Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Temporal dynamics of SARS-CoV-2 genome and detection of variants of concern in wastewater influent from two metropolitan areas in Arkansas

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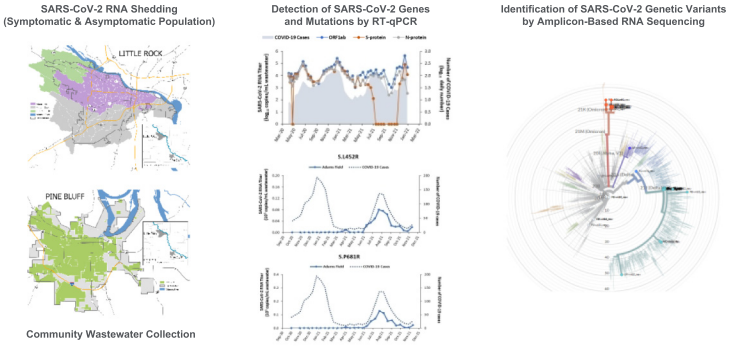
HIGHLIGHTS

- SARS-CoV-2 genomic changes in wastewater during major COVID-19 surges in Arkansas
- Changes in SARS-CoV-2 genome can affect the sensitivity of specific RT-qPCR assays.
- Allele-specific RT-qPCR assays detected key mutations in SARS-CoV-2 S-gene.
- Amplicon-based RNA sequencing detected SARS-CoV-2 variants of concern in wastewater.

GRAPHICAL ABSTRACT



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ABSTRACT

Although SARS-CoV-2 can cause severe illness and death, a percentage of the infected population is asymptomatic. This, along with other factors, such as insufficient diagnostic testing and underreporting due to self-testing, contributes to the silent transmission of SARS-CoV-2 and highlights the importance of implementing additional surveillance tools. The fecal shedding of the virus from infected individuals enables its detection in community wastewater, and this has become a valuable public health tool worldwide as it allows the monitoring of the disease on a populational scale. Here, we monitored the presence of SARS-CoV-2 and its dynamic genomic changes in wastewater sampled from two metropolitan areas in Arkansas during major surges of COVID-19 cases and assessed how the viral titers in these samples related to the clinical case counts between late April 2020 and January 2022. The levels of SARS-CoV-2 RNA were quantified by reverse-transcription quantitative polymerase chain reaction (RT-qPCR) using a set of TaqMan assays targeting three different viral genes (encoding ORF1ab polyprotein, surface glycoprotein, and nucleocapsid phosphoprotein). An allele-specific RT-qPCR approach was used to screen the samples for SARS-CoV-2 mutations. The identity and genetic diversity of the virus were further investigated through amplicon-based RNA sequencing, and SARS-CoV-2 variants of concern were detected in wastewater samples throughout the duration of this study. Our data show how changes in the virus genome can affect the sensitivity of specific RT-qPCR assays used in COVID-19 testing with the surge of new variants. A significant association was observed between viral titers

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in wastewater and recorded number of COVID-19 cases in the areas studied, except when assays failed to detect targets due to the presence of particular variants. These findings support the use of wastewater surveillance as a reliable complementary tool for monitoring SARS-CoV-2 and its genetic variants at the community level.

1. Introduction

In late 2019, a novel human coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was first detected in Wuhan, China, causing an outbreak of a coronavirus disease (COVID-19). This virus quickly spread to other countries, and on 11 March 2020, the World Health Organization declared the viral spread a pandemic (Cucinotta and Vanelli, 2020). The first known case of COVID-19 in the United States (USA) was reported in Washington State on 20 January 2020, and due to the widespread transmission across the country, the USA has become heavily affected by COVID-19, both in number of cases and deaths per capita (https://coronavirus.jhu.edu/map.html; Holshue et al., 2020).

COVID-19 is an ongoing and life-threatening disease in humans and has a wide spectrum of clinical presentations and outcomes (Huang et al., 2020). Even though respiratory symptoms represent the main clinical presentation of COVID-19, a significant proportion of patients develop gastrointestinal symptoms (Cheung et al., 2020; Cholankeril et al., 2020). SARS-CoV-2 enters the host cell through binding to the angiotensin-converting enzyme 2 receptor, which is abundantly expressed on the epithelial cell lining, not only on the lungs, but also in the gastrointestinal tract (Xu et al., 2020; Yan et al., 2020; Xiao et al., 2020). This mechanism supports the possibility of viral replication and consequent shedding through the feces, which also occurs in asymptomatic individuals (Holshue et al., 2020; Lamers et al., 2020; Wu et al., 2020a).

Despite the other sources of viral RNA shedding routes, such as sputum, saliva, and urine, stool has repeatedly been implicated as the primary contributor to the viral load of SARS-CoV-2 RNA in wastewater (Ahmed et al., 2020; Wannigama et al., 2021; Wolfe et al., 2021; Crank et al., 2022). About 40 % of infected individuals shed SARS-CoV-2 in feces, and although the likelihood of viral shedding in respiratory fluids is higher ($\approx$80 %), the volume of stool discharged daily to the sewer system is orders of magnitude higher (Roshandel et al., 2020; Khiabani and Amirzade-Iranaq, 2021; Crank et al., 2022).

The awareness that humans can become severely ill by new, highly pathogenic strains of coronaviruses highlights the importance of monitoring their circulation in the environment, especially as they are usually underdiagnosed. Although SARS-CoV-2 causes severe illness and death, a percentage of the infected population is asymptomatic. This, along with other factors, such as insufficient diagnostic testing, contributes to the silent transmission of SARS-CoV-2 (He et al., 2020; Kimball et al., 2020). Thus, consideration for additional tools has arisen with the goal to improve the strategies used for the detection of SARS-CoV-2. The shedding of detectable viral RNA in the feces of infected individuals enables its detection in community wastewater, and this has become a valuable public health tool worldwide as it allows the monitoring of the disease on a populational scale.

This approach is successfully being explored in the surveillance of SARS-CoV-2 in the community at many locations in the USA and other countries (Ahmed et al., 2020; D'Aoust et al., 2021; Haramoto et al., 2020; Kumar et al., 2020; La Rosa et al., 2020; Medema et al., 2020a; Peccia et al., 2020; Wu et al., 2020b; Gerrity et al., 2021; Graham et al., 2021; Prado et al., 2021; Takeda et al., 2021; Nagarkar et al., 2022). Additionally, research groups throughout the world have engaged in nationwide interlaboratory programs in wastewater monitoring of COVID-19, including Netherlands (Medema et al., 2020b), north America (Chik et al., 2021; Kirby et al., 2021; Pecson et al., 2021; Kumblathan et al., 2022), Australia (Ahmed et al., 2021), France (Cluzel et al., 2022; Wurtzer et al., 2022), other countries in the European Union (Agrawal et al., 2022; Wade et al., 2022), and countries in Africa, Asia, and South America (Street et al., 2020; Prado et al., 2021; Street et al., 2021; Takeda et al., 2021).

The emergence of new SARS-CoV-2 genetic variants has raised concerns not only about possibly increased pathogenicity of this virus, but also the impact on the effectiveness of the vaccines and sensitivity of detection. Viral mutations may occur as an adaptation to the host and evasion to the immune response. Coronaviruses, in particular, are known for the high frequency of genetic mutation and recombination (Woo et al., 2009).

As this virus continues to evolve and challenge the public health response, the development of more sensitive methods to reflect its spread in the communities is important. Hence combining prospective collection of wastewater surveillance as a tool to monitor the endemic and dynamics of COVID-19 at the community level would be beneficial.

In the current study, we optimized RT-qPCR and amplicon next-generation sequencing methods to detect and determine the abundance (viral titer) of SARS-CoV-2 RNA in wastewater samples in two metropolitan areas of Arkansas, Little Rock and Pine Bluff, for over 20 months. We show the viral genome dynamics during major surges of COVID-19 cases in the state of Arkansas and assess how the viral titers and variants in the wastewater samples related to the reported clinical COVID-19 cases count. The findings presented here might contribute and support federal agencies, such as the Centers for Disease Control and Prevention and the U.S. Environmental Protection Agency, in their ongoing efforts to monitor SARS-CoV-2.

2. Material and methods

2.1. Wastewater samples

One liter of 24-hour composite samples of wastewater influent was collected every other week in three water treatment facilities in Little Rock, AR (Little Rock Water Reclamation Authority: Adams Field, Fourche Creek, Little Maumelle) and one in Pine Bluff, AR (Pine Bluff Wastewater Utility) (Fig. 1). The wastewater influent flow and estimated size of the population for each service basin are presented in Table 1. The facility located in Pine Bluff also receives wastewater from the city of White Hall, which is considered part of the Pine Bluff metropolitan area.

The wastewater samples were collected from the headworks of each facility using a high frequency, automated, and refrigerated sampler. During the collection, wastewater samples were thoroughly mixed and transferred to high-density polyethylene bottles and kept on ice for transport, which happened mostly within the same day. One hundred and eighty-six samples were assessed between 28 April 2020 and 25 January 2022. Due to technical issues, no wastewater samples were collected on week 34 at Little Maumelle facility or week 76 at Fourche Creek facility.

