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Monitoring of SARS-CoV-2 Variants by Wastewater-Based Surveillance as a Sustainable and Pragmatic Approach—A Case Study of Jaipur (India)

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Abstract: Wastewater-based surveillance has been emerging as an efficient and advantageous tool to predict COVID-19 prevalence in the population, much earlier (7–28 days) than reported clinical cases, thus providing sufficient time to organize resources and optimize their use in managing COVID-19. Since the commencement of the COVID-19 pandemic, SARS-CoV-2 genetic lineages have emerged and are circulating all over the world. The assessment of SARS-CoV-2 variants of concern (VOCs) in wastewater has recently been proven to be successful. The present research demonstrates a case study utilizing an established approach to perform monitoring of SARS-CoV-2 variants from 11 distinct wastewater treatment plants across Jaipur (India) during the second peak period of COVID-19 (from 19 February 2021 to 8 June 2021). The sequences obtained were analyzed to detect lineage using the Pangolin tool and SNPs using the mpileup utility of Samtools, which reported high genome coverage. The mutation analyses successfully identified the penetration of the B.1. in the first two weeks of sampling (19–26 February), followed by the B.1.617.2 variant into Jaipur in the first week of March 2021. B.1.617.2 was initially discovered in India in October 2020; however, it was not reported until early April 2021.The present study identified the presence of B.1.617.2 in early March, which correlates well with the clinical patient's data (290 cases were reported much later by the government on 10 May 2021). The average total genome coverage of the samples is 94.39% when mapped onto the severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1; a complete genome (NC_045512.2) sequence and SNP analysis showed that 37–51 SNPs were identified in each sample. The current study demonstrates that sewage surveillance for variant characterization is a reliable and practical method for tracking the diversity of SARS-CoV-2 strains in the community that is considerably faster than clinical genomic surveillance. As a result, this method can predict the advent of epidemiologically or clinically important mutations/variants, which can help with public health decision making.

Keywords: COVID-19; next generation sequencing; SARS-CoV-2; sewage; variants of concerns

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1. Introduction

SARS-CoV-2 has had an ever-expanding epidemiology since its introduction in December 2019, with an increase in the number of patients and its spread. As of 17 November 2021, there have been 255,252,955 clinically confirmed SARS-CoV-2 cases registered worldwide, with 5,133,224 deaths [1]. Viruses evolve and diversify throughout time, and the

introduction of new variants such as the diversity of SARS-CoV-2 is an expected occurrence due to evolution and adaptability, as what has been seen internationally. Upon the suggestion of WHO's Technical Advisory Group on Virus Evolution, WHO recognized the variant B.1.1.529, known as Omicron, as a variant of concern on 26 November 2021 [2]. Most mutations that arise will not provide the virus with a selection advantage. Some alterations or combinations of mutations—such as greater transmissibility via increased receptor binding or the ability to avoid the host immune response by changing surface features recognized by antibodies [3,4]—may do so. Understanding the genetic nature of circulating SARS-CoV-2 in the community is critical in this scenario. Due to limited resources, it is essentially impossible to conduct a genomic surveillance of clinical patients on a large scale as the number of cases and mutations grows. In such cases, the wastewater-based epidemiological paradigm would cover community-wide genetic variant monitoring and would prove to be a practical, cost-effective, and important resource for analyzing SARS-CoV-2 mutations in the population [5]. The World Health Organization (WHO) has also acknowledged environmental sewage surveillance as a method of protecting public health by monitoring and detecting viral infections in circulation [6]. The use of genomic surveillance of wastewater could prove to be a valuable tool for detecting, identifying, predicting, and building an early warning system for the identification of variants of concern (VOCs) in circulation to support public health actions. Table 1 shows the list of currently designated variants of concerns, as defined by WHO [7,8].

Table 1. Currently designated variants of concern by WHO [9].

WHO Label	Pango Lineage	GISAID Clade	Additional Amino Acid Changes Monitored	Earliest Documented Samples	Date of Designation
Alpha	B.1.1.7	GRY	+S: 484K +S: 452R	United Kingdom, Sep-2020	18 December 2020
Beta	B.1.351	GH/501Y.V2	+S: L18F	South Africa, May-2020	18 December 2020
Gamma	P.1	GR/501Y.V3	+S: 681H	Brazil, Nov-2020	11 January 2021
Delta	B.1.617.2	G/478K.V1	+S: 417N +S: 484K	India, Oct-2020	VOI: 4 April 2021 VOC: 11 May 2021
Omicron	B.1.1.529	GRA	+S: R346K	Multiple countries, Nov-2021	VUM: 24 November 2021 VOC: 26 November 2021

The appearance of new SARS-CoV-2 variants with mutations linked to greater transmissibility, a weaker antibody response, or both [10–12] has recently attracted attention. SARS-CoV-2 mutations have been found across the world, raising worries about the efficiency of treatment and vaccinations. Because of its positive-sense single-stranded RNA genetic material, SARS-CoV-2 has a high mutation rate, posing a public health threat. Different highly infectious forms of SARS-CoV-2 that have emerged because of mutations in the SARS-CoV-2 genome have been identified as variants of concerns (VOCs) (Table 1). The B.1.1.7 lineage of SARS-CoV-2, for example, which was discovered in the United Kingdom (UK) in November 2020, is thought to be 40–80% more contagious than the initial strain [13]. Other SARS-CoV-2 lineages from Brazil (P.1), Southern African countries (B.1.351), and India (B.1.617.2) are also more transmissible than the early 2020 variants. Because Omicron has been designated as a variant of concern, WHO recommends that countries improve their surveillance and sequencing of cases, share genome sequences on publicly available databases such as GISAID, report initial cases or clusters to WHO, and conduct field investigations and laboratory assessments to better understand if Omicron has different transmission or disease characteristics, or how it affects the effectiveness of vaccines. In

terms of viral pathogenicity, virulence, and transmission, the variants of concern (VOCs) are crucial [2].

