar, H.; De, K. B.; Eland, M.; L.; Peaper, D. R.; Kelley, K.; Omerza, G.; Grieser, H.; Meak, S.; Afifi, S.; Morgan, S.; Marchbank, A.; Price, A.; Kitchen, C.; Gulliver, H.; Merrick, I.; Southgate, J.; Guest, M.; Munn, R.; Workman, T.; Connor, T.; Fuller, W.; Bresner, C.; Snell, L.; Patel, A.; Charalmous, T.; Nebbia, G.; Batra, R.; Edgeworth, J.; Robson, S.; Beckett, A.; Aanensen, D.; Underwood, A.; Yeats, C.; Abdubah, K.; Taylor, B.; Benegazzo, M.; Clark, G.; Smith, W.; Khakh, M.; Fleming, V.; Lister, M.; Howson-Wells, H.; Berry, L.; Boswell, T.; Joseph, A.; Willingham, I.; Jones, C.; Holmes, C.; Bird, P.; Helmer, T.; Fallon, K.; Tang; J.; Raviprakash, V.; Campbell, S.; Sheriff, N.; Blakey, V.; Williams, L.-A.; Loose, M.; Holmes, N.; Moore, C.; Carille, M.; Wright, V.; Sang, F.; Debebe, J.; Coll, F.; Signell, A.; Betancor, G.; Wilison, H.; Eldirdiri, S.; Kenyon, A.; Davis, T.; Pybus, O.; de Plessis, L.; Zarebski, A.; Raghwani, J.; Kraemer, M.; Francois, S.; Atwood, S.; Vasylyeva, T.; Zamudio, M. E.; Gutierrez, B.; Torok, M.; Hamilton, W.; Goodellow, I.; Hall, G.; Jahan, A.; Chaudhry, Y.; Homsilo, M.; Pinckert, M.; Georgana, I.; Moses, S.; Lowe, H.; Bedford, L.; Moore, J.; Stonehouse, S.; Fisher, C.; Awan, A.; Bojes, J.; Breuer, J.; Harris, K.; Brown, J.; Shah, D.; Atkinson, L.; Lee, J.; Storey, N.; Flaviani, F.; Alcolea-Medina, A.; Williams, R.; Vernet, G.; Chapman, M.; Levet, L.; Heaney, J.; Chatterton, W.; Pusok, M.; Xu-Mccrae, L.; Smith, D.; Ashston, M.; Young, G.; Holmes, A.; Randell, P.; Cox, A.; Madona, P.; Bolt, P.; Price, J.; Mookerjee, R.; Raggonet-Cronin, R.; Nascimento, F. J.; Jorgensen, D.; Siveroni, I.; Johnson, L.; Boyd, O.; Geidelberg, L.; Volz, E.; Rowan, A.; Taylor, G.; Smollett, K.; Loman, N.; Quick, J.; McMurray, C.; Stockton, J.; Nicholls, S.; Rowe, W.; Pophlawski, R.; Mcnally, A.; Nunez, R. M.; Mason, J.; Robinson, T.; O’Toole, E.; Watts, J.; Breen, C.; Cowell, A.; Slaga, G.; Machin, N.; Ahmed, S.; George, R.; Halstead, F.; Swapraksam, Y.; Hogned, W.; Iliningworth, C.; Jackson, C.; Thomson, E.; Shephard, A.; Asamaphan, P.; Niebel, M.; Li, K.; Shah, R.; Jesudason, N.; Tong, L.; Broos, A.; Mair, D.; Nichols, J.; Carmichael, S.; Nomikou, K.; Aranday-Cortes, E.; Johnson, N.; Starinskij, I.; da Silva