Before processing, the wastewater samples were pasteurized at 56 °C for 30 min to reduce biosafety risk from bioaerosol-generating procedures during wastewater sample handling and stored at 4 °C until further processing, which occurred mostly within 24 h of collection.

2.2. Wastewater sample seeding and viral concentration

Bovine coronavirus (BCoV) was used as a surrogate for human coronavirus to assess viral recovery and to serve as a control of sample processing (centrifugation, viral concentration, RNA extraction, and RT-qPCR).

A cell-adapted BCoV strain 88 (BCoV-88) was cultivated in human rectal tumor (HRT)-18 cells and 1 mL at 2×10^6 copies was used to spike the pasteurized wastewater samples (1:200). The BCoV-seeded wastewater samples (200 mL) were centrifuged at 10,000 $\times g$ for 30 min at 4 °C in a fixed-angle rotor JLA-16.250, Avanti JXN-30 centrifuge (Beckman Coulter, Brea, CA) (Mullis et al., 2012). The supernatant was collected and incubated with 10 % polyethylene glycol (PEG) 8000 (Fisher Scientific, Hampton, NH) and 2.2 % sodium chloride (MilliporeSigma,

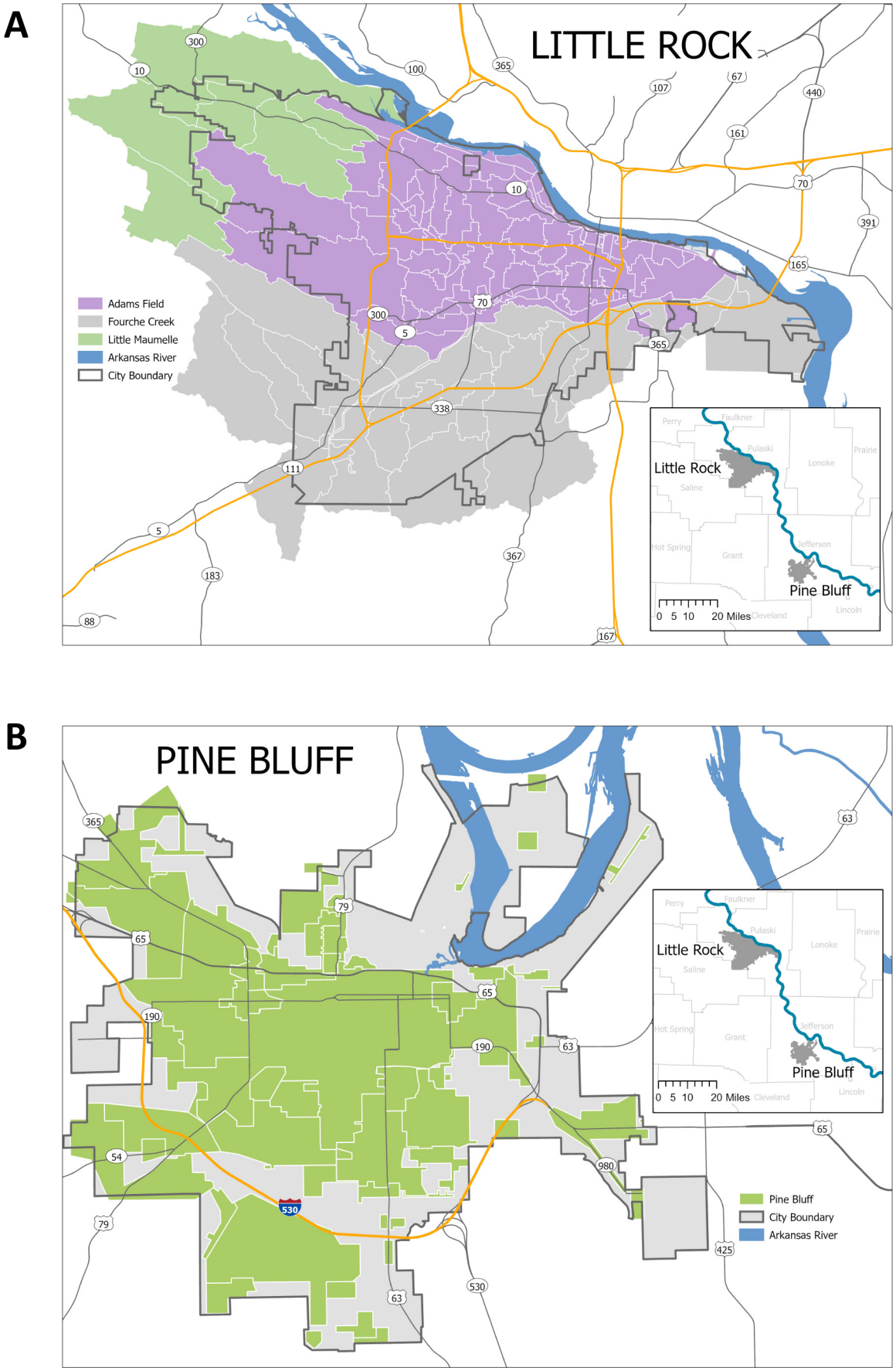


Fig. 1. Service basins of each wastewater treatment facility in this study within Little Rock **(A)** and Pine Bluff **(B)**, AR. The geographic information shown here was provided by Little Rock Water Reclamation Authority and Pine Bluff Wastewater Utility.

Table 1
Wastewater influent flow and estimated size of the population sampled in each water treatment facility included in the study[a].

Wastewater treatment facility[b]	Location	Population size	Average influent flow (cubic meters per day)[b]
Adams Field	Little Rock	135,769	79,115.1 ± 3785.4
Fourche Creek	Little Rock	50,239	35,582.9 ± 3406.9
Little Maumelle	Little Rock	10,285	7570.8 ± 378.5
Pine Bluff	Pine Bluff	≈50,000	30,283.3 ± 3028.3

[a] Information provided by Little Rock Water Reclamation Authority and Pine Bluff Wastewater Utility.
[b] These data represent the average flow of the wastewater samples collected during the period of this study and are expressed as mean ± standard error of the mean.

Saint Louis, MO) at 4 °C overnight, followed by centrifugation at 10,000 ×*g* for 30 min at 4 °C. Similar to other studies reporting the detection of SARS-CoV-2 in wastewater, the PEG method was used for viral concentration (Wu et al., 2020b; Graham et al., 2021; Kumar et al., 2020).

2.3. Nucleic acid isolation and spike-in

Different RNA extraction protocols were tested to optimize the extraction protocol. Three untreated wastewater samples and one Milli-Q water negative control sample were processed under different conditions for initial method development. Following incubation at 56 °C for 30 min, these samples were centrifuged at 10,000 ×*g* for 30 min at 4 °C in a fixed-angle rotor JLA-16.250 and processed with or without filtration (Stericup Quick Release-HV Sterile Vacuum Filtration System 0.45 μm pore size, MilliporeSigma) before viral concentration. Two commercial kits were tested for viral RNA extraction: **A.** MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit (Applied Biosystems, Foster City, CA) and **B.** MagMAX™ Microbiome Ultra Nucleic Acid Isolation Kit (Applied Biosystems). Kit A is recommended for RNA isolation of SARS-CoV-2 from biofluid samples, while Kit B is intended for fecal samples and includes an additional step with lysis buffer and zirconia beads. Only Kit B was successful on the detection of the SARS-CoV-2 genes being assessed in wastewater samples using RT-qPCR (Supplementary Table 1). Samples submitted to filtration gave a higher threshold cycle (Ct) than samples that were not filtered; therefore, this step was removed from our protocol. Additionally, the resuspension of the PEG precipitate in lysis buffer instead of phosphate buffered saline yielded a higher concentration of RNA because it eliminated one dilution step.

The pellet resulting from PEG precipitation was resuspended in 1.2 mL lysis buffer, provided with Kit B. Samples were transferred to a microtube containing zirconia beads and processed as indicated by the manufacturer's protocol. Viral RNA was extracted from 400 μL of lysate using the King Fisher™ Flex purification system (Thermo Fisher Scientific, Waltham, MA). One hundred thousand copies of TaqMan® Universal RNA Spike In/Reverse Transcription (Xeno) Control (Applied Biosystems) were added to the binding solution per sample to monitor RNA recovery and RT-qPCR inhibition. Nuclease-free water was included as a negative extraction control. Purified RNA samples were eluted in 50 μL elution buffer and stored at −80 °C.

