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Use of wastewater surveillance for early detection of Alpha and Epsilon SARS-CoV-2 variants of concern and estimation of overall COVID-19 infection burden

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A decline in diagnostic testing for SARS-CoV-2 is expected to delay the tracking of COVID-19 variants of concern and interest in the United States. We hypothesize that wastewater surveillance programs provide an effective alternative for detecting emerging variants and assessing COVID-19 incidence, particularly when clinical surveillance is limited. Here, we analyzed SARS-CoV-2 RNA in wastewater from eight locations across Southern Nevada between March 2020 and April 2021. Trends in SARS-CoV-2 RNA concentrations (ranging from 4.3 log10 gc/L to 8.7 log10 gc/L) matched trends in confirmed COVID-19 incidence, but wastewater surveillance also highlighted several limitations with the clinical data. Amplicon-based whole genome sequencing (WGS) of 86 wastewater samples identified the B.1.1.7 (Alpha) and B.1.429 (Epsilon) lineages in December 2020, but clinical sequencing failed to identify the variants until January 2021, thereby demonstrating that ‘pooled’ wastewater samples can sometimes expedite variant detection. Also, by calibrating fecal shedding (11.4 log10 gc/infection) and wastewater surveillance data to reported seroprevalence, we estimate that ~38% of individuals in Southern Nevada had been infected by SARS-CoV-2 as of April 2021, which is significantly higher than the 10% of individuals confirmed through clinical testing.
1. Introduction

As of March 2021 in the United States, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) had resulted in 30 million confirmed COVID-19 cases and 536,000 deaths (https://coronavirus.jhu.edu/map.html, accessed 3/30/21). In up to 70% of all COVID-19 infections, individuals show mild to no symptoms (Buitrago-Garcia et al., 2020; Johansson et al., 2021; Oran and Topol, 2020), highlighting the possibility that the aforementioned case count may significantly underestimate the true number of infections. Diagnostic tests are known to produce false negative results (Kanji et al., 2021; Kucirka et al., 2020; Woloshin et al., 2020), suggesting that infections may also go undetected due to methodological limitations. As the number of individuals seeking diagnostic testing declines, combined with the relaxation of COVID-19 mitigation measures (e.g., capacity limits and mask mandates) (Anderson et al., 2020; Hatef et al., 2021), known and emerging SARS-CoV-2 variants of concern (VOCs) and interest (VOIs) have the potential to trigger new waves of infection (Abdelnabi et al., 2021; Abdool Karim and de Oliveira, 2021; Harvey et al., 2021). In the U.S., this has occurred multiple times throughout the COVID-19 pandemic, including the winter of 2020/2021 with the Alpha case counts and SARS-CoV-2 concentrations within the individual sewersheds. Over a one-month period, weekly grab sampling at a manhole also detected an ongoing outbreak at a community shelter. Next, we sequenced and analyzed 86 wastewater samples and 575 clinical samples from Southern Nevada during the same time period, ultimately using wastewater to detect the introduction of the Alpha (B.1.1.7) and Epsilon (B.1.429) lineages in the community up to one month prior to clinical confirmation. Finally, by calibrating fecal shedding and wastewater surveillance data to reported seroprevalence, we developed an approach for estimating the total number of infections in individual sewersheds and across Southern Nevada between March 2020 and April 2021.

2. Methods

2.1. Sample collection

Wastewater surveillance was performed weekly at seven treatment facilities (described as Facilities 1–7) that collectively encompass the vast majority of the population of the Las Vegas metropolitan area. The average daily flow at the treatment facilities ranged from 0.8 million gallons per day (mgd) to 100 mgd, and the corresponding sewersheds served 16,000 to 872,000 individuals, respectively (Table 1). Due to a combination of factors related to study design and/or practical limitations, sample types included grab primary effluent at Facility 1 (collected at ~10:00 am), 24-h flow-weighted composite influent at Facilities 2–4, and grab influent at Facilities 5–7 (collected at ~8:00 am). Weekly monitoring at these facilities (every Monday) commenced on different dates: March 2020 for Facility 1, August 2020 for Facilities 2–6, and December 2020 for Facility 7. Weekly sampling at Facility 8 (i.e., a community shelter manhole) was conducted during a four-week span from November 23, 2020 through December 14, 2020 and involved collection of three grab samples spaced 5 min apart (collected at ~6:00 am) to generate a manual composite.