Filipe, A.; Robertson, D.; Orr, R.; Hughes, J.; Vattipally, S.; Singer, J.; Nickbaksh, S.; Hale, A.; Macfarlane-Smith, L.; Harper, K.; Carden, H.; Taha, Y.; Payne, B.; Burton-Fanning, S.; Waugh, S.; Collins, J.; Eltringham, G.; Rushston, S.; O’Brien, S.; Bradley, A.; Maclean, A.; Mollert, G.; Blacow, R.; Tempton, K.; McHugh, M.; Dewar, R.; Westehng, E.; Dervisvic, S.; Stanley, R.; Meader, E.; Coupland, L.; Smith, L.; Graham, C.; Barton, E.; Padgett, D.; Scott, G.; Swindells, E.; Greenaway, J.; Nelson, A.; McCann, C.; Yew, W.; Andessor, M.; Petos, T.; Justice, A.; Eyre, D.; Crook, D.; Sloan, T.; Duckworth, N.; Walsh, S.; Chauhan, A.; Grayshber, S.; Bicknell, K.; Willsie, S.; Elliott, S.; Lloyd, A.; Impey, R.; Levene, N.; Monagahan, L.; Bradley, D.; Wyatt, T.; Allara, E.; Pearson, C.; Osman, H.; Bosworth, A.; Robinson, E.; Muir, P.; Vidon, I.; Hopes, R.; Pymont, H.; Hutchings, S.; Curran, M.; Parmar, S.; Lackenby, A.; Mbisa, T.; Platt, S.; Miah, S.; Bibby, D.; Manto, C.; Hobb, J.; Chand, M.; Dabera, G.; Ramsay, M.; Bradshaw, D.; Thornton, A.; Myers, R.; Schaefer, U.; Groves, N.; Gallaghar, E.; Lee, D.; Williams, D.; Ellaby, N.; Harrison, I.; Hartman, H.; Manesis, N.; Patel, V.; Bishop, C.; Chalker, V.; Ledesma, J.; Twogih, K.; Holden, M.; Shaaban, S.; Birchley, A.; Adams, D.; Davies, A.; Gaskin, A.; Plimmer, A.; Gatica-Wilcox, B.; McKerr, C.; Moore, C.; Williams, C.; Heyburn, D.;
Admission, and Vaccine Effectiveness. Kermack, L.; Gupta, R.; Ludden, C.; Peacock, S.; Palmer, S.; Cormie, C.; Gill, H.; Dias, J.; Higginson, E.; Maes, M.; Young, J.; Hesketh, A.; Blane, B.; Girgis, S.; Leek, D.; Sridhar, S.; Forrest, S.; Ashcroft, F.; Moles-Garcia, E.; Cumley, N.; Smith, C.; Bucca, G.; Pandey, S.; Berry, L.; Jones, K.; Richter, A.; Beggs, A.; Best, A.; Cotic, M.; Bayzid, N.; Westhorpe, A.; Hartley, J.; Jannoo, R.; Lowe, Wilson-Davies, E.; Williams, R.; Kristiansen, M.; Roy, S.; Williams, C.; Saeed, K.; Mahanama, A.; Samaraweera, B.; Silveira, S.; Pelosi, E.; Johnson, K.; Liggett, S.; Baker, P.; Bonner, S.; Essex, S.; Lyons, R.; Ratcliffe, L.; Simpson, D.; Molnar, Z.; Kay, G.; Trotter, A.; Alikhan, N.-F.; de Oliveira Martins, L.; Le-Viet, SummerhaYes, S.; Taylor, S.; Cottrell, S.; Jones, S.; Edwards, S.; L., (52) Sheikh, A.; McMenamin, J.; Taylor, B.; Robertson, C. SARS-CoV-2 Delta Variant, Key Spike Mutations and Immune Escape. Front. Immunol. 2021, 12, 751778.