2.4. Viral RNA detection using reverse-transcription quantitative polymerase chain reaction (RT-qPCR)

The presence of SARS-CoV-2 RNA was assessed by RT-qPCR with a set of TaqMan™ RT-PCR assays (TaqMan™ 2019-nCoV Assay Kit v1, Applied Biosystems) targeting three different viral genes [encoding open reading frame 1ab polyprotein (ORF1ab), surface glycoprotein (S-protein), and nucleocapsid phosphoprotein (N-protein)], and one endogenous control (Human RNase P RPPH1 gene). A volume of 2.5 μL RNA was used as template in a final 10 μL reaction volume. Synthetic DNA target sequences for each of the assays were used as positive controls (TaqMan™ 2019-nCoV Control Kit v1, Applied Biosystems), as recommended by manufacturer. Nuclease-free water was included as a no-template control.

As negative controls for SARS-CoV-2, Milli-Q water and tap water collected in Little Rock and Pine Bluff were processed in the same way as the wastewater samples (centrifugation, viral concentration, and RNA extraction), and tested in concurrently with the wastewater samples by RT-qPCR.

To examine potential cross-reactivity between BCoV and SARS-CoV-2, BCoV-seeded Milli-Q water was tested with the assays for the SARS-CoV-2 genes (ORF1ab, S-protein and N-protein). In addition, 2019-nCoV Control (Applied Biosystems) was assayed for BCoV. No cross-reaction was observed (undetermined Ct values in all cases).

RT-qPCR was performed on a QuantStudio™ 7 Flex (Applied Biosystems) using a one-step kit (TaqPath™ 1-Step RT-qPCR Master Mix, Life Technologies, Carlsbad, CA). The thermocycling conditions were: UNG incubation at 25 °C for 2 min, reverse transcription at 50 °C for 15 min, polymerase activation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 3 s and annealing/extension at 60 °C for 30 s. Each reaction was performed in duplicate. The amplification curves were analyzed using QuantStudio™ Real-Time PCR software version 1.3 (Applied Biosystems).

Biases due to changes in wastewater dilution and human waste input over the period of surveillance were corrected by quantifying pepper mild mottle virus (PMMoV), a plant pathogen highly abundant in domestic wastewater with potential use as an enteric virus process indicator (Rosario et al., 2009; Symonds et al., 2018). This normalization also accounts for biases caused by sampling, storage, and processing. PMMoV has been used consistently as an internal reference for SARS-CoV-2 in wastewater in other studies (D'Aoust et al., 2021; Wu et al., 2020b; Gerrity et al., 2021; Graham et al., 2021). Therefore, the SARS-CoV-2 titers measured in this study were normalized to PMMoV relative levels in each sample.

TaqMan gene expression assays used for the RT-qPCR detection of BCoV and PMMoV are shown on Table 2. The sequences used for the detection of SARS-CoV-2 (TaqMan™ 2019-nCoV Assay Kit v1) and Xeno™ Control (Assay ID Ac00010014_a1) are proprietary information and have not been disclosed by the manufacturer.

2.5. Standard curves and viral recovery rate

A standard curve was built with RNA extracted from BCoV-88/HRT-18-seeded Milli-Q water (1:200) submitted to the same processing methods (centrifugation, viral precipitation, RNA extraction, and RT-qPCR) as the wastewater samples. The starting genome copy number was 2.0×10^6 genome equivalents/mL. Ten-fold serial dilutions were prepared corresponding to 5.21×10^4 to 0.00521 genome equivalents/sample.

The percentage of viral recovery was estimated by comparing the number of copies of BCoV detected by RT-qPCR (number of copies recovered) in seeded wastewater samples versus the known number of copies used to seed these samples: % viral recovery = number of viral RNA copies recovered/number of viral RNA copies seeded × 100 (Ahmed et al., 2020; D'Aoust et al., 2021).

To determine the viral titer of the SARS-CoV-2 RNA in the wastewater samples, standard curves were generated for each of TaqMan 2019-nCoV assay (ORF1ab, S-protein and N-protein) by amplification of viral RNA extracted from Milli-Q water seeded with known amounts of the heat-inactivated SARS-CoV-2 isolate USA-WA1/2020 (NR-52286, Biodefense and Emerging Infections Research Resources Repository, Manassas, VA) (Harcourt et al., 2020). The starting genome copy number (as estimated

Table 2
TaqMan gene expression assays used for the detection of BCoV and PMMoV by RT-qPCR[a].

Gene	Position[b]	Sequence (5′ → 3′)	Product (bp)	Source
BCoV Orf1ab	15,615–15,637	F: GCGTCCAAAGGCTATATTGCTAA	91	Izzo et al., 2012
	15,705–15,682	R: CCCAACATTTGGATTCTGACATAA		
	15,644–15,661	Probe: TGCCTTTCAACAGGTATT		
PMMoVgp2	1878–1901	F: GAGTGGTTTGACCTTAACGTTTGA	68	Haramoto et al., 2013
	1945–1926	R: TTGTCGGTTGCAATGCAAGT		Zhang et al., 2006
	1906–1921	Probe: CCTACCGAAGCAAATG		Zhang et al., 2006

[a] BCoV, Bovine coronavirus; PMMoV, Pepper mild mottle virus; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; Orf1ab, open reading frame 1ab polyprotein-encoding gene, PMMoVgp2, replication-associated protein-encoding gene; F, forward oligonucleotide; R, reverse oligonucleotide.
[b] Oligonucleotide position is referred to the complete genome of BCoV (GenBank accession number: NC_003045.1) and PMMoV (GenBank accession number: NC_003630.1).

by digital PCR performed by the manufacturer) was 3.75×10^8 genome equivalents/mL. Ten-fold serial dilutions were prepared corresponding to 1.95×10^5 to 0.195 genome equivalents/sample.

The estimated viral RNA copies were calculated for each set of TaqMan 2019-nCoV assay (ORF1ab, S-protein and N-protein) and BCoV assay by plotting Ct values onto standard curves for each of the genes analyzed, where $\log_{10}$ (copy number) = (Ct – Y intercept)/slope. Linear regression analysis yielded a trend line equation that was used to derive the quantity of viral RNA in each of the samples from the Ct values. Additionally, ten-fold serial dilutions were prepared with RNA isolated from wastewater samples to verify amplification efficiency of the PMMoV and Xeno Control assays.

2.6. Detection of SARS-CoV-2 mutations using RT-qPCR

A subset of wastewater samples collected between October 2020 and January 2022 and that tested positive for SARS-CoV-2 was screened for the presence of selected mutations on the S-gene (Table 3). Sixteen commercially available probe-based genotyping TaqMan™ SARS-CoV-2 Mutation Panel assays (Applied Biosystems) were used. The allelic discrimination of each assay was determined by two probes with a minor groove binder, a non-fluorescent quencher, and the following 5′ reporter dyes: one VIC™ dye-labeled probe to detect the reference sequence and one FAM™ dye-labeled probe to detect the mutation sequence, as described by the manufacturer. The assays were combined with TaqPath™ 1-Step RT-qPCR Master Mix, CG (Life Technologies) and the RT-qPCR was performed with a QuantStudio™ 7 Flex (Applied Biosystems), following the manufacturer's protocol. The thermocycling conditions were pre-read at 60 °C for 30 s, reverse transcription at 50 °C for 10 min, polymerase activation at 95 °C for 2 min, 45 cycles of denaturation at 95 °C for 3 s and annealing/extension at 60 °C for 30 s, followed by post-read stage at 60 °C for 30 s. Data analyses and genotype calling were performed using the QuantStudio™ Real-Time PCR software version 1.3 (Applied Biosystems). For genotyping assessment, each mutation was differentiated by comparing its relative Ct values to the reference (wild type) SARS-CoV-2 S-gene assay that served as a benchmark.

2.7. SARS-CoV-2 genome sequencing

Selected wastewater samples that tested positive for SARS-CoV-2 around the COVID-19 surges, as determined with ORF1ab-gene and N-gene RT-qPCR assays, were used for SARS-CoV-2 next-generation sequencing analysis. A total RNA of 73 samples collected on Weeks 36–42, 50, 60–68, and 76–92 across the four wastewater treatment facilities was extracted from wastewater samples using MagMAX™ Microbiome Ultra Nucleic Acid Isolation Kit (Applied Biosystems), as described in Section 2.3, except these samples were not seeded with BCoV. For better quality of the sequencing libraries, the eluted RNA was treated with DNase (TURBO DNA-free™ kit, Invitrogen, Waltham, MA), following the manufacturer's protocol.

Total RNA concentrations were quantified using a NanoDrop 2000c spectrometer (Thermo Fisher Scientific). RNA libraries for amplicon RNA-sequencing were prepared from total RNA using AmpliSeq Library PLUS for Illumina library preparation kits and AmpliSeq for Illumina SARS-CoV-2 Research Panel (Illumina, San Diego, CA), which contains 247 amplicons in two pools targeting the SARS-CoV-2 genome. The panel includes >99 % coverage of the SARS-CoV-2 genome (≈30 kb) and covers all potential genotypes. Reverse transcription was performed using 200 ng of total RNA from a SARS-CoV-2-positive wastewater concentrated sample

Table 3
List of TaqMan™ SARS-CoV-2 mutation panel assays used for the genotyping RT-qPCR[a].