The clinical monitoring, which includes whole genome sequencing from infected patients' nasopharyngeal samples, is an effective method, although it is costly, labor-intensive, and time-consuming. It also has serious flaws, such as only including genomes of symptomatic patients, which, according to a recent study [14–16], accounts for only two-thirds of the population, with the remaining one-third of SARS-CoV-2 infections being asymptomatic and not covered by clinical genomic monitoring. As a result, finding variants in wastewater could be a more efficient, quicker, and practical way to track the appearance and spread of new variants in a community. Several research works have attempted to sequence the SARS-CoV-2 genome and detected genetic variations from wastewater samples in several locations, including Montana, USA [16], California, USA [17], Switzerland [18], London [19], Canada [20], and India [21], among others. To effectively control the COVID-19 pandemic in a coordinated manner, it is critical to promote this unique strategy on a much larger scale globally and to build a repository of dominant variants in circulation.

With almost 5000 cases recorded in Jaipur during the second wave of COVID-19, it was vital to analyze the wastewater from the city to understand the infection dynamics and focus on the variations circulating in the community. To highlight the necessity of variant surveillance, we conducted an amplicon-based metagenomic landscape of SARS-CoV-2 in the wastewater of the Jaipur region during the period of the second wave, from 19 February to 8 June 2021.

The study's goal was to see if the SARS-CoV-2 RNA was present in wastewater streams using NGS technology (Illumina NextSeq500) for sequencing, which could be used to look for mutations. The current investigation could provide crucial information about circulating variations in the community far before a clinically confirmed discovery, as well as investigate the possibility of WBE as a cost-effective and practical surveillance technique.

2. Methodology

2.1. Sample Collection and Transportation

For the monitoring of the SARS-CoV-2 variants, influent samples were collected from 11 municipal wastewater treatment plants (WWTPs) located across Jaipur city. Figure 1 depicts the geographic locations of the study's sampling sites, which cover roughly 60–70% of the city's sewerage network. Table 2 summarizes the details of WWTPs. The samples were collected weekly between 19 February and 8 June 2021 in this temporal investigation. All the samples were collected as one-liter grabs in sterile bottles and brought to the Dr. B. Lal Institute of Biotechnology's Environmental Biotechnology Laboratory in Jaipur for further research and analysis. As previously described [22–25], samples were kept at 4 °C for 24 h after sampling. For sample collection, appropriate precautions, including ambient temperatures, were considered. During the whole sample process, the concerned staff wore standard personal protective equipment (PPE).

Table 2. Characteristics of WWTP sampling sites.

Site No.	Sampling Location	Type of Secondary Treatment Technology	Type of Tertiary Treatment	Dosage and Contact Time of Tertiary Treatment	Design Capacity (m^3/Day)	Flow Rate (Average. MLD)	Number of Connected Residents (Approximately)
Site 1	Brahmpuri, Jaipur 26.9373° N, 75.8250° E	SBR	No treatment	NA	27,000 m^3/day	~8	>59,000
Site 2	Central Park Garden, Jaipur 26.9048° N, 75.8073° E	SBR	Cl_2 (Bleach Powder)	4 ppm by dropping system	1000 m^3/day	~1	>7000

Table 2. *Cont.*

Site No.	Sampling Location	Type of Secondary Treatment Technology	Type of Tertiary Treatment	Dosage and Contact Time of Tertiary Treatment	Design Capacity (m^3/Day)	Flow Rate (Average. MLD)	Number of Connected Residents (Approximately)
Site 3	Ramniwas Garden, Jaipur 26.8963° N, 75.8100° E	MBBR	UV	NA	1000 m^3/day	~1	>7000
Site 4	MNIT, Jaipur 26.8640° N, 75.8108° E	MBBR	Cl_2 (Hypochlorite)	2.5–3 ppm, 30 min	1000 m^3/day	~1	>2000
Site 5	Jawahar Circle Garden, Jaipur 26.5029 ^{0}N, 75.480^0 E	MBBR	UV	NA	1000 m^3/day	~1	>7000
Site 6	Dravyavati River, Jaipur 26.7980° N, 75.8039° E	SBR	Cl_2 (Hypochlorite)	3–5 ppm, 30 min	65,000 m^3/day	~65	>480,000
Site 7	Dhelawas, Jaipur 27.3735° N, 75.8926° E	ASP	No treatment	3 ppm, 30 min	65,000 m^3/day	~62.5	>480,000
Site 8	Paldi Meena, Jaipur 26.8759° N, 75.8945° E	SBR	No treatment	NA	3000 m^3/day	0.6–0.7	~5000
Site 9	Ralawata, Jaipur 26.76873° N, 75.93092° E	ASP	Cl_2 (Hypochlorite)	10 kg per hour	30,000 m^3/day	20–22	~170,370
Site 10	Kho Nagorian, Jaipur 26.84063° N, 75.88546° E	SBR	No treatment	NA	50,000 m^3/day	~45.5	>480,000
Site 11	Dr. B. Lal Institute of Biotechnology Institute's Campus WTWP 26.85697° N, 75.82749° E	Biokube™	No treatment	NA	7.5 m^3/day	7.5 KLD	~500

Note: MNIT = Malaviya National Institute of Technology, MBBR = Moving Bed Biofilm Reactor, SBR = Sequencing Batch Reactor, ASP = Activated Sludge Process-plug flow process, Biokube™ = Plug & Play type packaged sewage treatment plant—Mars 4000, Cl_2 = Chlorine disinfection, UV = Ultraviolet disinfection, MLD = million liters per day, NA = Not applicable.

