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Identification of a rare SARS-CoV-2 XL hybrid variant in wastewater and the subsequent discovery of two infected individuals in Nevada

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HIGHLIGHTS

- Wastewater surveillance can provide information about common and rare variants.
- High sequencing depth and genome coverage can reveal actionable SNPs from wastewater samples.
- Recombinant variants can be identified in wastewater.
- Sequencing data from wastewater can lead to the identification of variants in infected individuals.

ABSTRACT

The identification of novel SARS-CoV-2 variants can predict new patterns of COVID-19 community transmission and lead to the deployment of public health resources. However, increased access to at-home antigen tests and reduced free PCR tests have recently led to data gaps for the surveillance of evolving SARS-CoV-2 variants. To overcome such limitations, we asked whether wastewater surveillance could be leveraged to detect rare variants circulating in a community before local detection in human cases. Here, we performed whole genome sequencing (WGS) of SARS-CoV-2 from a wastewater treatment plant serving Las Vegas, Nevada in April 2022. Using metrics that exceeded 100× depth at a coverage >90% of the viral genome, we identified a variant profile similar to the XL recombinant lineage containing 26 mutations found in BA.1 and BA.2 and three private mutations. Prompted by the discovery of this rare lineage in wastewater, we analyzed clinical COVID-19 sequencing data from Southern Nevada and identified two cases infected with the XL lineage. Taken together, our data highlight how wastewater genome sequencing data can be used to discover rare SARS-CoV-2 lineages in a community and complement local public health surveillance.

1. Introduction

Since the start of the COVID-19 pandemic in 2020, timely diagnoses of clinical cases have been hampered by strained healthcare systems, intermittent supply chain issues, and challenges resulting from delays in contact tracing and limited genome sequencing (Vandenberg et al., 2021). Coupled with

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the observation that a large fraction of individuals infected by COVID-19 show mild to no symptoms, clinical testing limitations have resulted in an underestimation of infections across the United States (Buitrago-Garcia et al., 2020). Recent declines in caseloads and/or testing rates, easing of mitigation measures (e.g., lifting of mask mandates and social distancing practices), and reductions in funding have raised questions of whether public health surveillance is still adequate to rapidly detect new COVID-19 surges and deploy public health resources. This may lead to the inability to detect novel and evolving COVID-19 variants that could be highly transmissible, cause severe disease, and evade vaccine-induced or natural immunity.

Wastewater monitoring programs have been leveraged recently as an early warning system to complement public health surveillance for COVID-19 (Bivins et al., 2022; Gerrity et al., 2021; McClary-Gutierrez et al., 2021; Vo et al., 2022a). As of 2022, the U.S. Centers for Disease Control and Prevention’s (CDC) National Wastewater Surveillance System (NWSS) was analyzing and sequencing wastewater from approximately 500 sites across 38 states (Kirby et al., 2021). Such programs can detect SARS-CoV-2 circulating in a community regardless of whether individuals are tested (Ahmed et al., 2020; Betancourt et al., 2021; Medema et al., 2020; Vo et al., 2022b), and because wastewater represents a ‘pooled’ sample of the local community, it may be able to alert public health officials to the introduction or circulation of a variant of interest (VOI) or concern (VOC) even before its detection in a clinical setting (Smyth et al., 2022). These discoveries are possible for some respiratory pathogens (e.g., SARS-CoV-2 and influenza) because their nucleic acid is often found in the fecal material or urine of infected individuals, even if they are asymptomatic (Jones et al., 2020; Wolfe et al., 2022). As a complement to public health data, wastewater analyses reveal the magnitude of viral levels relative to historical data for a given location, potentially highlighting changes in testing coverage. For example, a growing discrepancy between rising viral loads in wastewater and confirmed cases may point to an undetected surge in infections requiring expanded public health surveillance.