2.2. Quantification of SARS-CoV-2 in Southern Nevada wastewater

Sample processing and qPCR analysis followed a modification of our previously published protocols (Gerrity et al., 2021). Instead of a combined sample concentration approach with tangential hollow fiber ultrafiltration (HFUF) and centrifugal ultrafiltration, 10-L samples from Facilities 1 and 4 were processed with HFUF alone (REXCEED-255, 30 kDa, Asahi Kasei Medical Co., Japan), and 150-mL samples from all other locations were processed with Centricon centrifugal ultrafiltration (Centricon Plus-70, 100 kDa, Millipore Sigma, Burlington, MA, USA). This was meant to ensure experimental continuity (Gerrity et al., 2021), while allowing for additional sample throughput for the sites added in the current study. SARS-CoV-2 RNA concentrations were reported as averages (+1 standard deviation) of duplicate qPCR reactions across four SARS-CoV-2 gene target assays (orf1a, E,Sarbeco, N1, and N2). All concentrations were adjusted for equivalent sample volume and sample-specific recovery of spiked bovine coronavirus (BCoV) (summarized later). All other details were described previously (Gerrity et al., 2021).
Our previous findings demonstrated that wastewater concentrations of SARS-CoV-2 RNA were impacted by a number of factors beyond COVID-19 incidence, including sewershed characteristics (e.g., size and flow rate), sample type, and sample time (Gerrity et al., 2021). The grab primary effluent at Facility 1 consistently underestimates concentrations in the corresponding composite influent and composite primary effluent, presumably because it reflects influent wastewater arriving at the facility between 5:00 am and 6:00 am (Gerrity et al., 2021). To account for this diurnal variability and ultimately provide a more accurate infection estimate using the model described later, the observed concentrations for Facility 1 were increased by a factor of 3.5—the median from the prior study (Gerrity et al., 2021). On the other hand, the mid-morning grab influent samples at Facilities 5 and 6 were not significantly different from split samples of composite influent (p = 0.62 and p = 0.14, respectively) based on paired t-tests (N = 25 each). Therefore, no adjustment for diurnal variability was warranted for those facilities. Facility 7 does not collect composite samples so it was not possible to conduct a statistical evaluation of diurnal variability to determine whether the mid-morning grab sample was representative of concentrations throughout the day.

2.3. Detection of variants of concern (VOCs) in Southern Nevada

Sequencing libraries were constructed using the CleanPlex SARS-CoV-2 FLEX Panel from Paragon Genomics per manufacturer’s instructions. More than 10 ng of total RNA was processed for first-strand cDNA synthesis. Libraries were sequenced using an Illumina NextSeq 500 sequencer with either mid-output or high-output v2.5 (300 cycles) flow cells. Upon sequencing, Illumina adapter sequences were trimmed from read pairs using cutadapt version 3.2 (Martin, 2011). Sequencing reads were mapped to the SARS-CoV-2 reference genome (NC_045512.2) using bwa mem, version 0.7.17-r1188 (Li, 2013). Paragon Genomics CleanPlex SARS-CoV-2 FLEX tiled-amplion primers were trimmed from the aligned reads using fgbio TrimPrimers version 1.3.0 in hard-clip mode and variants were called by iVar variants v1.3 (Grubaugh et al., 2019). Genome coverages were calculated using samtools coverage v1.10 (Li et al., 2009).

Variants were called from the aligned sequences by searching for each of the 14 non-synonymous mutations, three deletions, and six synonymous mutations commonly observed in B.1.1.7 samples (Table S4). B.1.1.7-positive samples were identified using defined criteria, including observation of multiple sites and prioritization of mutations of functional concern. We first required the detection of the mutation responsible for spike N501Y (23603 A>T). Because N501Y is also found in the unrelated VOC lineages P.1 and B.1.351 (Faria et al., 2021), we also required the detection of the spike ΔH69/ΔV70 deletion (21765-21770 deletion) or receptor binding domain (RBD) mutant A570D (23271 C>A). Using these criteria, we identified seven wastewater samples with N501Y and either ΔH69/ΔV70 or A570D, in addition to many other known B.1.1.7 mutations (Fig. 3A).

We also interrogated the wastewater samples for evidence of the highly transmissible B.1.429 lineage, searching our variant calls for five non-synonymous mutations in the genome (Zhang et al., 2021). One additional mutation not described in the original report (Zhang et al., 2021)—N protein T205I—was added to the search, due to it being reported as another characteristic mutation of B.1.429 (Bourassa et al., 2021). For B.1.429 assignment in wastewater, we required the detection of the spike mutation Δ452R (22917 T>G) and either of the additional spike mutations S13I (21600 G>T) or W152C (22018 G>T). Using these criteria, B.1.429 was detected in five wastewater samples (Fig. 3B).

2.4. Processing of clinical samples and whole genome sequencing

Clinical samples were collected and confirmed for the presence of SARS-CoV-2 RNA using RT-PCR at the Southern Nevada Public Health Laboratory, as described previously (Hartley et al., 2020; Tillett et al., 2021). WGS on samples collected in December 2020 was performed by the UNLV lab using the CleanPlex SARS-CoV-2 FLEX Panel (Paragon Genomics) on an Illumina NextSeq 500 sequencer. Samples collected from January to April 2021 were sequenced by the Nevada State Public Health Laboratory using a Clear Labs platform; the ClearLabs SARS-CoV-2 test is an automated NGS library preparation and sequencing platform that uses a modified ARTIC v3 library preparation and performs ONT sequencing on a GridION (Oxford Nanopore). Genome consensus sequences were assembled using Medaka via the ARTIC pipeline (Lu et al., 2020), and mutations and deletions were identified using NextClade. Sequences were then classified by all defined viral lineages categorized both by Pangolin lineage classifications and major NextStrain (Hadfield et al., 2018) clades.