Figure 1. Geographical locations of wastewater treatment plant sampling sites as presented on the map: 1. Brahmpuri, 2. Dravyavati River, 3. Ramniwas Garden, 4. Central Park Garden, 5. MNIT, 6. Dr. B. Lal Institute of Biotechnology, 7. Paldi Meena, 8. Jawahar Circle, 9. Kho Nagorian, 10. Dhelawas, 11. Ralawata.

2.2. Sample Preparation for SARS-CoV-2 Detection Using RT-qPCR

The samples for RNA isolation were produced with minor changes to the methodology previously reported [25,26]. The wastewater samples were exposed to UV light for 30 min to sterilize the surface before being thoroughly mixed. Furthermore, 1 mL of the sample was aliquoted and centrifuged at 7000× *g* for 30 min (to remove debris and undesirable components), with the supernatant being processed for RNA extraction, as described in [27]. The viral RNA was extracted from the treated wastewater samples using the automated KingFisher™ Flex equipment, as reported in [24], using the MagMAX Viral/Pathogen Nucleic Acid Isolation Kit (Applied Biosystems, Waltham, MA USA), according to the manufacturer's instructions. The aliquots of eluted RNA in the plates were then sealed and stored at −20 °C until needed.

2.3. SARS-CoV-2 Quantitative and Qualitative Detection

The presence of SARS-CoV-2 RNA was detected qualitatively and quantitatively in total RNA isolated from wastewater samples using a CFX 96 Thermal Cycler (Bio-Rad, Hercules, CA, USA) RT-PCR machine and two commercially available kits. Kit 1 contained 2019-nCoV MOM (prepared master mix), 5X Real-time One-step Buffer, Real-time One-step Enzyme, and exogenous Internal Control for qualitative detection of SARS-CoV. Kit 2 contained 2019-nCoV MOM (prepared master mix), 5X Real-time One-step Buffer, Real-time One-step Enzyme, and exogenous Internal Control for qualitative detection of SARS-CoV. (IC). Kit 1 was designed to read the E gene, N gene, and RdRp gene on the Cal Red 610 and Quasar 670 fluorophore channels, with FAM and HEX as internal controls. A total of 11 uL of extracted RNA was mixed with 14 uL of RT-PCR master mix to make the PCR reaction. The reaction protocol was as follows: 1 cycle at 50 °C for 20 min, 1 cycle at 95 °C for 15 min, 45 cycles of denaturation at 94 °C for 15 s, combined annealing and extension for 30 s at 58 °C, plate read, and detection. The Bio-Rad CFX Manager software version 3.1 was used to analyze the PCR run (Bio-Rad Laboratories). The identification of a minimum of two genes (out of three) in a sample was judged positive based on Ct values, according to the manufacturer's instructions.

InnoDetect One Step COVID-19 (Kit 2) was also used to further quantify the presence of the SARS-CoV-2 viral genome in wastewater samples, with two different plasmid DNA consisting of the N gene and the ORF1ab gene, separately used to prepare a standard curve (ranging from 10 pg/uL to 0.01 fg/uL), as per the manufacturer's instructions. The quantification of the individual genes in the samples was then based on these standard curves. To make a primary stock with a concentration of 40 ng/uL, RNase-free water was employed. For individual identification, kit 2 included a master mix, primer probes (N gene, ORF1ab, and RNaseP), and three fluorophore channels (HEX/VIC, FAM, and ROX/Texas Red, respectively). SARS-CoV-2 viral RNA was utilized as a positive control, while DNase RNase-free water was used as a negative control. A reverse transcription stage at 42 °C for 15 min and 1 cycle, cDNA initial denaturation at 95 °C for 3 min and 1 cycle, denaturation at 95 °C for 15 s, and combined annealing and extension at 60 °C for 40 s were all followed by plate read and detection. Positive samples had a quantifiable presence of either of the two genes (N or ORF1ab) or both genes. The samples that tested positive for SARS-CoV-2 were chosen for further analysis.

2.4. Sample Pre-Processing for NGS RNA Extraction: Virus Filtration and Concentration

The virus was inactivated by transferring the samples to 50 mL Tarsons falcon (code 546041) tubes in a biosafety cabinet (BSL2), which was followed by a 70% ethanol spray over the surface of the falcon tubes and 30 min of UV light exposure for surface sterilization. After UV irradiation, the samples were placed in a water bath at 60 °C and incubated for 90 min to ensure that the virus was heat-inactivated. After the coronavirus was inactivated, the samples were brought to room temperature and filtered using a vacuum filter assembly via a 0.45 µ membrane. Each sample's filtrate was placed in a new 50 mL falcon containing 0.9 g sodium chloride (NaCl) and 4 g polyethylene glycol (PEG). By gently mixing the

ingredients, the contents were dissolved. After that, the PEG and NaCl-containing samples were centrifuged at 4 °C for 30 min at 7000 rpm. After that, the pellet was re-suspended in 1X Phosphate Buffer Saline (PBS) and processed for RNA extraction.