Mutation[b]	World Health Organization label	Associated variants	Earliest documented samples
S.D614G.GAT.GGT	Various	B.1.1.207, P.1, B.1.1.33, B.1.1.7, B.1.177, B.1.258, B.1.351, B.1.525, Mink variant	Nigeria, United Kingdom, South Africa, Brazil/Amazon
S.delH69V70	Alpha	B.1.1.7, B.1.258, B.1.525	United Kingdom
S.delY144	Alpha	B.1.1.7	United Kingdom
S.E484K.GAA.AAA	Beta, Gamma, Eta	P.1,B.1.1.33, B.1.351, B.1.525	South Africa, Brazil
S.L18F.CTT.TTT	Gamma	P.1, B.1.351	South Africa, Brazil/Amazon
S.L452R.CTG.CGG	Delta, Epsilon	B.1.617, B.1.617.1, B.1.617.2, B.1.617.3, B.1.429	India, California
S.P681H.CCT.CAT	Alpha	B.1.1.207, B.1.1.7	Nigeria, United Kingdom
S.P681R.CCT.CGT	Delta	B.1.617.1, B.1.617.2, B.1.617.3	India
S.S477N.AGC.AAC	lota	B.1.526	New York
S.T20N.ACC.AAC	Gamma	P.1	Brazil/Amazon
S.T478K.ACA.AAA	Delta	B.1.617.2	
S.EFR156-158G[c]	Delta	B.1.617.2	India
S.N501Y.AAT.TAT	Alpha, Beta, Gamma	P.1, B.1.1.7, B.1.351	United Kingdom, South African, Brazil/Amazon
S.K417N.AAG.AAT	Beta	B.1.351	South Africa
S.G339D.GGT_GAG	Omicron	B.1.1.529	Various countries
S.Q493R.CAA_CGA	Omicron	B.1.1.529	Various countries

[a] Information obtained from the TaqMan SARS-CoV-2 Mutation Panel by Thermo Fisher Scientific.
[b] Naming convention: gene name, amino acid mutation, reference codon and mutant codon; except for S.EFR156-158G and the multi-nucleotide deletions (S.delH69V70 and S.delY144). S, spike protein-encoding gene.
[c] S.EFR156-158G: two amino acid deletions (E156del and F157del) and a substitution (R158G) (Fatihi et al., 2021; Kannan et al., 2021).

and the AmpliSeq cDNA Synthesis for Illumina kit (Illumina), according to the manufacturer's recommendations, using the following program: 42 °C for 30 min and 85 °C for 5 min. The cDNA of SARS-CoV-2 was then amplified by PCR with the two AmpliSeq for Illumina SARS-CoV-2 Research Panel primer pools (Illumina) and AmpliSeq HiFi Mix (Illumina) using the following program: 99 °C for 2 min, 19 cycles of 99 °C for 15 s and 60 °C for 4 min. Each PCR product was partially digested with FuPa Reagent (Illumina), ligated with AmpliSeq CD Indexes Set A for Illumina (Illumina), and purified with Agencourt AMPure XP beads (Beckman Coulter Life Sciences, Indianapolis, IN), following the manufacturer's recommendations. Next, the index-ligated PCR products were amplified with Lib Amp Mix (Illumina) and Library Amp primers (Illumina) using the following program: initial denaturation 98 °C for 2 min, 9 cycles of 98 °C for 15 s and 64 °C for 1 min. After the final purification with Agencourt AMPure XP beads, the libraries were quantified using Qubit 2.0 fluorometer (Invitrogen) and the fragment sizes were analyzed on a 2100 Agilent Bioanalyzer (Agilent, Santa Clara, CA) using High Sensitivity DNA Reagents and chips kits (Agilent). The libraries were normalized to a 4 nM concentration, pooled, denatured, and diluted in HT1 buffer (Illumina), according to the Illumina's standard sequencing protocol. Dual-indexed pair-end sequencing with a 150-bp read length was carried out on the Illumina NextSeq 500 sequencing system (Illumina).

FASTQ files were uploaded into on BaseSpace Sequence Hub (Illumina) for bioinformatic analysis. After adapter trimming, raw sequencing data were aligned to a SARS-CoV-2 reference genome and the coverage of targeted regions, variant calling, and consensus sequences were generated with the Illumina DRAGEN COVID Lineage application and Explify Respiratory Pathogen ID/AMR Panel (RPIP) software within BaseSpace Sequence Hub using the Illumina's suggested parameters.

The SARS-CoV-2 isolate Wuhan-Hu-1 sequence (MN908947.3) was used as the reference genome. For variants detection, the following parameters were used: minimum coverage of 10×, Phred score > 30, and a minimum frequency threshold of 50 %. Lineage/clade analysis of SARS-CoV-2 wastewater samples with >80 % genome coverage was performed using Pangolin and NextClade tools, v1.13.1, https://clades.nextstrain.org/ (Aksamentov et al., 2021). The relative abundance of SARS-CoV-2 lineages was estimated in wastewater samples by using the bioinformatic package Freyja (https://github.com/andersen-lab/Freyja) from the Andersen Lab at the Scripps Research Institute (Karthikeyan et al., 2022).

2.8. Epidemiological data and statistical analysis

Data for 7-day moving COVID-19 clinical case count averages for each day from April 2020 until January 2022 for persons residing in the Little Rock and Pine Bluff metropolitan areas were obtained from the Arkansas Department of Health. Cases, as defined by a positive antigen or RT-qPCR-based test specific for SARS-CoV-2, were attributed to a date in time based on when the sample was collected. If the sample collection date was missing, the date the result was reported was used. An epi-curve was generated from these data for each city location. Epi-curves are graphical representations of the number of cases by date and are utilized to determine the status of an outbreak (Reintjes and Zanuzdana, 2010).

General linear models were utilized to analyze the association between the log-transformed SARS-CoV-2 copy number normalized to PMMoV from each flow site separately and the average number of COVID-19 detected cases by city (Little Rock and the larger Pine Bluff metropolitan area) by date. A trend analysis was performed comparing the slopes of the wastewater SARS-CoV-2 copy number values to the slopes of the 7-day moving COVID-19 case number average to contextualize the burden of reported COVID-19 cases represented within the wastewater samples and to aid in interpretation of findings. Relationships were considered significant at p-value < 0.05. Of note, point estimates of the actual community burden were not calculated because data were not available for the prevalence of COVID-19 cases that account for asymptomatic and symptomatic presentations.

In response to the COVID-19 pandemic, the Centers for Disease Control and Prevention have launched the National Wastewater Surveillance System and have published guidelines on wastewater surveillance data reporting and analytics, which were incorporated into the methodology utilized in this project (https://www.cdc.gov/healthywater/surveillance/wastewater-surveillance/data-reporting-analytics.html). These included using normalized log-transformed values and an independent variable for date of collection. Analyses were done utilizing the Statistical Analysis System - SAS® software, version 9.4.

3. Results

3.1. RT-qPCR performance, estimated viral recovery, and controls

The RT-qPCR standard curves presented a linear range of quantification. The slope, R^2, and amplifications efficiency for each set of assays were, respectively: BCoV (−3.47, 1.00, 94.2 %), PMMoV (−3.37, 1.00, 98.1 %), Xeno™ Control (−3.24, 0.99, 103.3 %), ORF1ab (−3.33, 0.99, 99.6 %), S-protein (−3.41, 1.00, 96.4 %), and N-protein (−3.31, 1.00, 100.4 %).

BCoV was used as a matrix control to estimate the viral recovery rates from wastewater, with mean ± standard deviation of 29.6 ± 7.6 %, where Adams Field: 23.3 ± 14.4 %, Fourche Creek: 25.9 ± 15.0 %, Little Maumelle: 40.3 ± 20.7 %, and Pine Bluff: 35.2 ± 21.9 %.

PMMoV was detected in all wastewater samples with mean Ct value ± standard deviation of 20.7 ± 1.0 (Supplementary Table 2). The data were normalized to PMMoV relative levels in each sample, which allowed for a correction for the concentration of human feces in wastewater and was important for interpretation and comparison of samples over time.

When we compared the non-normalized data to the data normalized to PMMoV, a difference was noticed, especially in the facilities that serve a larger population. On the other hand, while comparing data normalized to the PMMoV and data normalized to the flow, the results were very consistent.

The TaqMan® Universal RNA Spike In/Reverse Transcription (Xeno) Control sequence was amplified (TaqMan® Assay Ac00010014_a1) in all wastewater samples with mean Ct value ± standard deviation of 26.8 ± 0.4. The nuclease-free water spiked with the Xeno Control was used to set-up a reference point (Ct value = 27.1 ± 0.47) (Staley et al., 2012; Ahmed et al., 2018). No indication of PCR inhibition was observed as the difference between the wastewater sample Ct values and the reference Ct value was smaller than two (dCt < 2) in all cases.