2.5. Estimating total COVID-19 infections in Southern Nevada

In order to develop a total infection estimate for Southern Nevada (see Fig. S1 for a schematic illustrating the overall process), we first performed a numerical integration of the wastewater SARS-CoV-2 concentrations observed at each facility (Table S2), coupled with the corresponding average daily flow rates (Table 1). These total wastewater loads (Table S1) were then divided by the total fecal load per infected individual over the course of an infection. Based on a preliminary fecal shedding model for SARS-CoV-2 (Gerrity et al., 2021; Wöfle et al., 2020), we initially assumed a daily feces production rate of 126 g per person per day and a fecal load of 8.9 log10 gene copies (gc) per gram, which was assumed to decrease steadily over approximately 25 days (Fig. 1). After numerical integration, this resulted in a total fecal load of 11.1 log10 gc over a typical infection period. Modifications to this initial approach are described in the Results section. Note that the trajectory in Fig. 1 represents a simplification of fecal shedding and is primarily meant to provide a ‘real-world’ interpretation of the calibrated total fecal load. Also, this calibrated shedding parameter actually represents the total amount of SARS-CoV-2 shed through feces,

### Table 1

Summary of sewershed characteristics and estimated and confirmed infections in Southern Nevada.

| Facility | Sewershed characteristic | Sample type | Estimated infections | % of sewershed population | Reported infections (confirmed) | % of sewershed population (confirmed) | Ascertaintment ratio |
|----------|--------------------------|-------------|----------------------|--------------------------|-------------------------------|--------------------------------------|---------------------|
|          | Average daily flow (mgd) |             |          |                          |                               |                                      |                     |
| 1        | 100                      | 872,009     |         |                         |                               |                                      |                     |
| 2        | 42                       | 757,418     |         |                         |                               |                                      |                     |
| 3        | 20                       | 255,008     |         |                         |                               |                                      |                     |
| 4        | 5                        | 86,330      |         |                         |                               |                                      |                     |
| 5        | 15                       | 133,977     |         |                         |                               |                                      |                     |
| 6        | 6                        | 114,532     |         |                         |                               |                                      |                     |
| 7        | 0.8                      | 16,399      |         |                         |                               |                                      |                     |
| 8        | N/A                      | N/A         | <100    | Shelter manhole         | N/A                           | N/A                                  | N/A                 |
| All      | 189                      | 2,235,673   |         |                         | 843,675                       | 226,775                             | 10%                 | 3.7                 |

*a Initial monitoring dates: Facility 1 = 03/09/2020, Facility 2/3/5/6 = 08/31/2020, Facility 4 = 04/07/2020, Facility 7 = 12/14/2020, Facility 8 = 11/23/2020.

*b See main text regarding adjustment for diurnal variability (Facility 1 viral loads were increased by a factor of 3.5).

*c See main text regarding adjustment for grab sample outlier on 12/28/20 (8.7 log10 gc/L).

**d Total for all zip codes served by Southern Nevada's wastewater treatment facilities as of 4/12/21 (overall total for Southern Nevada = 238,555).
the late-December SARS-CoV-2 spike at Facility 7 (8.7 log10 gc/L) was adjust for diurnal variability (described earlier (Gerrity et al., 2021)) and datasets: (1) Facility 1 concentrations were increased by a factor of 3.5 to con

data. Two other modi

cations were made to the wastewater surveillance datasets: (1) Facility 1 concentrations were increased by a factor of 3.5 to adjust for diurnal variability (described earlier (Gerrity et al., 2021)) and (2) the late-December SARS-CoV-2 spike at Facility 7 (8.7 log10 gc/L) was adjusted downward to match the following week's concentration. Including the spike resulted in a seemingly erroneous total infection estimate and poor alignment between modeled and observed wastewater concentrations for Facility 7. The high concentration was assumed to be related to grab sampling coupled with the small size of that sewershed, leading to a presumably non-representative SARS-CoV-2 concentration in that particular sample.

Sewershed-specific populations and case counts were derived from data published by the Southern Nevada Health District (SNHD, 2021). Specifically, zip code-level data were allocated to sewersheds by cross-referencing zip code locations against jurisdictional maps. The vast majority of zip codes in Southern Nevada align by municipality/jurisdiction, thus each zip code can be assigned to a specific facility and sewershed. Sewershed-specific ascertainment ratios (i.e., X-fold infection undercounts) were then calculated as wastewater-derived infection estimates divided by confirmed case counts. These ascertainment ratios were then used to revise a previously published MATLAB (MathWorks, Natick, MA) model (Gerrity et al., 2021) for estimating wastewater concentrations based on confirmed case counts (code provided in Text S1). The original model used a different fecal shedding parameter and also assumed a single ascertainment ratio of 2 for all sewersheds (i.e., 50% ‘asymptomatic’). The revised code increased daily case counts to adjust for the ascertainment ratio and then entered the corresponding number of infected individuals into a 25-day shedding sequence that followed the aforementioned trajectory (Fig. 1). The total daily SARS-CoV-2 load for each sewershed was then divided by the average daily flow rate for the corresponding wastewater treatment facility to arrive at the expected concentration for each day.