2.5. Total RNA Extraction

ZymoBIOMICS®96Magbead DNA/RNA Kit R2136 was used to extract total RNA according to the manufacturer's instructions. Using the simple MAG extractor, 3 mL of lysis buffer containing concentrated sewage was extracted. Extracted NA were eluted in a 55 L elution buffer and stored at 70 °C until appropriate samples were pooled and transmitted to an NGS service provider (Eurofins, Bangalore, India, Company Headquarters Luxembourg, France) for sequencing.

2.6. Sample Pooling for NGS

The wastewater samples were collected continuously from Sites 1–11 for 11 weeks. The samples found positive each week were then processed to extract total RNA. A total of 51 samples out of more than 110 influent samples collected were found positive for SARS-CoV-2 presence. These 51 RNA samples were pooled week-wise to make composite RNA samples for 11 weeks, starting from 19 February to 10 June, as described in Table 2.

2.7. Qualitative and Quantitative Analysis of RNA Samples

The quality and quantity of the viral RNA samples were checked by Nanodrop, and results are shown in Table S1.

2.8. Preparation of 2 × 150 NextSeq500 ARTIC Library

NGSeq ARTIC SARS-CoV-2 Kit and Illumina TruSeq Nano DNA Library Prep Kit were used to construct paired-end sequencing libraries from the QC-passed and selected viral RNA samples. In a nutshell, isolated viral RNA was reverse transcribed to cDNA before being used in a PCR experiment with the oligo mix pools provided. Both amplicon pools were combined and purified using AMPure XP beads before being fragmented with a Covaris M220. End Repair Mix was used to repair the fragmented amplicons by eliminating the 3′ overhangs and filling in the 5′ overhangs with 5′–3′ polymerase activity, followed by adapter ligation to the fragments. The low rate of chimaera (concatenated template) creation is ensured by this method. AMPure XP beads were used to size the ligated products. As specified in the kit methodology, the size-selected products were PCR amplified using the index primer, and index adapters were ligated to the ends of the DNA fragments, preparing them for hybridization onto a flow cell.

2.9. Quantity and Quality Check (QC) of Library on Agilent 4200 Tape Station

The PCR enriched libraries were purified with AMPure XP beads and evaluated on an Agilent Technologies 4200 Tape Station system with a high-sensitivity D1000 Screen tape, as detailed in Table S2.

2.10. Cluster Generation and Sequencing

The PE illumina libraries were loaded onto NextSeq500 for cluster creation and sequencing after getting the Qubit concentration for the libraries and the mean peak sizes from the Agilent TapeStation profile. On the NextSeq500, paired-end sequencing allowed the template fragments to be sequenced both forward and backward. After re-synthesis of the reverse strand during sequencing, the adapters were designed to facilitate selective cleavage of the forward strands. The reverse strand was then utilized to sequence the fragment from the opposite end.

2.11. Bioinformatic Analysis

The lineage detection was performed by using Pangolin tools. SNP analysis was conducted using the mpileup utility of Samtools (v 0.1.18). Multiple sequence alignment

was generated using CLUSTAL W, and the visualization and analysis were completed using Jalview 2.11.1.1.

3. Results

3.1. SARS-CoV-2 Gene Concentrations in Wastewater and the Quality and Depth of the Data Obtained

Three SARS-CoV-2 genes (E, RdRP, and N gene regions) were found in all 110 wastewater influents during this 5-month study, with quantities ranging from 1×10^4 to 1×10^6 gene copies/L of wastewater. The SARS-CoV-2 gene concentrations were relatively steady at the start of the study, grew fast in mid-March, peaked in April and May, then plateaued in June, according to the general observed trend. This pattern matches the trend of the daily COVID-19 new confirmed cases in Jaipur [28,29]. Table S3 shows the results of genome concentrations in several samples across different WWTP concentrations. Sample pooling (from several WWTPs) is described in Table 3, and high-quality clean reads were acquired by processing raw sequences with Trimmomatic reads.

Table 3. Description of samples and raw data reads.

Sample No.	Sample Coding	Sample Collection Date	Type of Sample (Total No. of Samples)	Sample Pooled from WWTPs	Total Number PE of Reads
1	JPR 1	27 February 2021	Individual RNA	3	1,409,611
2	JPR 2	20 February 2021	Pooled RNA (2)	9 and 2	1,552,223
3	JPR 3	12 March 2021	Pooled RNA (2)	5 and 7	1,570,305
4	JPR 4	19 March 2021	Pooled RNA (3)	7, 4, 5	1,579,011
5	JPR 5	1 April 2021	Pooled RNA (7)	4, 2, 3, 1, 5, 8, 4	1,385,609
6	JPR 6	9 April 2021	Pooled RNA (7)	5, 2, 8, 6,4, 1, 3	1,507,711
7	JPR 7	20 April 2021	Pooled RNA (6)	5, 8, 4, 2, 3, 1	1,352,690
8	JPR 8	1 May 2021	Pooled RNA (5)	5, 4, 1, 6, 8	1,588,702
9	JPR 9	8 May 2021	Pooled RNA (5)	4, 6, 5, 11, 1	1,606,125
10	JPR 10	15 May 2021	Pooled RNA (6)	2, 7, 5, 4, 6, 8	1,414,505
11	JPR 11	24 May 2021	Pooled RNA (6)	9, 7, 10, 6	1,194,455

3.2. Lineage Identification and Multiple Sequence Analysis with Other Reference Sequences

The high-quality reads of the samples were subsequently aligned to the severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1, complete genome (NC_045512.2) reference sequence using BWA MEM (version 0.7.17). Consensus sequence was extracted using Samtools mpileup. The mostly probable lineage of the sample consensus sequence was assigned using the Pangolin tool. The identified lineage for the samples is provided in Table 4.