Positive and negative controls were included in all RT-qPCR runs. All positive controls (TaqMan™ 2019-nCoV Control) performed appropriately (Ct < 30) as recommended by manufacturer, while no amplification was observed for negative controls, which included a negative extraction control, a no-template control, and a SARS-CoV-2 negative control. The Ct values of the negative controls were undetermined in all cases.

3.2. Detection of SARS-CoV-2 viral RNA in untreated wastewater samples

Wastewater samples collected from four water treatment facilities (three in Little Rock and one in Pine Bluff) were tested for the presence of SARS-CoV-2 RNA during the period of 92 weeks (April 2020–January 2022). The wastewater data obtained for Little Rock and Pine Bluff correspond to, respectively, about 50 % of the population of Pulaski County, AR and approximately 74.8 % of the population of Jefferson County, AR.

The three SARS-CoV-2 genes (ORF1ab, S-gene, and N-gene) tested in this study were detected by RT-qPCR in wastewater samples in all locations monitored (Fig. 2). From April 2020 through March 2021, there was a total of seven undetected Ct values across the assays (ORF1ab: 2; S-protein: 4; N-protein: 1), all toward the first six months of the pandemic (Supplementary Table 2). Starting at the end of May 2021, a gradual increase in the number of samples with undetermined Ct values for the S-gene was observed at every location. The S-gene drop-out was detected first at Pine Bluff and last in Little Maumelle (Supplementary Table 2). Over the 92 weeks of the study, the viral titers (average of all three SARS-CoV-2 genes normalized to PMMoV) in wastewater influent ranged from 4.70×10^2 copies/mL

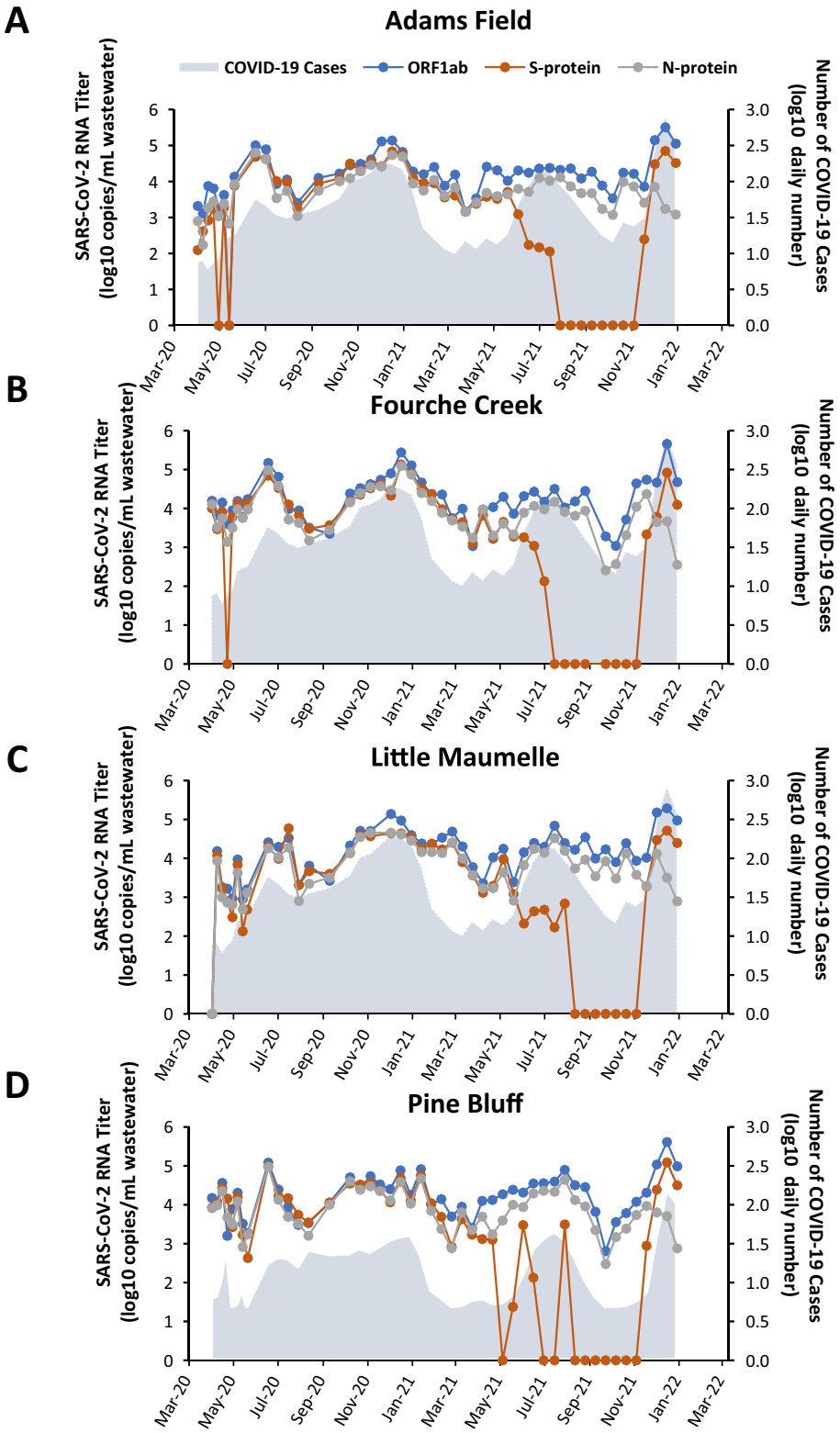


Fig. 2. Temporal dynamics of SARS-CoV-2 genes in wastewater and COVID-19 case count in Little Rock, AR and Pine Bluff, AR. The primary y-axis presents the levels of the viral RNA by gene in wastewater samples in each treatment facility (**A.** Adams Field, **B.** Fourche Creek, **C.** Little Maumelle, **D.** Pine Bluff) over the period of 92 weeks. The SARS-CoV-2 titers were normalized to PMMoV and are presented as viral RNA $\log_{10}$ copy number/mL of wastewater shown per each gene (ORF1ab, S-protein, and N-protein). The secondary y-axis shows the daily number of COVID-19 cases in Little Rock, AR and Pine Bluff, AR, as reported by the Arkansas Department of Health during the same period. These data reflect positive RT-qPCR and/or rapid antigen results in individuals tested for SARS-CoV-2 and are shown as 7-day moving average. The outlier seen on 15 May 2020 in Pine Bluff was due to a COVID-19 outbreak in a correctional facility.

to 1.82 × 10^5 copies/mL (Supplementary Table 3). During this period, the highest mean Ct value detected for SARS-CoV-2 assays was 39.7 and the lowest was 26.7 (Supplementary Table 2).

Fig. 2 shows the abundance and dynamics of the three SARS-CoV-2 genes analyzed in the wastewater samples collected in each treatment facility over the 92-week period (shown in the primary y-axis) in comparison to the COVID-19 case count reported by the Arkansas Department of Health in the respective metropolitan areas (shown in the secondary y-axis). Three epidemic surges have occurred during the period of this study, and they were observed in both clinical (Supplementary Fig. 1) and wastewater (Fig. 2) surveillances. The first surge occurred in the late December 2020/early January 2021, followed by the summer 2021, and late December 2021/early January 2022. The highest levels of SARS-CoV-2 RNA in wastewater samples were observed on 11 January 2022 in all four study locations (Fig. 2), while the highest number of COVID clinical cases was reported in mid-January 2022 (Supplementary Fig. 1).

During the rise of COVID-19 infections in the summer of 2021 in Arkansas (predominantly Delta variant, https://www.gisaid.org/hcov19-variants), there was a consistent decline in the detection of the S-gene in the wastewater samples in all locations (Fig. 2). In mid-December 2021, the S-gene titers started to increase gradually once again in the wastewater samples in all four sewer catchments (Fig. 2), followed by another epidemic surge of COVID-19 cases (mainly Omicron variant) in Arkansas in January 2022 (Supplementary Fig. 1). Additionally, while the levels of ORF1ab and S-protein genes increased in wastewater samples in all study locations from late December 2021, levels of the N-gene started to decline (Fig. 2).

The SARS-CoV-2 RNA titers for the ORF1ab and S-protein genes obtained from samples collected in all four wastewater treatment facilities showed a significant linear association (p-value < 0.05) with the COVID-19 cases reported in the area (Table 4). When assessing the data through December 2021, the viral RNA titers for all genes presented a high correlation to the number of cases (data not shown). By including additional timepoints collected in January 2022, when N-gene levels in wastewater declined, this gene did not correlate to the number of COVID-19 cases (Table 4).

3.3. Detection of SARS-CoV-2 mutants in wastewater samples

Probe-based assays designed to target selected mutations in the S-gene commonly featured in the Delta variant were used to investigate further the failure to detect the S-gene during the summer 2021 surge. According to the genotyping RT-qPCR data, five S-gene mutations (L452R, P681R, T478K, EFR156-158G, and D614G) were identified in samples showing S-gene drop-out during this period (Supplementary Table 4). These mutations, with the exception of D614G, were first detected by RT-qPCR in wastewater samples collected in June 2021 (Supplementary Table 4). An association between the decline in S-gene levels and the detection of two of these mutations (L452R and P681R) was observed in wastewater samples collected in all four locations (Fig. 3). Additional mutations (S477N, L18F, and T20N) were also investigated, but were not detected in any of these samples tested from October 2020 to October 2021 (Supplementary Table 4).