2.6. Human subjects statement

The University of Nevada Las Vegas Institutional Review Board (IRB) reviewed this project and determined it to be exempt from human subject research according to federal regulations and university policy.

3. Results

3.1. Quantification of SARS-CoV-2 in Southern Nevada wastewater

During the 13-month monitoring period, SARS-CoV-2 RNA was detected in nearly all samples, and recovery-adjusted concentrations ranged from a minimum of 4.3 log10 gc/L for Facility 1 at the onset of the pandemic to a maximum of 8.7 log10 gc/L for Facility 7 during the winter 2020/2021 surge (Table S2). Fig. 2 summarizes the site-specific average BCoV recoveries and also illustrates the relationship between wastewater SARS-CoV-2 concentrations and confirmed COVID-19 case data reported at the zip code level by the Southern Nevada Health District. With the exception of
Facility 7, the data demonstrate that trends in wastewater SARS-CoV-2 concentrations align with trends in COVID-19 incidence, even at the sewershed level. Trends were less apparent for Facility 7 (Fig. 2H), presumably due to its sample type (i.e., grab rather than composite), small sewershed size, and shorter monitoring period, all of which make this system more susceptible to short-term fluctuations in SARS-CoV-2 load. However, there was an extreme concentration spike at Facility 7 in late December (8.7 log10 gc/L) that could potentially be explained by the surge in COVID-19 incidence.

Fig. 2. Wastewater surveillance across Southern Nevada. Summary of new daily cases (based on official reporting date), recovery-adjusted average (+ 1 standard deviation) SARS-CoV-2 wastewater concentrations, and modeled SARS-CoV-2 wastewater concentrations in each sewershed. (A) Data from March 2020 through April 2021 for Facility 1. (BH) Data for the expanded monitoring effort from August 2020 through April 2021 for Facilities 1–7. The two open circles for Facility 3 indicate non-detects/inconclusive results and represent sample-specific limits of quantification (LoQs). Daily case data represent population-standardized 7-day moving averages.
Limited grab samples were also collected from a manhole serving a community shelter (Facility 8). SARS-CoV-2 RNA was detected in all four weekly samples collected between November 23, 2020 and December 14, 2020, with concentrations steadily increasing from $4.6 \log_{10} \text{gc/L}$ to $6.8 \log_{10} \text{gc/L}$ during this time (Table S2). Subsequent communication with shelter personnel confirmed that a COVID-19 outbreak occurred within the complex during this monitoring period.

3.2. Whole genome sequencing (WGS) of viral genomes from wastewater: Identification of the Alpha (B.1.1.7) lineage

SARS-CoV-2 VOCs are known to include a series of mutations affecting their virulence and ability to evade antibody response (Deng et al., 2021). Given declining testing rates and the challenges with contact tracing (Becker et al., 2021), we asked whether viral variants could be identified through the analysis of wastewater samples. Using an amplicon-based NGS platform, we performed WGS of the SARS-CoV-2 genome in 86 wastewater samples collected in Southern Nevada between November 30, 2020 and February 22, 2021. Illumina sequencing yielded an average of 2.7 million $2 \times 150$ basepairs per wastewater sample (Table S3). Mean aligned genome coverage spanned 29,800 of the 29,903 nucleotides in the SARS-CoV-2 genome, or ~ 99% (Table S3); mean depth of coverage ranged between 3000- and 37,000-fold, with a median depth of 15,000-fold across all samples (Table S3). In addition, we sequenced three RNA controls, including a B.1.1.7 patient-derived RNA sample, a synthetic B.1.1.7 RNA sample, and a synthetic lineage 19B RNA sample.

We first detected the B.1.1.7 lineage using WGS on December 21, 2020 at Facility 5. This sample satisfied all minimum criteria for B.1.1.7 identification, including the N501Y, ΔH69/ΔV70, and A570D mutations (Fig. 3A), at frequencies between 10 and 27% (Table S5). We additionally detected the spike Y144 deletion and spike single nucleotide variations (SNVs) at P681H, T716I, and S982A. The B.1.1.7 lineage was also detected at Facilities 1, 2, and 6 in samples collected on February 8, 2021, with varying numbers of observed mutations. Seven of the 23 definitional mutations were observed in the Facility 1 sample, all at or below 50% frequency, while 21 and 18 mutations could be identified in the Facility 2 and 6 samples, respectively. On February 15, 2021, B.1.1.7 was detected for a second time in Facility 6 wastewater, this time with nine mutations identified, and for the first time at Facility 7, with seven mutations identified. On February 22, 2021, B.1.1.7 was detected for a second time at Facility 5, with six mutations identified. Among these seven total samples that satisfied all minimum criteria for B.1.1.7 identification, between six and 18 additional B.1.1.7 mutations were observed (Fig. 3A and Table S5).