Table 4. Lineage identification in 11 pooled samples from different WWTPs.

Sample	Lineage
JPR 1	B.1
JPR 2	B.1
JPR 3	B.1.617.2
JPR 4	B.1.617.2
JPR 5	B.1.617.2
JPR 6	B.1.617.2
JPR 7	B.1.617.2
JPR 8	B.1.617.2
JPR 9	B.1.617.2
JPR 10	B.1.617.2
JPR 11	B.1.617.2

3.3. Analysis of Phylogeny and SNPs in the Samples

This analysis was performed by comparing the obtained sequences to a consensus sequence from the ancestral SARS-CoV-2 Wuhan city variant. However, since these samples were not just a single patient sample but rather a pool of viral RNA obtained from various WWTPs of the city at a given time point, it was important to assess if this data obtained could be applied and used to monitor the emerging variants and phylogenies in the whole city and aid in genome surveillance initiatives. To find out if the phylogenetic analysis and ancestry could be studied from the sequence information obtained from these samples, multiple sequence alignment was performed by using Clustal Omega [30], and phylogenetic trees were obtained by the neighbor joining method (Figure 2). The alignments were first completed to check if the sequences obtained could be analyzed amongst themselves without any external reference sequence (Figure 2a). The analysis was repeated using several reference sequences, viz., the Delta variant obtained in January 2021 from India in the state of Tamil Nadu, Delta variant from Netherlands in the month of June 2021, Delta variant sequence obtained from Italy in September 2021, and a completely different variant sequence, the Mu variant, obtained from Columbia, USA in April 2021 (Figure 2b). This analysis demonstrated the usability of the genomic sequence obtained from the wastewater samples, which can be used as effectively in variant surveillance, at par with the patient data.

The sequences obtained from the pooled samples not only gave robust reads but were also aligned amongst themselves with the different variant sequences. Since all the samples were classified as B.1.617.2, except for the first two time point samples, it was a worthy question to look at the genetic variations in these samples over the period of sampling. By comparing them with NC_045512.2, SNP detection was carried out, and the following SNPs could be identified with their genomic coordinates, reference-alternate bases associated genes, and amino acid changes.

Out of these, a total of 423 gene-related SNPs were detected from all the samples, out of which a total sum of 254 belonged to the ORF1ab polyprotein gene. Out of these, 61 were unique SNPs found across 11 of the samples analyzed. In these 11 samples, there were 12 types of SNP mutations which did not change the amino acid phenotype in the sequence (Ser549Ser, Phe924Phe, and Thr5960Thr in all samples; Tyr4227Tyr and Leu6666Leu in ten; Leu4386Leu and Gly334Gly in five; Gly6068Gly, Asn6333Asn, and Ser2500Ser in three; and Pro1692Pro and Thr1773Thr in two samples were detected in the 11 pooled samples). Table 5 describes the SNPs in 11 samples. Amongst the SNPs which led to a change in the amino acid sequences, six SNPs were present in all the samples; these were Ala2529Thr, Ala3209Val, Val3718Ala, Pro4715Leu, Pro5401Leu, and Pro5971Leu. Furthermore, there were also a few SNPs which were not present in all the samples but only in a few out of 11 samples sequenced, i.e., Pro1640Leu (91% of samples), Gly5063Ser (72%), Pro5401Leu (63%), Thr3750Ile (36.4%), Arg6676Gln (36.4%), Thr5036Met (27.4%), Ala385Val (18.2%), Lys798Asn(18.2%), Gln1021His (18.2%), Pro2046Leu (18.2%), Thr3255Ile (18.2%), Thr5477Ile (18.2%), and His6547Tyr (18.2%).

Three SNP variants were found to be linked with ORF3A, out of which Ser26Leu was found in every sample, Gly100Val was found in 18%, and Ala110Ser in only 9% of instances. It was interesting to note that the only three polymorphisms in ORF7a (Val82Ala, Leu116Phe, and Thr120Ile) which were detected in 11 wastewater samples were present in 72% of the samples and were highly correlated and always present together as a set. No other SNPs were detected in the samples. Furthermore, the ORF8 gene was linked with three types of SNPs detected in the study. Out of these, Ala65Ser was uniformly detected in all the samples, while Phe120Leu and Asp119Val were present in only 27% and 18% of the samples, respectively. Unlike ORF7a, these SNPs were correlated with respect to their associated gene. Strangely, only two out of 11 samples showed any SNPs related to gene M during our analysis. Three types of SNPs were detected: Asn66Lys (two samples), Val70Ile (unique detection), and Pro71His (two samples). In the Membrane protein M gene, five types of SNPs were detected. Out of these, Ile82Thr was present in 91% of the

samples sequenced but the other four SNPs were unique mutations found only in one of the 11 samples. In the E gene, a total of two samples only showed SNP mutations, two of which were present together, Asn66Lys and Pro71His. In addition to these, only one more uniquely present SNP, Val70Ile, was detected.