(53) Cherian, S.; Potdar, V.; Jadhav, S.; Yadav, P.; Gupta, N.; Das, M.; Rahkrit, P.; Singh, S.; Abraham, P.; Panda, S.; Team. SARS-CoV-2 Spike Mutations, L452R, T478K, E484Q and P681R, in the Circulating SARS-CoV-2 Variants Based on Multiple PCR-MALDI-TOF MS. medRxiv (Infectious Diseases except HIV/AIDS), June 12, 2021, 2021.06.08.21258523.

(54) Zhao, F.; Wang, X.; Hou, X.; Qin, T.; Meng, F.; Xu, X.; Li, T.; Zhou, H.; Kan, B.; Lu, J.; Xiao, D. A Novel Strategy for the Detection of SARS-CoV-2 Variants Based on Multiple PCR-MALDI-TOF MS. medRxiv (Infectious Diseases except HIV/AIDS), June 12, 2021, 2021.06.08.21258523.

(55) Cherian, S.; Potdar, V.; Jadhav, S.; Yadav, P.; Gupta, N.; Das, M.; Rahkrit, P.; Singh, S.; Abraham, P.; Panda, S.; Team. SARS-CoV-2 Spike Mutations, L452R, T478K, E484Q and P681R, in the Circulating SARS-CoV-2 Variants Based on Multiple PCR-MALDI-TOF MS. medRxiv (Infectious Diseases except HIV/AIDS), June 12, 2021, 2021.06.08.21258523.

(56) Wilhelm, A.; Toptan, T.; Pallas, C.; Wolf, T.; Goetsch, U.; Gottschalk, R.; Vehreschild, M. J. G. T.; Ciesek, S.; Widera, M. Antibody-Mediated Neutralization of Authentic SARS-CoV-2 B.1.617.2 Variants Harboring L452R and T478K/E484Q. Viruses 2021, 13 (9), 1695.

(57) Johnson, R.; Sharma, J. R.; Ramharack, P.; Mangwana, N.; Kinne, C.; Viraragavan, A.; Glanzmann, B.; Louw, J.; Abdelatif, N.; Reddy, T.; Surujial-Naicker, S.; Nkambule, S.; Mahlangeni, N.; Webster, C.; Mdhuli, M.; Gray, G.; Mathae, A.; Preiser, W.; Muller, C.; Street, R. Tracking the Circulating SARS-CoV-2 Variant of Concern in South Africa Using Wastewater-Based Epidemiology. Sci. Rep. 2022, 12 (1), 1182.

(58) Yaniv, K.; Ozer, E.; Lewis, Y.; Kushmaro, A. RT-QPCR Assays for SARS-CoV-2 Variants of Concern in Wastewater Reveals Compromised Vaccination-Induced Immunity. Water Res. 2021, 207, 117808.

(59) Merad, M.; Cieza, D.; Neher, R. A.; Deng, X.; Gu, W.; Federman, S.; Chiu, C.; Duchin, J. S.; Moore, D. C.; Tsiodras, S.; Scorilas, A.; Vasiliou, V.; Dimopoulos, M.-A.; Famulare, M.; Huang, M.-L.; Nalla, A.; Pepper, G.; Reinhardt, A.; Chennubhotla, J.; Hodcroft, E. B.; Huddleston, J.; M., 2021.04.30.21242526.

(60) Neher, R. A.; Deng, X.; Gu, W.; Federman, S.; Chiu, C.; Duchin, J. S.; Moore, D. C.; Tsiodras, S.; Scorilas, A.; Vasiliou, V.; Dimopoulos, M.-A.; Famulare, M.; Huang, M.-L.; Nalla, A.; Pepper, G.; Reinhardt, A.; Chennubhotla, J.; Hodcroft, E. B.; Huddleston, J.; M., 2021.04.30.21242526.
(69) Islam, A. B. M. Md. K.; Khan, Md. A.-A.-K.; Ahmed, R.; Hossain, Md. S.; Kabir, Md. C. T.; Islam, Md. S.; Siddiki, A. M. A. M. Z. Transcriptome of Nasopharyngeal Samples from COVID-19 Patients and a Comparative Analysis with Other SARS-CoV-2 Infection Models Reveal Disparate Host Responses against SARS-CoV-2. *Journal of Translational Medicine* **2021**, *19* (1), 32.