![Fig. 3. Variants of concern detected by wastewater sequencing surveillance. (A) Known B.1.1.7 and (B) B.1.429 lineage mutations were observed in wastewater sampled between December 2020 and February 2021. Observed variant frequencies are binned as (green) <50% and (blue) 50–100%. Single nucleotide variants (SNVs) with amino acid coding consequences and deletions are indicated and adapted along the genome map. Variant positions were called using iVar variants v1.3, with a 3% minimum frequency and Fisher’s exact test $p < 0.05$ requirement.](image-url)
3.3. Identification of the Epsilon (B.1.429) lineage

We first detected this VOC in a sample collected from Facility 8 (i.e., the manhole for the community shelter) on December 14, 2020. In this earliest sample, all six of the defining B.1.429 mutations were observed, at frequencies ranging between 7 and 80% (Table 6). The fact that this VOC was only detected in the last of four samples collected at Facility 8 suggests that the outbreak at the community shelter likely consisted of multiple SARS-CoV-2 variants. On December 28, 2020, B.1.429 was also detected at Facility 5—the same location where B.1.1.7 was first identified. In three additional wastewater samples (Table 6), we detected the spike protein allele of concern L452R as early as December 7, 2020, but without additional variant observations, association of these samples to B.1.429 could not be made confidently.

3.4. No detection of B.1.351, P.1, and B.1.427 lineages in wastewater

At the time of this study, other VOCs in the United States included B.1.351, P.1, and B.1.427. Therefore, we investigated whether these VOCs could be identified in wastewater samples collected between November 2020 and February 2021. These lineages bear mutations of particular concern at spike protein N501Y (shared with B.1.1.7) and E484K (23012 G>A; not shared with B.1.1.7). The full descriptions of B.1.351 and P.1 in nucleotide and amino acid consequence are provided in Table S4. The E484K mutation was not detected in the sequenced wastewater samples. As such, our data do not support the detection of B.1.351 nor P.1 in Southern Nevada wastewater (as of February 2021). Finally, an additional B.1.427 variant had been identified as a VOC and shares many mutations with the original B.1.429, but B.1.427 also includes two additional mutations (Orf1a.S3158T and Orf1b.P976L), neither of which was observed in the Southern Nevada wastewater samples.

3.5. Whole genome sequencing (WGS) of viral genomes from clinical samples

We also sequenced SARS-CoV-2 genomes from 823 individuals that tested positive for the virus by qPCR in Southern Nevada between December 2020 and April 2021. Using a Clear Labs WGS sequencing platform, we assembled 575 samples for which 90% of bases were called (>27,000 out of 29,903 bases), mean coverage ranged from 40 to 15,000-fold, and median depth was 730-fold.

Genotyping of SARS-CoV-2 clinical samples collected in December 2020 (n = 13) identified only the major clades 20A, 20C, and 20G (Fig. 4A). By January 2021 (Fig. 4B), clinical sampling reached the greatest depth of the study period (n = 246), resulting in the first six clinical observations of VOC B.1.1.7/20I and 81 samples (33%) from the B.1.427 + 429 lineage. Interestingly, these observations occurred one month after their detection in the corresponding wastewater samples. In February 2021 (Fig. 4C), the proportion of identified B.1.427 + 429 increased to 44% of clinical samples (65/149), B.1.1.7/20I was again observed, and VOI lineage B.1.526 was found in five samples. All major clades observed in January were also found in the February collections. Summed together, VOCs and VOIs comprised approximately half of the genotypes observed in the study area by February 2021 (48%; 73/149). Sampling of clinical specimens was lower in the first two weeks of April 2021 (n = 38; Fig. 4E), but the proportion of VOCs and VOIs increased further (79%; 30/38), representing the majority of all SARS-CoV-2 lineages in Southern Nevada.

As noted in the previous section, B.1.351, P.1, and B.1.427 were not detected in Southern Nevada wastewater during the study period. This is consistent with the clinical data for B.1.351, for which there were no detections. The clinical data did capture a small number of P.1 cases in March 2021 (1%; Fig. 4D) and April 2021 (3%; Fig. 4E), but there was no corresponding wastewater sequencing data for those months. Therefore, P.1 may not have been detected in wastewater simply because it was not present as of February 2021. Alternatively, insufficient sensitivity for wastewater sequencing may have resulted in false negatives, or those infected individuals may have been isolated to septic systems. This further highlights the complementary nature of clinical and wastewater surveillance. Finally, it was not possible to confidently distinguish B.1.429 and B.1.427 in wastewater, potentially due to insufficient sensitivity for the characteristic mutations. However, it is unclear whether distinguishing these particular variants would actually be necessary or beneficial in a public health context.