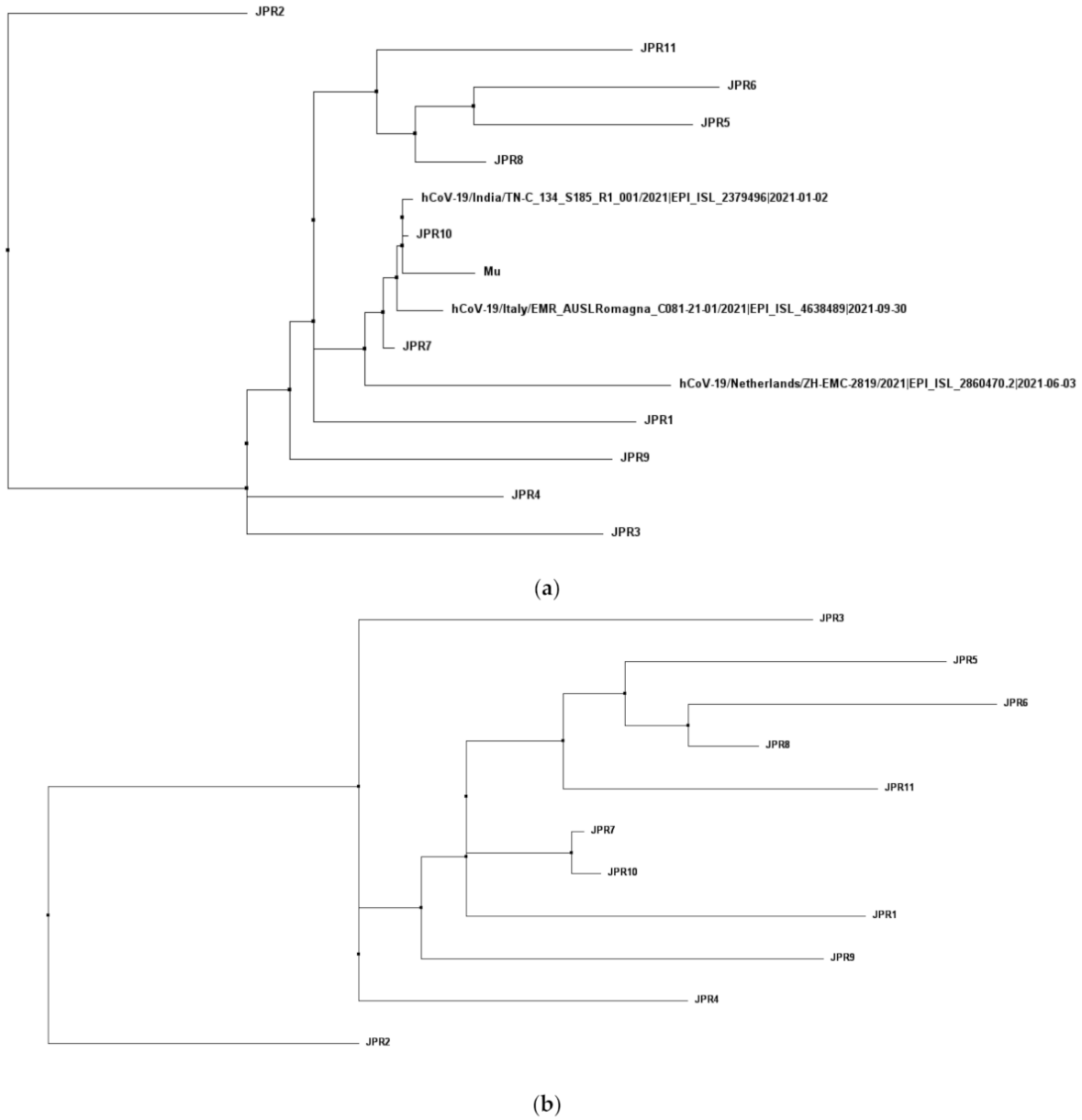


Figure 2. Phylogenetic analysis with the sequences obtained from wastewater samples. (**a**) Phylogenetic tree of 11 samples collected from Jaipur between 27 February (JPR1) and the end of May 2021 (JPR11). JPR2-4 corresponds to samples collected weekly from March, JPR5-7 from April, and JPR 8-11 from May. (**b**) Shows the phylogeny of the collected samples with GISAID-submitted sequences from different time points as a reference. Mu stands for the sequence collected in April for the Mu variant, observed for the first time in Columbia, USA.

Table 5. SNP identification in samples.

Sample	Number of SNPs Detected	Number of Genic SNPs Detected
JPR 1	39	35
JPR 2	44	41
JPR 3	40	36
JPR 4	51	47
JPR 5	39	36
JPR 6	41	37
JPR 7	42	39
JPR 8	45	42
JPR 9	40	36
JPR 10	44	40
JPR 11	37	34
Total	462	423

Out of 423 instances of gene-related SNPs, the N and S genes had 33 and 65 linked detected SNP sites across all the samples, respectively. The N gene had eight unique SNP mutations across the samples. These were Ile337Ile, Thr379Ile, Arg385Lys, Arg185Leu, Asp63Gly, Gln281Lys, Ser327*, Arg189Ser. Although Asp377Tyr and Arg203Met were present in 91% and 82% of the samples, the presence of both in the same sample was only 72%. On the contrary, two of the SNP types, Asp402Asn and Asp402Val, although present in only about 27%, were always present together. The S gene had 13 different types of SNPs associated with it. Out of these, Ile472Ile, Gln218His, Gln1208His, Pro426Leu, and Gly142Asp were uniquely identified. Furthermore, Thr19Arg, Leu452Arg, Thr478Lys, and Pro681Arg were the ones which were found in all the samples, and Asp614Gly was found in 91%. Ala222Val, Asp950Asn, and Lys1191Asn all occurred approximately in 18% of the samples; there was no correlation in sample overlap amongst them. There were additional 39 SNPs which could be detected in the intergenic regions of various samples.