The D614G mutation was mainly present in all samples tested during the epidemic surges in this study (Supplementary Table 4). In addition, some other key mutations in the S-gene (H69-V70 and Y144 deletions, and P681H), usually observed in the Alpha variant, were detected in samples collected between April and June 2021 (Supplementary Table 4).

Allele-specific assays targeting mutations related to the Omicron variant were used in wastewater samples collected between October 2021 and January 2022. Following the recovery of the S-gene detection, observed in mid-December 2021, other S-gene mutations (G339D, Q493R, K417N, and N501Y) were identified in wastewater samples from all study locations in late December 2021 (Supplementary Table 4). The genotyping RT-qPCR data were validated by amplicon RNA sequencing (Supplementary Table 5).

Table 4
Linear regressions between SARS-CoV-2 RNA titers per gene in wastewater samples collected in each water treatment facility and the 7-day moving average of the daily number of positive COVID-19 cases in Little Rock, AR and Pine Bluff, AR.

Water treatment facility	ORF1ab		S-gene		N-gene	
	p-Value	R^2	p-Value	R^2	p-Value	R^2
Adams Field	<0.0001	0.84	<0.0001	0.51	0.2042	0.06
Fourche Creek	<0.0001	0.69	0.0003	0.32	0.1803	0.07
Little Maumelle	<0.0001	0.74	0.0002	0.34	0.236	0.04
Pine Bluff	<0.0001	0.67	<0.0001	0.43	0.7168	0.03

3.4. SARS-CoV-2 RNA sequencing

Seventy-three wastewater samples collected during the main COVID-19 surges were used for next-generation sequencing analysis of the SARS-CoV-2 amplicon. Amplicon sequencing confirmed the presence of SARS-CoV-2 RNA in all samples (Supplementary Table 5). Forty-seven of the sequenced samples reached a genome coverage >90 % SARS-CoV-2 and successfully generated consensus sequences. Several samples showed low mean amplicon coverage depth and genome coverage, which could be associated with RNA degradation and/or low viral abundancy in the wastewater samples.

Phylogenetic analysis was performed to assess whether consensus sequences, including partial sequences, from wastewater samples in each location could be associated with variants of concern. Of the sequenced samples with sufficient coverage, 24 samples from all four locations were grouped into clades 19A, 20A, 20C, and 20G (Supplementary Table 5 and Fig. 4A). Twenty-five wastewater samples collected during the 62–86-week period in all four locations were classified as SARS-CoV-2 Delta variant clade 21A and/or 21J (Supplementary Table 5, Fig. 4A and B). Beginning in December 2021, the Omicron variant was detected in the samples collected from all four wastewater treatment facilities and reached 100 % prevalence by mid-January 2022 (Fig. 4B). These data match the SARS-CoV-2 variants of concern reported in COVID-19 patients by the Arkansas Department of Health during the same period (Fig. 4C).

Sequences with incomplete genome coverages tend to cluster close to the root of the tree (Fig. 4A). In addition to known variants of concern, such as Delta and Omicron-specific (Supplementary Table 5), multiple nonsynonymous mutations were detected that may result in amino acids changes and indicate the presence of other variants of SARS-CoV-2 virus in these communities (Supplementary Table 5). The data generated by RNA sequencing are available at the National Center for Biotechnology Information (NCBI) public repository under the BioProject accession number PRJNA865728 and is a component of the GenomeTrakr umbrella for SARS-CoV-2 wastewater data (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA865728/).

4. Discussion

The current study shows the temporal dynamics of SARS-CoV-2 genes in wastewater sampled from two metropolitan areas in Arkansas during major surges of COVID-19 cases. During the period assessed, three SARS-CoV-2 variants of concern (Alpha, Delta, and Omicron) responsible for the epidemic outbreaks in the area were identified in wastewater in the study locations. These same variants were found in COVID-19 patients from Arkansas during the same period and the viral titers in wastewater correlated to the number of COVID-19 cases in the areas studied.

The presence of SARS-CoV-2 RNA was monitored in wastewater influent using RT-qPCR, while the viral genome dynamics was monitored using amplicon-based high-throughput sequencing. Four sewer catchments with different population sizes, three in Little Rock (Pulaski County, AR) and one in Pine Bluff (Jefferson County, AR), were monitored during the COVID-19 pandemic between 28 April 2020 and 25 January 2022. The period of the study spanned major surges of COVID-19 cases in these two metropolitan areas. The levels of SARS-CoV-2 RNA titers were estimated by quantification of three different regions of the virus' genome (encoding ORF1ab, S-protein, and N-protein). Several performance controls were incorporated, in accordance with guidelines by the Centers for Disease

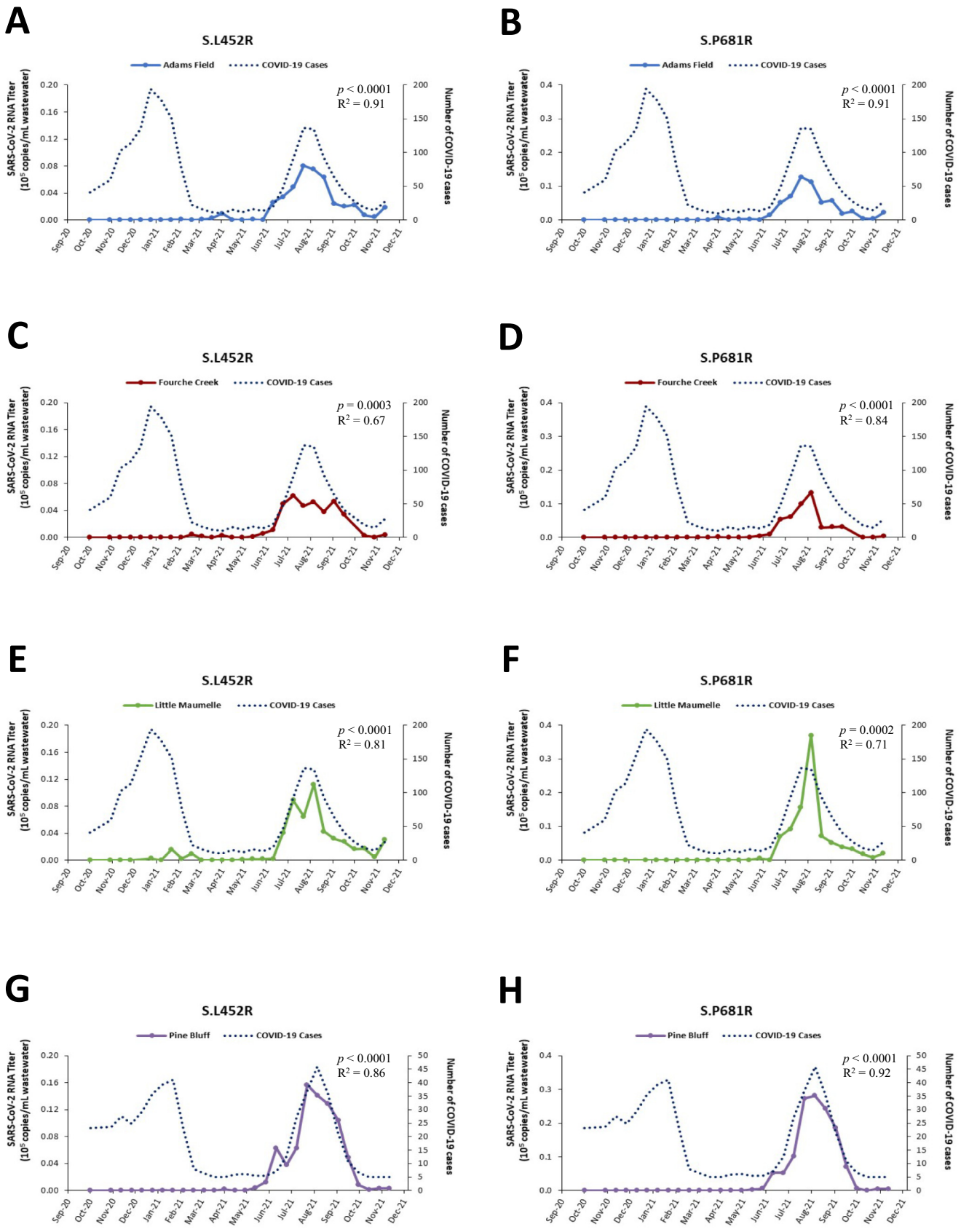
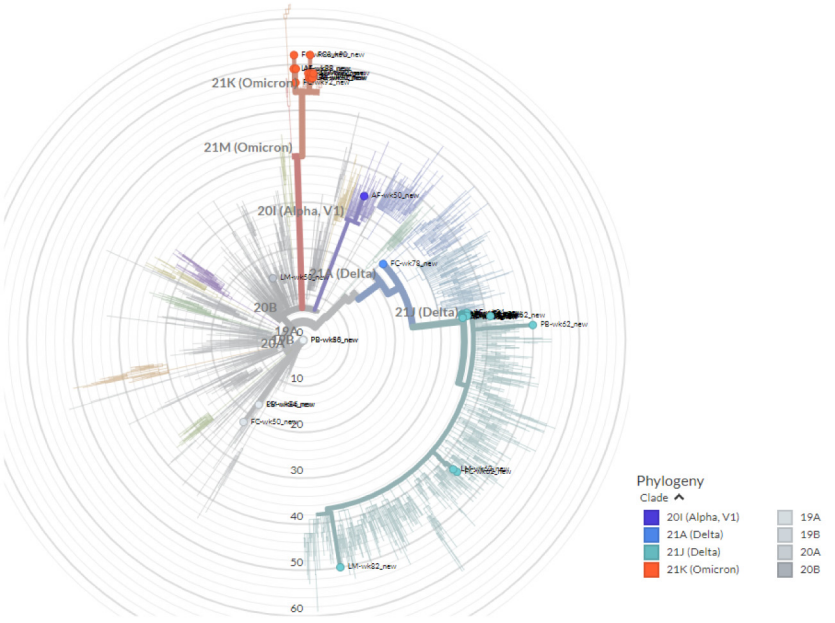
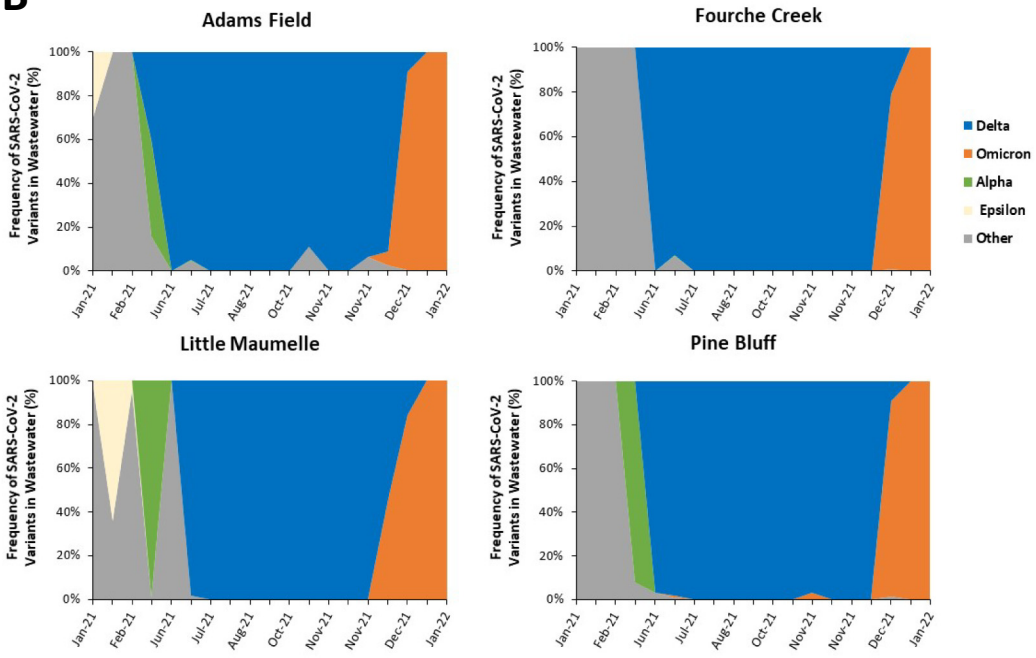