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**Fig. 4.** Whole genome sequencing of SARS-CoV-2 in clinical samples. Genotyping of SARS-CoV-2 clinical samples led to the identification of major clades in Southern Nevada in (A) December 2020, (B) January 2021, (C) February 2021, (D) March 2021, and (E) April 2021. B.1.1.7 and B.1.427/B.1.429 were identified in clinical samples starting in January 2021; both were detected in local wastewater starting in December 2020. The variant of concern lineages accounted for at least 60% of all infections by April 2021.
3.6. Estimating total COVID-19 infections in Southern Nevada

Since the onset of the pandemic, multiple wastewater surveillance, seroprevalence, and probability analysis studies have highlighted discrepancies between estimated COVID-19 infections and confirmed cases (Angulo et al., 2021). Due to limited testing capacity early in the pandemic, the high frequency of mildly symptomatic and asymptomatic infections, and the potential for false negative results, confirmed COVID-19 case counts likely underestimate the true number of infections. In fact, studies indicate the actual infection total may have been up to 20 times higher than the confirmed case count in some areas at certain points in the pandemic (Angulo et al., 2021).

By coupling observed wastewater concentrations across all facilities (Fig. 2), wastewater flow rate (Table 1), and an initial assumption of 11.1 log_{10} gc/infection (Fig. 1), our preliminary calculations indicated that 73% of Southern Nevada had been infected at some point between March 2020 and April 2021, with three of the seven sewersheds yielding infection ratios (or COVID-19 prevalence) >100%. Because there was no reason to believe that nearly the entire population had been infected one or more times by that point in the pandemic, our initial assumption for fecal shedding was assumed to be too low. To resolve this issue, we modified the total SARS-CoV-2 load by calibrating our wastewater calculations to the U.S. Centers for Disease Control and Prevention (CDC’s) reported seroprevalence for Nevada, which was approximately 25% as of spring 2021 (CDC, 2021; https://covid.cdc.gov/covid-data-tracker/#national-lab). In other words, we increased the total fecal load per infection until the wastewater-derived estimate for total infections in Southern Nevada aligned with the CDC’s seroprevalence estimate of 25% (Fig. 1).

Exact calculation to the CDC seroprevalence data resulted in an estimated number of infections for Facility 4 that was slightly less than the confirmed total for that sewershed. Therefore, the fecal shedding estimate had to be higher than the initial assumption of 11.1 log_{10} gc/infection, but lower than the revised assumption of 11.6 log_{10} gc/infection. The total SARS-CoV-2 load was then calibrated further to achieve an ascertainment ratio of at least 1.0 for Facility 4. This final calibration resulted in a revised total SARS-CoV-2 load of 11.4 log_{10} gc/infection, which can be described with a decay approach (initial fecal load = 9.2 log_{10} gc/g) or by assuming a constant fecal load (7.9 log_{10} gc/g) over 25 days (Fig. 1). Interestingly, wastewater surveillance efforts in university dormitories (Schmitz et al., 2021) suggest that fecal shedding may be as high as 9.8 log_{10} gc/g, which supports the higher shedding estimate in the current study.

Using this modified fecal load, our calculations suggest ~38% of Southern Nevada was infected at some point between March 2020 and April 2021, with sewershed-specific estimates ranging from 7% for Facility 4 to 59% for Facility 5 (Table 1). In contrast, clinical testing suggests that only 10% of Southern Nevada had been infected during that same time period. The resulting sewershed-specific ascertainment ratios (i.e., wastewater-derived infection estimates divided by confirmed infections) ranged from 1.0 for Facility 4 to 7.7 for Facility 5. These ascertainment ratios were then used in conjunction with the updated fecal shedding trajectory (Fig. 1) to revise a previously published model for estimating wastewater SARS-CoV-2 concentrations based on confirmed case counts (Gerrity et al., 2021). The resulting sewershed-specific modeled concentrations aligned closely with the observed concentrations (Fig. 2), thereby indicating that the calibrated fecal shedding parameter and the calculated ascertainment ratios effectively corrected for the clinical undercount of COVID-19 infections.

4. Discussion

Molecular tools, including qPCR, ddPCR, and NGS, have proven effective for studying patterns of SARS-CoV-2 transmission in individual buildings, such as dormitories and community shelters, or across entire communities by surveilling large wastewater treatment facilities (Ahmed et al., 2020; Betancourt et al., 2021; Bivins et al., 2020; Grits-Christoph et al., 2021; Gerrity et al., 2021; Hartley et al., 2020; Nemudryi et al., 2020; Tillett et al., 2021). Tracking SARS-CoV-2 VOCs is currently a major priority throughout the world, although efforts are expected to be hindered due to declining rates of diagnostic testing. With growing infection levels across more resilient subpopulations (Monod et al., 2021), specifically younger adults and children that are more likely to be mildly symptomatic or completely asymptomatic (Davies et al., 2020), and the emergence of less virulent strains of SARS-CoV-2, we anticipate a widening discrepancy between total infections and confirmed case counts (i.e., increasing ascertainment ratios). This scenario presents an opportunity—or even an urgent need—for communities to implement wastewater surveillance as a complement to clinical surveillance and outbreak mitigation strategies (McClary-Gutierrez et al., 2021).