3.4. Comparisons of SNPs with the Delta and Delta Plus Variants

There are several key mutations in single nucleotide bases that have been reported to characterize the Delta and Delta Plus variants. The most widely inspected probably belong to the surface glycoprotein gene of the Spike protein. The characteristic mutations which are present in Delta strains as compared to the ancestral variant are Thr19Arg, (Val70Phe*), Thr95Ile, Gly142Asp, Glu156-, Phe157-, Arg158G, (Ala222Val*), (Trp258Leu*), (Lys417Asn*), Leu452Arg, Thr478Lys, Asp614Gly, Pro681Arg, and Asp950Asn, out of which the three shown with an "*" here even more prevalent in the Delta plus variant.However, the SNPs found and those overlapping with known SNPs for the S gene were Thr19Arg, Thr478Lys, Leu452Arg, Ala222Val, Gly142Asp, Asp614Gly, Asp950Asn, and Pro681Arg only; other SNPs from the known list and the observed list did not match for the S gene. Two observations worth noting are: Firstly, the Lys417Asn mutation was not observed in any of the samples; however, 18% of the samples showed a Lys1191Asn SNP change. Secondly, the Pro681Arg SNP was observed in 100% of the samples, which is an SNP site present in the Mu variant as Pro681His.

In addition to the S gene, there are other mutations which are reported in non-S loci for the Delta and Delta Plus variants, e.g., ORF1ab, M, N, ORFs 3a, 7a, and 8, E, etc. The signature mutations in these sites were also compared with the SNPs detected in the Jaipur samples and are summarized in the Table 6.

Table 6. Comparison of the SNPs present at non-spike loci in the Delta and Delta plus variants with the Jaipur samples.

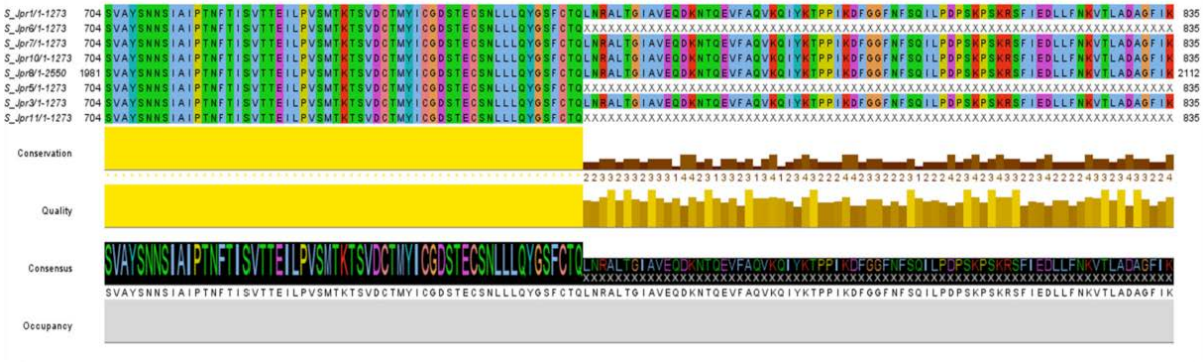
Genes	SNPs Overlapping with Delta and Delta Plus	SNPs Which Were Absent in Jaipur Samples	Total Types of Non-Unique Detected SNPs	Total Unique SNPs Detected
ORF1ab	Thr3646Ala	Ala1146Thr	30	61
	Thr3255Ile	Ala3209Val		
	Val3718Ala	Val2930Leu		
	Pro2046Leu	Pro2287Ser		
	Pro1640Leu	Ala1306Ser		
		Pro2046Leu		
		Thr3750Ile		
ORF3a	Ser26Leu		2	1
M	Ile82Thr		1	4
N	Arg203Met	Gly215Cys	4	8
	Asp63Gly			
	Asp377Tyr			
ORF7A	Thr120Ile	Thr40Ile	3	0
	Val82Ala			