Fig. 3. Rise of two mutations in SARS-CoV-2 S-gene (L452R and P681R) as detected by RT-qPCR in wastewater samples collected in Adams Field **(A–B)**, Fourche Creek **(C–D)**, Little Maumelle **(E–F)**, and Pine Bluff **(G–H)**. The primary y-axis presents the levels of the viral RNA by mutant in wastewater samples. The SARS-CoV-2 titers were normalized to PMMoV and are presented as viral RNA copy number/mL of wastewater. The secondary y-axis shows the daily number of COVID-19 cases in Little Rock, AR (**A–F**) or Pine Bluff, AR (**G–H**), as reported by the Arkansas Department of Health during the same period.

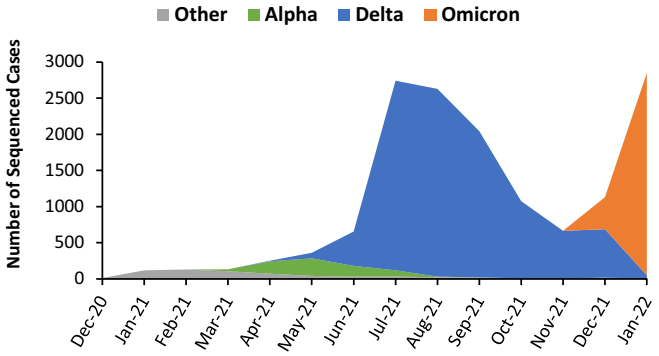
A


21K (Omicron)
21M (Omicron)
20I (Alpha, V1)
21A (Delta)
21J (Delta)
20B
20A
20C
10
20
30
40
50
60
Phylogeny
Clade
20I (Alpha, V1)
21A (Delta)
21J (Delta)
21K (Omicron)
19A
19B
20A
20B

B


Adams Field
Fourche Creek
Little Maumelle
Pine Bluff
Frequency of SARS-CoV-2 Variants in Wastewater (%)
100%
80%
60%
40%
20%
0%
Jan-21
Feb-21
Jun-21
Jul-21
Aug-21
Oct-21
Nov-21
Nov-21
Dec-21
Jan-22
Delta
Omicron
Alpha
Epsilon
Other

C


Other
Alpha
Delta
Omicron
Number of Sequenced Cases
3000
2500
2000
1500
1000
500
0
Dec-20
Jan-21
Feb-21
Mar-21
Apr-21
May-21
Jun-21
Jul-21
Aug-21
Sep-21
Oct-21
Nov-21
Dec-21
Jan-22

Control and Prevention, such as matrix recovery and process control (BCoV), fecal human control (PMMoV), and molecular process control (universal RNA spike in). In addition, we assessed how the viral titers in the wastewater samples related to the clinical COVID-19 case count reported by the Arkansas Department of Health.

During the period of the study, the numbers of confirmed COVID-19 cases increased from 490 to 94,048 in Pulaski County, AR and from 162 to 17,367 in Jefferson County, AR. In general, the SARS-CoV-2 RNA levels in wastewater showed similar trends as COVID-19 case count during the period being evaluated. Although the positive association seemed stronger during the first epidemic surge, more detailed epidemiologic investigations are needed for assessments in particular periods as the trends might differ during each epidemic surge due to a number of factors, including the dynamic genetic diversity of SARS-CoV-2 virus, percentage of clinical testing, underreporting due to self-testing, and vaccination rate, among others. In addition, a time lag analysis may play an important role when assessing the correlation between wastewater and clinical surveillance data. A similar trend on the lower correlation between wastewater and clinical data during the second surge of infections has been observed and suggested to be due to a decrease in clinical testing (Tomasino et al., 2021). Yaniv et al. (2021) have suggested a decrease in the level of SARS-CoV-2 RNA in wastewater when vaccination rate reached over 50 %.

Similar to what was seen in the clinical surveillance, surges of SARS-CoV-2 RNA in wastewater samples were observed around the holidays, summer break, and at periods when the physical distancing and mask requirements were relaxed. Vaccination for COVID-19 began in Arkansas in mid-December 2020, initially for health care workers, first responders, and residents of long-term care facilities, and became available to the entire population 16 years of age and older by April 2021. The number of COVID-19 cases reported in the state reached the first peak in January 2021. A decline in the number of cases was expected due to the immunization (Rosenberg et al., 2021); however, the relatively low vaccination rate in the state, associated with the new variant surge and lift in the mask-wearing mandate (March 2021), had an impact on the rising number of cases and hospital admissions seen in Arkansas in July 2021. At this time, the vaccination rates were 41.3 % (Pulaski County) and 26.5 % (Jefferson County). These numbers increased to 52.7 % (Pulaski County) and 43.9 % (Jefferson County) by December 2021, when the SARS-CoV-2 Omicron variant was first reported in the United States and rapidly became the predominant circulating variant responsible for the largest COVID-19 surge to date (CDC COVID-19 Response Team, 2021). This variant was associated with increased transmissibility and partial immunity evasion (CDC COVID-19 Response Team, 2021).