The emergence of VOCs poses a clear threat to ongoing public health measures, particularly therapeutic strategies and vaccination efforts, considering that viral mutations have the potential to increase transmission and evade protection from natural or vaccine-induced immunity. For example, early in the pandemic, 55% of Pfizer or Moderna vaccine recipients showed reduced antibody titers to the B.1.427/B.1.429 variants in plaque reduction neutralization tests (Deng et al., 2021). In addition, the B.1.427/B.1.429 variants were 4- to 6-fold more resistant to antibodies from prior infection and 2-fold more resistant to antibodies from vaccination (Garcia-Beltran et al., 2021). Given that some monoclonal antibody treatments were shown to be ineffective against the B.1.427/B.1.429 variants, the U.S. government even recommended against distributing Eli Lilly’s bamlanivimab to California, Arizona, and Nevada (“Coronavirus Disease, 2019 (COVID-19) Treatment Guidelines”). The U.S. Food and Drug Administration eventually revoked the emergency use authorization for this treatment entirely, due to its reduced efficacy against B.1.427/B.1.429 variants (“Coronavirus Disease, 2019 (COVID-19) Treatment Guidelines”).

Our WGS data demonstrate that wastewater surveillance was able to detect the B.1.1.7 and B.1.429 variants in Southern Nevada as early as December 2020, approximately one month earlier than clinical testing. In fact, B.1.1.7 and B.1.429 were detected in the same sewershed within one week of each other, which suggests these variants were likely circulating in that community at the same time. The detection of these variants also coincided with a dramatic surge in confirmed cases in Southern Nevada. With greater sampling depth, these variants may have been detected in clinical samples as well, but sample limitation—only 13 samples were made available for public health surveillance in December 2020—likely hindered their early detection. Therefore, our data demonstrate the value of wastewater surveillance as an early warning system under resource-limited or sample-limited conditions, specifically by providing a pooled sample that effectively increases community sampling depth at significantly reduced costs. With advanced notice, public health officials can be prepared to respond to emerging variants and mitigate their impacts, for example by discontinuing use of ineffective therapeutic strategies. With higher resolution sampling campaigns (e.g., strategic selection of manholes or sewer collector/trunk lines), detection of VOCs in wastewater could potentially identify zip codes for targeted sequencing of clinical samples, to more rapidly identify VOC-infected individuals, and to prioritize contact tracing efforts. Interpretation of wastewater sequencing data can also be improved, considering the wide range of observed mutation frequencies (e.g., <10% to >90%) in individual samples. For example, benchmarking tests can be performed to assess the degree of PCR-induced bias in allele frequency data.

Due to the high costs associated with whole genome sequencing, municipal wastewater surveillance efforts may often be limited to detection and quantification of SARS-CoV-2 by qPCR or ddPCR. Even this more basic application of wastewater surveillance may yield critically important information for public health officials and policy makers. Importantly, facility and sewershed-scale wastewater concentrations provide an unbiased assessment of infection trends and the efficacy of COVID-19 mitigation measures. In resource-limited settings, such as community shelters, nursing homes, or even prisons, wastewater surveillance may also provide an early warning system for disease outbreaks within vulnerable populations.

It is important for public health officials to understand how sewershed characteristics and demographics affect interpretation of wastewater surveillance.
surveillance data, or how these factors might impact transmission. In this study, estimated prevalence (37 ± 3%) and ascertainment ratios (3.4 ± 0.4) were generally similar for the sewersheds represented by primary effluent or composite influent samples and having the largest service areas (i.e., Facilities 1–3). These three sewersheds comprise approximately 85% of the Southern Nevada population and encompass a diversity of demographics, thereby offering a reasonable approximation of community-wide public health conditions. On the other hand, the composite influence samples from Facility 4 resulted in low estimates for prevalence (7%) and ascertainment ratio (1.0), which suggests both a low infection rate and high clinical testing coverage. Facility 4 primarily serves two zip codes with high median household incomes (> $90,000) and older, retirement-age populations (15–20% in the 65–74 age range; https://healthsouthernvada.org, accessed 3/30/21), each of which may have contributed to more favorable public health outcomes. With respect to Facility 7, the wastewater-derived infection estimate supported its relatively low confirmed prevalence, thereby increasing confidence in the clinical surveillance data. This could potentially be explained by the sewersheds’ more isolated geographic location, which may have helped control the spread of COVID-19 in that area.