Next-generation sequencing tools and quantitative polymerase chain reaction (qPCR)-based approaches have been developed to track the emergence and transmission of new SARS-CoV-2 lineages (Vo et al., 2022a; Crits-Christoph et al., 2021; Fontenele et al., 2021; Nemudryi et al., 2020). While several VOCs such as Alpha, Epsilon, Delta, and Omicron share mutations across each lineage, these VOCs can be identified in clinical samples by the presence of unique mutations. However, since the pooled samples offered by wastewater surveillance may contain degraded nucleic acid contributed by numerous people infected by diverse variants, confident identification of VOCs can be challenging. Current NGS approaches for wastewater sequencing typically generate reads that are less than ~400 nucleotides in length, which poses a challenge for phasing genomes in these pooled samples. To resolve this limitation, new bioinformatic pipelines have been developed to estimate the relative lineage abundances from a sequencing dataset (Karthikeyan et al., 2022; Valierris et al., 2022). In addition, Oxford Nanopore Technologies developed a “Midnight” library preparation chemistry that spans ~1200 nucleotides (Pembaur et al., 2021).

Studies demonstrate that even in a pooled sample containing multiple variants, VOCs with unique characteristic mutations can be identified with confidence (Vo et al., 2022a; Chappleboim et al., 2021). But more recently, new variants have been reported to be hybrid or recombinant viruses sharing mutations from two existing lineages (Chakraborty et al., 2022; Colson et al., 2022). These recombinant strains may emerge when a co-infection by two or more lineages leads to mixing of viral genomes during replication, which then results in the creation of a new sub-lineage. For SARS-CoV-2, there are >1000 possible recombinants that have been reported (He et al., 2022), and preliminary studies indicate that some recombinant variants may be more transmissible than the parent lineages (Li et al., 2020).

In this study, we investigated whether routine wastewater surveillance involving WGS could be deployed to detect rare hybrid variants circulating in an urban community. Because rare variants resulting in low sequencing frequency can sometimes be overlooked, we asked whether a specific sequencing depth was required to replicate WGS results from the same sample. Collectively, these findings demonstrate that sequencing of wastewater samples at high depth can overcome the pooling challenge to identify novel, rare, hybrid variants circulating in a community.

2. Methods and materials

2.1. Wastewater sample collection and quantification of SARS-CoV-2

Since March 2020, weekly sampling has been performed at wastewater treatment plants (WWTPs) serving the majority of the Las Vegas metropolitan area (Gerrity et al., 2021; Vo et al., 2022a). This study focuses on the detection of a recombinant hybrid from a single facility with an average daily flow of ~100 million gallons per day (mgd) serving ~872,000 individuals, hereafter described as WWTP1. Results from two other WWTPs (WWTP2 and WWTP3) were also included for comparison (Table S1). Grab primary effluent samples were collected from the facility every Monday at ~10:00 am using an autosampler. Sample processing for qPCR analysis was performed as described previously (Gerrity et al., 2021), while processing for WGS involved a Wizard Enviro Wastewater TNA kit (Promega Corp). 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, S, Sarbco, N1, and N2) after adjustment for equivalent sample volume and sample-specific recovery of spiked bovine coronaviruses (BGoV) (Gerrity et al., 2021).

2.2. Detection of SARS-CoV-2 mutations in wastewater samples

WGS of wastewater samples was performed using the CleanPlex SARS-CoV-2 FLEX Panel from Paragon Genomics as previously described but with several modifications (Vo et al., 2022a; Vo et al., 2022b). Briefly, >10 ng of total RNA was processed for first-strand cDNA synthesis using the LunaScript RT SuperMix kit (NEB). cDNA was then used for pool 1 and 2 amplification, and barcoded libraries were generated using the Paragon Genomics protocol. Libraries were sequenced using an Illumina NextSeq 500 sequencer with mid-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-amplicon primers were trimmed from the aligned reads using fgbio TrimPrimers version 1.3.0 in hard-clip mode and variants were called by Var variants v1.3 (Grubaugh et al., 2019). Genome coverages were calculated using samtools coverage v1.10 (Li et al., 2009) (Table S1). Sequencing yielded an average of 2.6 million 2 x 150 basepairs per wastewater sample, with mean coverage of ~97% and mean depth ranging between 502- and 2978-fold across all samples (Table S1).