(70) Betancourt, W. Q.; Schmitz, B. W.; Innes, G. K.; Prasek, S. M.; Pogreba Brown, K. M.; Stark, E. R.; Foster, A. R.; Spriessler, R. S.; Harris, D. T.; Sherchan, S. P.; Gerba, C. P.; Pepper, I. L. COVID-19 Containment on a College Campus via Wastewater-Based Epidemiology, Targeted Clinical Testing and an Intervention. *Science of The Total Environment* **2021**, *779*, 146408.

(71) Chan, A. Y.; Kim, H.; Bell, M. L. Higher Incidence of Novel Coronavirus (COVID-19) Cases in Areas with Combined Sewer Systems, Heavy Precipitation, and High Percentages of Impervious Surfaces. *Science of The Total Environment* **2022**, *820*, 153227.

(72) Zulli, A.; Pan, A.; Bart, S. M.; Crawford, F. W.; Kaplan, E. H.; Cartter, M.; Ko, A. I.; Sanchez, M.; Brown, C.; Cozens, D.; Brackney, D. E.; Peccia, J. Predicting Daily COVID-19 Case Rates from SARS-CoV-2 RNA Concentrations across a Diversity of Wastewater Catchments. *FEMS Microbes* **2022**, *2*, xtab022.

(73) Huisman, J. S.; Scire, J.; Caduff, L.; Fernandez-Cassi, X.; Ganesanandamoorthy, P.; Null, A.; Scheidegger, A.; Stachler, E.; Boehm, A. B.; Hughes, B.; Knudson, A.; Topol, A.; Wigginton, K. R.; Wolfe, M. K.; Kohn, T.; Ort, C.; Stadler, T.; Julian, T. R. Wastewater-Based Estimation of the Effective Reproductive Number of SARS-CoV-2. *Environ. Health Perspect.* **2022**, *130*, 057011.

(74) Medema, G.; Heijnen, L.; Elsinga, G.; Italiaander, R.; Brouwer, A. Presence of SARS-Coronavirus-2 RNA in Sewage and Correlation with Reported COVID-19 Prevalence in the Early Stage of the Epidemic in The Netherlands. *Environ. Sci. Technol. Lett.* **2020**, *7* (7), 511–516.

(75) Bibby, K.; Bivins, A.; Wu, Z.; North, D. Making Waves: Plausible Lead Time for Wastewater Based Epidemiology as an Early Warning System for COVID-19. *Water Res.* **2021**, *202*, 117438.

(76) Ahmed, W.; Bivins, A.; Smith, W. J. M.; Metcalfe, S.; Stephens, M.; Jenkinson, A. V.; Moore, F. A. J.; Bourke, J.; Schlebusch, S.; McMahon, J.; Hewitson, G.; Nguyen, S.; Barcelon, J.; Jackson, G.; Mueller, J. F.; Ehret, J.; Hosegood, I.; Tian, W.; Wang, H.; Yang, L.; Bertsch, P.; Tynan, J.; Thomas, K. V.; Bibby, K.; Graber, T. E.; Ziels, R.; Simpson, S. L. Detection of the Omicron (B.1.1.529) Variant of SARS-CoV-2 in Aircraft Wastewater. *Science of The Total Environment* **2022**, *820*, 153171.

(77) Lee, W. L.; Gu, X.; Armas, F.; Wu, F.; Chandra, F.; Chen, H.; Xiao, A.; Leifels, M.; Chua, F. J. D.; Kwok, G. W.; Tay, J. Y.; Lim, C. Y.; Thompson, J.; Alm, E. J. Quantitative Detection of SARS-CoV-2 Omicron Variant in Wastewater through Allele-Specific RT-QPCR. *medRxiv (Infectious Diseases (except HIV/AIDS)*, January 14, 2022, 2021.12.21.21268077..