Early in a pandemic, wastewater-derived ascertainment ratios can provide an unbiased assessment of health disparities and an opportunity for strategic public health action, including targeted testing (e.g., mobile test sites), public information campaigns, contact tracing, and other mitigation efforts. For example, Facilities 5 and 6 exhibited both high prevalence and high ascertainment ratios based on wastewater surveillance data, potentially highlighting opportunities for targeted intervention. With better characterization of fecal shedding, ascertainment ratios could even be estimated with confidence on a rolling basis to provide public health officials with real-time information with high spatial and temporal resolution.

In later stages of a pandemic, estimating total infection levels by sewershed may provide critical information for strategic vaccine rollouts. Specifically, wastewater-derived infection estimates may provide an indication of how many vaccinations are needed in a given area to achieve herd immunity targets. Our analysis suggests that nearly 850,000 people in Southern Nevada had been infected through mid-April 2021, in contrast with the ~240,000 confirmed infections. Assuming previously infected individuals retain adequate levels of protection (Pilz et al., 2022), herd immunity targets of 70–90% would require vaccinations of an additional 715,000 to 1.3 million SARS-CoV-2 naïve individuals. As of mid-April 2021, approximately 500,000 vaccinations had been completed in Southern Nevada. Moreover, sewershed-specific estimates of prevalence and ascertainment ratio could be used to inform vaccine distribution. Simply in a susceptibility context, confirmation of low prevalence (e.g., Facility 4) would suggest a higher risk of outbreak potential as communities relax mitigation measures. These areas could potentially be prioritized during the early rollout phase. There are other risk factors beyond susceptibility that must be considered, including high density living arrangements (e.g., correctional facilities) and vulnerable populations (e.g., long-term care facilities), but wastewater-derived metrics can at least better inform these complex decisions. Importantly, these recommendations would have to be re-evaluated over time to account for waning immunity and the emergence of new VOCs, particularly those with the ability to evade protection and “reset” herd immunity targets (e.g., Omicron).

Due to the lack of robust fecal shedding data in the literature, our study relied on seroprevalence to calibrate our fecal shedding assumptions (Wölfel et al., 2020). This offers a starting point for others to assess COVID-19 incidence based on wastewater surveillance data, but it also highlights the critical need for fecal shedding data for SARS-CoV-2 and for other pathogens of interest in the future. Until these data are available, our study serves as a proof-of-concept for estimating total COVID-19 infections using wastewater-based epidemiology. This study also highlights several additional factors that should be considered when implementing wastewater surveillance for public health decision-making efforts. Grab influent samples proved to be adequate for SARS-CoV-2 detection and general trend analysis, but these samples also appeared to be more susceptible to intermittent spikes in concentration that might be influenced by factors other than COVID-19 incidence (e.g., diurnal variability) (Gerrity et al., 2022). This can have significant implications for wastewater-based epidemiology, particularly for small systems. Based on this experience, our data suggest that wastewater-based epidemiology efforts should rely on 24-hour composite influent samples, if possible, and follow-up sampling whenever a dramatic change in concentration is observed (e.g., the Facility 7 spike). Grab samples may be adequate when monitoring a large sewershed or when collected from a system with high levels of dispersion, such as a sewer collection system with significant mixing or after a primary clarifier.

Looking to the future, two challenges are likely to constrain clinical surveillance of SARS-CoV-2 in the U.S. and around the world: (1) declining rates of reportable diagnostic testing as individuals become unwilling or unable to visit testing sites (Becker et al., 2021) or turn to at-home testing options and (2) lack of access to archived samples from diagnostic labs (Abbasi, 2021). This poses particular concerns for identification and characterization of variants of concern or interest. Our data suggest that implementation of wastewater surveillance, particularly with a combination of qPCR, ddPCR, and NGS tools, can mitigate the impacts of these constraints and lead to the development of an early warning system for SARS-CoV-2, or other pathogens in the future. Overall, such measures can help determine what response to an outbreak is appropriate and evaluate the progress of mitigation or containment efforts. Our data illustrate how wastewater and clinical analyses can be integrated in a large community to evaluate trends in SARS-CoV-2 infections, estimate ascertainment ratios and assess testing adequacy, and rapidly detect the introduction of VOCs at the facility and community-scale.

CRediT authorship contribution statement

Van Vo, Richard L. Tillett, Katerina Papp: Methodology, Supervision, Formal analysis, Writing - original draft.
Shirley Shen, Richard Gu, Andrew Gorgalski, Danielle Siao, Rayma Markland, Ching-Lan Chang, Hayley Baker: Methodology, Resource, Writing - review & editing.
Jingchun Chen, Martin Schiller, Walter Q. Betancourt, Mark Pandorl: Resource, Writing - review & editing.
Erin Buttery, Michael A. Picker: Methodology, Resource, Writing - review & editing, Formal analysis, Project administration, Supervision
Daniel Gerrity, Edwin C. Oh: Formal analysis, Funding acquisition, Project administration, Visualization, Supervision, Investigation, Writing - original draft.
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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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