Variants were called from the aligned sample by searching for synonymous and nonsynonymous mutations that have been identified in all variants to date, but this study focused on the variants that were most prevalent in Southern Nevada in April 2022, namely the various Omicron subvariants. For BA.1 and BA.2, we searched for mutations that were unique to each lineage and also for mutations shared with other Omicron subvariants (Table S2). For identification of the recombinant XL sublineage, we searched for the following unique mutations found in the ORF1a gene: Leu204Phe, Val1887Ile, and Ser29815yn (Table S2).

2.3. Processing and whole genome sequencing of clinical samples

Clinical samples were collected and confirmed for the presence of SARS-CoV-2 RNA using RT-qPCR at the Southern Nevada Public Health Laboratory (SNPHL), as described previously (Vo et al., 2022a). WGS was performed by SNPHL using COVIDseq (Illumina), achieving 97.13 % reference coverage and 5953-fold mean read depth. Based on visual inspection of sequencing reads using Integrative Genomics Viewer (IGV) (Robinson et al., 2011), an interactive tool for the visual inspection of genomic data, reported mutations had sufficient sequencing support. The clinical sample, sample 22,600791, was assigned to lineages using Pangolin version 4.0.5 (PUSHER-v1.3), and additional analyses were performed using USHER (Ultrafast Sample placement on Existing tRee) (Turakhia et al., 2021).
2.4. 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 and discussion

3.1. Quantification and whole genome sequencing of SARS-CoV-2 in wastewater

In the first 4 months of 2022, recovery-adjusted SARS-CoV-2 concentrations in Facility 1 wastewater dropped from a maximum of $6.8 \log_{10} \text{gc/L}$ in January—the peak of the initial Omicron (BA.1) surge—to a minimum of $4.5 \log_{10} \text{gc/L}$ in April (Fig. 1A). A similar trend was observed in all Southern Nevada sewersheds during that timeframe (Wastewater Surveillance, 2022). Despite the local decline in wastewater concentrations, a number of Omicron subvariants were detected in clinical samples throughout the world, raising concerns about the possibility of immune evasion and subsequent infection surges.

During the week of April 18, 2022, sequencing of wastewater samples satisfied all minimum criteria for both BA.1 and BA.2 identification, including the Thr376Ala, Lys417Asn, and Asn501Tyr mutations in the spike gene (Fig. 1B and Table S2).

3.2. Identification of the XL recombinant lineage in wastewater from Southern Nevada

Recombination is a common phenomenon in coronaviruses and is a process that leads to the accumulation of mutations and increased transmissibility in viral hybrid strains (Li et al., 2020; Burel et al., 2022). For

Fig. 1. Quantification and whole genome sequencing of SARS-CoV-2 in wastewater and identification of the XL recombinant. (A) Summary of new daily cases (based on official reporting date) and recovery-adjusted SARS-CoV-2 wastewater concentrations from Jan 2, 2022 to May 2, 2022 in WWTP 1. (B) Variant prevalence (according to Freyja (Karthikeyan et al., 2022)) for a WWTP 1 sample collected on April 18, 2022 and sequenced in duplicate. (C) Characterization of the XL recombinant lineage observed in the April 18, 2022 wastewater sample from WWTP 1. 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 as shown previously (Vo et al., 2022a). Observed variant frequencies are shown as (green) $<10 \% \text{ SNP frequency and } >100 \times \text{ read depth and (blue) } >90 \% \text{ SNP frequency and } >100 \times \text{ read depth.}$ The wastewater and clinical samples are listed below the SARS-CoV-2 genome schematic.
example, recombination of Alpha and Delta (Sekizuka et al., 2022) VOCs and Delta and Omicron (Colson et al., 2022) VOCs have been documented. In addition, new Omicron-Omicron recombinants have also been described recently (Chakraborty et al., 2022; Rahimi and Talebi Bezmin Abadi, 2022). Since several hybrid strains are often connected at a breakpoint, a major technical question in identifying hybrid strains using a tiled-amplicon approach is whether genomes can be phased across the breakpoint using amplicons that are ~200–300 nucleotides in length. In response to reporting of recombinant variants in the United States during March 2022, we investigated whether such recombinant genomes were present in our samples and searched for unique mutations that have been annotated in recombinant strains. Using this approach, we detected two nucleotide changes in ORF1a from one of three samples collected on April 18, 2022 (i.e., from WWTP1 but not WWTP2 or WWTP3). These coding changes—Leu204Phe and Val1887Ile—had mutation frequencies of 3.2 % and 8.7 %, respectively. Based on previous SARS-CoV-2 sequencing experience, single nucleotide polymorphisms (SNPs) with low frequencies (<10 %) can sometimes be missed or misinterpreted, justifying replicate sequencing to confirm rare mutations or lineages. Duplicate sequencing of this WWTP1 sample yielded results that were >90 % identical but also yielded a third unique non-coding mutation characteristic of the XL recombinant (CoV-lineages, 2022) —Thr9208Cys—with a SNP frequency of 5.1 %.