From April 2020 until March 2021, all three SARS-CoV-2 genes were detected in nearly every wastewater sample of the study across the four treatment stations, and the signal and pattern of all three SARS-CoV-2 genes was comparable among them; however, during the surge of COVID-19 cases in the summer of 2021, the S-gene was detected less frequently, likely due to the new variant B.1.617 (Delta), which was identified by then as the predominant circulating in the state, according to the Arkansas Department of Health (95 % in July 2021). In our study, the probe-based genotyping detected main S-gene mutations of the Delta variant (L452R, P681R, T478K, and EFR156-158G), which were also confirmed by amplicon-based RNA-sequencing in wastewater samples collected in all four sewer catchments between June and December 2021. Approximately eleven key mutations characteristic of the Delta variant were identified through amplicon-based RNA-seq.

The mutations featured in the Delta variant were initially detected in the wastewater samples in Little Rock and Pine Bluff areas in June 2021, whereas this variant was first identified in clinical samples in the state in July 2021. According to the Arkansas Department of Health, the prevalence of Delta variant in the state of Arkansas increased from 3 % in April 2021 to 99 % in August 2021, based on clinical cases. The levels of these key mutation genes progressively decreased in wastewater toward the end of 2021, when the S-gene detection started to increase once again concurrent with the rise of Omicron variant cases.

In late December 2021, signature mutations characteristic of the S-gene in the Omicron BA.1 variant were detected in wastewater samples in all study locations and, in parallel, the N-gene detection was lower when compared to the other targets (ORF1ab and S-protein genes). Allele-specific RT-qPCR assays initially detected G339D, Q493R, K417N, and N501Y substitutions in wastewater sampled in late December 2021. The amplicon-based RNA-sequencing identified approximately 20 mutations commonly seen in the Omicron variant, lineage BA.1 in wastewater samples collected in all four study locations between 29 December 2021 and 25 January 2022. Four main mutations in N-gene (E31, G204R, P13L, and R203K) were identified in the samples collected during this period and might have led to reduced sensitivity of the assay targeting the N-gene. Interestingly, the detection of Delta and Omicron variants in wastewater samples in the current study seem to have preceded the detection in clinical samples, which has also been observed by others (Karthikeyan et al., 2021; Agrawal et al., 2022; Barbé et al., 2022; Karthikeyan et al., 2022; Wurtzer et al., 2022).

The frequent mutations in SARS-CoV-2 seem to affect the sensitivity of detection assays over time (Wang et al., 2020; Brejová et al., 2021; Tao et al., 2021; Zannoli et al., 2022). The ORF1ab and N-genes might be more stable than the S-gene, but they are also undergoing mutations as shown here and in other studies (Kannan et al., 2021; Spurbeck et al., 2021; Tao et al., 2021; Agrawal et al., 2022; Popping et al., 2022; Zannoli et al., 2022). The current study confirms that it is crucial for the efficacy and accuracy of detection tests to be constantly evaluating and modifying the molecular assays accordingly to address emerging mutations (Wang et al., 2020; Ascoli, 2021; Spurbeck et al., 2021; Popping et al., 2022). In addition, the use of assays targeting multiple SARS-CoV-2 genes is important to guarantee higher accuracy in detection with the surge of new variants (Popping et al., 2022; Zannoli et al., 2022).

Our wastewater RT-qPCR data showed changes in the pattern of the S- and N-gene detection when mutations occurred, and that itself could be an early warning signal to investigate for new variants in the population. Moreover, the use of the genotyping RT-qPCR method for detection of known viral mutations, as shown in the current study and others (Barbe et al., 2022; Wurtzer et al., 2022), can be a useful and cost-effective monitoring tool in wastewater surveillance when tracking emerging variants. This type of approach has also been applied in clinical surveillance and is encouraged when genes commonly targeted for COVID-19 diagnosis lose sensitivity due to primer/probe mismatch caused by frequent mutations in genes (Boršová et al., 2021; Neopane et al., 2021).

The results of RT-qPCR assays must be carefully interpreted due to potential mutations in the primer and probe binding genome regions in new viral variants. These issues could also be successfully overcome by

Fig. 4. Temporal dynamics of SARS-CoV-2 variants as assessed by sequencing of wastewater and clinical samples. **A.** Phylogenetic analysis of SARS-CoV-2 viral genome consensus sequences detected in wastewater samples collected in Little Rock, AR and Pine Bluff, AR wastewater treatment facilities. Lines with dots corresponding to the legend color indicate samples sequenced in the study. SARS-CoV-2 consensus sequences were generated using Explify Respiratory Pathogen ID/AMR Panel (RPIP) software. Clades were assigned by using the NextClade tool (https://clades.nextstrain.org). Phylogenic tree shows the evolutionary relationship among SARS-CoV-2 variants circulating in wastewater in these locations. **(B)** Diversity of SARS-CoV-2 lineages based on amino acid changes in wastewater samples in Little Rock and Pine Bluff, AR overtime. The y-axis shows the percentage of each Variant of Concern, and "Other" refers to all lineages not designated as Variants of Concern based on the number of mutation-positive samples. **(C)** Distribution of SARS-CoV-2 variants among selected COVID-19 cases in the state of Arkansas. These data were obtained from 14,789 specimens sequenced for variant detection and do not represent all COVID-19 cases in the state between the period of December 2020 and January 2022, https://www.gisaid.org/hcov19-variants.

using the amplicon sequencing approach. In addition, targeted RNA-sequencing of wastewater samples can be a cost efficient and highly specific tool for viral variant surveillance, especially to identify and detect new and not yet characterized viral variants at the local community level. Despite limitations, the RNA genome sequencing of wastewater samples has the potential to detect known variants of the virus circulating in local communities and provide information about the genetic diversity of SARS-CoV-2 during the COVID-19 pandemic (Crits-Christoph et al., 2021; Fontenele et al., 2021; Agrawal et al., 2022; Barbé et al., 2022). Even though the method is effective on detecting SARS-CoV-2 variants in wastewater in periods of high viral load, the abundance of the virus seems to affect the sensitivity of the variant call by RNA-seq.

This study supports the use of wastewater surveillance as a reliable complementary tool for monitoring of SARS-CoV-2 and its new genomic variants at the community level. As the titers of the SARS-CoV-2 genes detected in wastewater reflect the viral shedding in the feces of infected individuals, continuing wastewater surveillance should provide information on the efficacy of interventions, such as vaccination, use of facial masks, air filtration, and physical distancing, to decrease virus transmission (Chu et al., 2020).

4.1. Future considerations

Early detection and continuous monitoring of SARS-CoV-2 in wastewater have the potential to help state and federal agencies to implement appropriate approaches to restrain the spread of COVID-19 in the community, decrease the burden of patient admissions in healthcare facilities in case of future pandemic waves, and provide insight on the efficacy of public health interventions. However, there are many challenges in the field, and one should be careful in the interpretation of the data. The application of combined performance controls, as exemplified in the current study and in a number of articles on wastewater monitoring of COVID-19, is important for the achievement of reliable data in wastewater surveillance (D'Aoust et al., 2021; Gerrity et al., 2021; Li et al., 2021; Ahmed et al., 2022; Nagarkar et al., 2022). Moreover, mathematical models have been recently proposed to smooth biases and variability associated with SARS-CoV-2 genome quantification in wastewater, which are valuable especially in light of the growing interlaboratory efforts worldwide on wastewater surveillance (Cluzel et al., 2022; Courbariaux et al., 2022; Wurtzer et al., 2022).

In addition, due to the implications of the viral genome changes on the sensitivity of assays used in gene detection through RT-qPCR, further analyses are underway to investigate the correlation between wastewater and clinical data throughout each particular epidemic surge. There is still a substantial knowledge gap regarding the pattern of fecal virus shedding, as well as the proportion of infected individuals shedding the virus in stool and the time frame the shedding occurs. Additionally, consideration on whether a particular variant presents a different rate and pattern of fecal shedding should be investigated. Further analyses are needed for a more accurate prediction and effective use of temporal dynamics of SARS-CoV-2 titer in wastewater on forecasting the number of COVID-19 cases and the spread of the disease in the communities. Long term monitoring of SARS-CoV-2 titers in wastewater will allow a better evaluation of its use as an early indicator of trends for the spread of COVID-19 in local communities.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.157546.

Disclaimer

This article reflects the views of the authors and does not necessarily reflect those of the U.S. Food and Drug Administration or the Arkansas Department of Health. Any mention of commercial products is for clarification only and is not intended as approval, endorsement, or recommendation.

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Volodymyr P. Tryndyak: Methodology, Validation, Investigation, Formal analysis, Data Curation, Writing - Original Draft, Visualization.

Luísa Camacho: Conceptualization, Methodology, Validation, Writing - Review & Editing.

Mohammed S. Orloff: Conceptualization, Methodology, Formal analysis, Validation, Writing - Review & Editing.

Austin Porter: Conceptualization, Data curation, Writing – Review and Editing.

Kelley Garner: Software, Formal analysis, Data Curation, Writing – Review and Editing.

Lisa Mullis: Resources.

Marli Azevedo: Conceptualization, Methodology, Writing - Review & Editing.

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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