Given the presence of all three unique XL mutations in the wastewater sample, we next asked whether we could identify clinical cases in Southern Nevada that were infected by the same XL recombinant. On April 19, 2022, a clinical sample was sequenced by the Southern Nevada Public Health Laboratory and passed all quality control parameters (see methods). The sample was assigned to a candidate recombinant clade with one parent in Omicron (BA.1) and the other parent in Omicron (BA.2). A single breakpoint at 8392 nucleotides in the genome was identified, and mutations associated with both BA.1 and BA.2 were found and separated by the breakpoint (Clinical sample 1: Fig. 1C). The purified RNA was sent to CDC for confirmation of the XL recombinant lineage. Subsequent analysis of GISAID revealed that another Nevadan, who was tested on April 9, 2022, was also assigned to the XL lineage (Clinical sample 2: Fig. 1C).

Using Nextclade, we determined the lineage components of the recombinant XL genome by taking each fragment that was separated at the breakpoint position. We positioned each fragment (1–8393 and 8394 to the 3’ end) onto a reference tree and visualized the parents of the recombinant genomes with auspice. While the 1–8393 fragment clustered with BA.1, the 3’ end clustered with BA.2, demonstrating that this XL lineage was a hybrid between the Omicron/BA.1 and Omicron/BA.2 VOCs (Fig. 2).

4. Conclusion

While it has subsequently been shown that the XL recombinant likely does not exhibit higher transmissibility than BA.1 or BA.2 (outbreak.info, 2022), this study illustrates how detection of rare mutations in wastewater can potentially provide advanced notice of rare recombinant SARS-CoV-2 genomes circulating in the local community. A limitation to our study is that we do not know whether either clinically confirmed XL case actually contributed to the WWTP where the XL genome was detected. Our wastewater results may in fact be associated with a larger number of individuals infected by the XL variant. Nonetheless, the wastewater data shows concordance with clinical observation of lineages. Recombinant genomes pose significant bioinformatics challenges even for identification in clinical samples, but our study highlights how high depth (>100-fold depth) at >80 % genome coverage can overcome these challenges in pooled wastewater samples.

CRediT authorship contribution statement

Van Vo, Anthony Harrington: Methodology, Supervision, Formal analysis, Writing – original draft.
Salman Afzal, Katerina Papp, Ching-Lan Chang, Hayley Baker, Perseveranda Aguilar, Erin Buttery, Michael A. Picker: Methodology, Resource, Writing – reviewing & editing.
Cassius Lockett, Horng Yuan Kan: Resource, Writing – reviewing & editing.
Daniel Gerrity, Edwin C. Oh: Formal analysis, Funding acquisition, Project administration, Visualization, Supervision, Investigation, Writing – original draft.
Anthony Harrington, Van Vo, and Salman Afzal are co-first authors and contributed equally.

Data availability

Data will be made available on request.
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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.160024.

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