3.5. Variations in Various Genes Identified in the WBE Samples

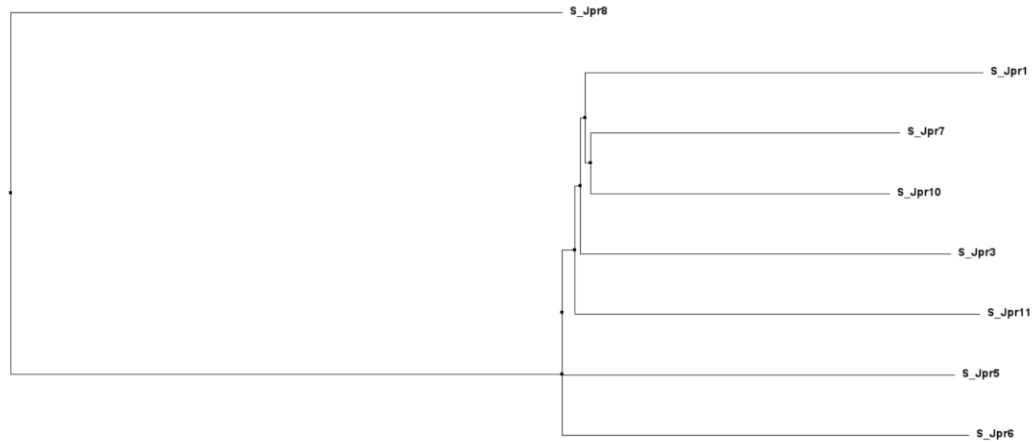
Since the SNPs were variably present in the genes of different samples, multiple sequence alignment was performed with the predicted peptide sequences to identify the extent of variations across various sample sequences. Figure 3 depicts the regions of variations and phylogenetic relationships of the S, N, M, and ORF1ab genes. As can be observed from the phylogenetic relationships, the higher number of SNPs was reflected by the higher variability in the alignment, which in turn was associated with the more diversified clades of the tree. The existence of such diversity in the same time frame in a single city might allow for an understanding of the evolution of variants and possibly hold some key insights into the host–pathogen interactions. Figure 3 shows the analysis of gene variations of the S, M, N, and ORF1ab genes.

3.6. Comparing the Sequences Obtained in Context of Patient Samples of the City Obtained during the Same Time Frame

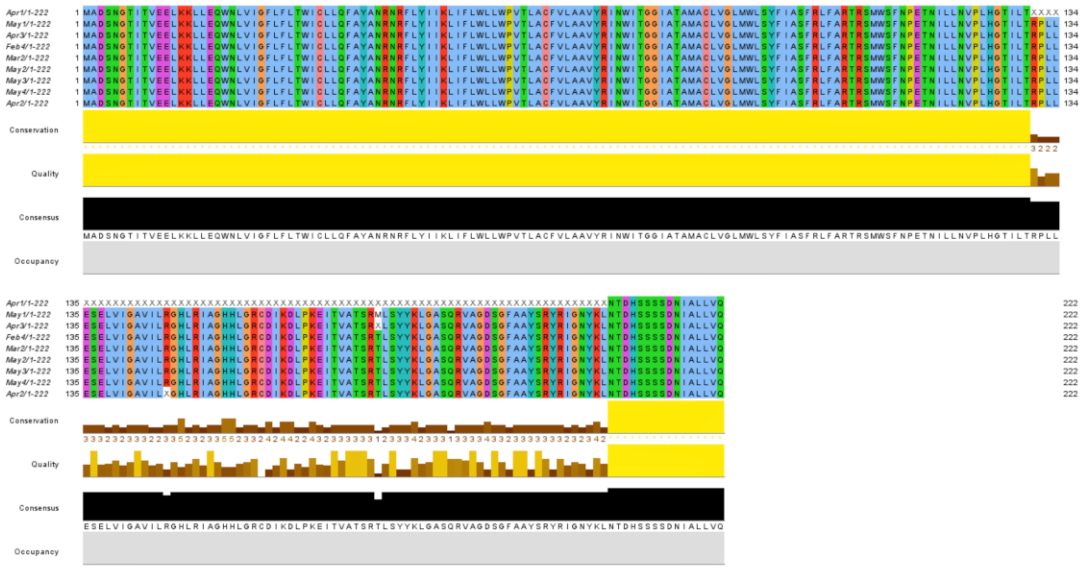
Since the wastewater sample genome sequences showed phylogenetic trees that were well-aligned with the different timeline reference sequences, an attempt was made to investigate if the sequences showed any correlation between the community viral variants and the hospital patient viral variants prevalent in the city at the time of sampling. The phylogenetic tree was calculated for the wastewater genome sequences with eight patient samples and a Delta variant reference sequence. These patient samples were collected between the same window of wastewater sampling in the city of Jaipur (from February 2021 to May 2021). The Phylogenetic tree is depicted in Figure 4.



(**a**)

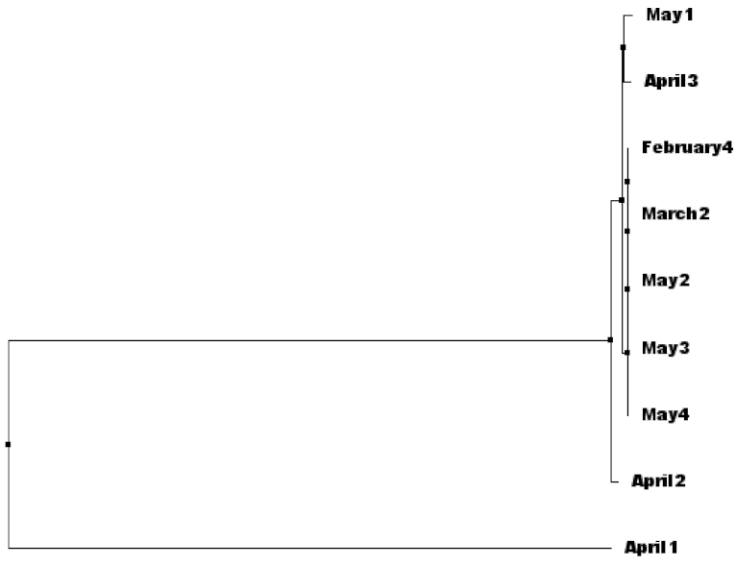


(**b**)

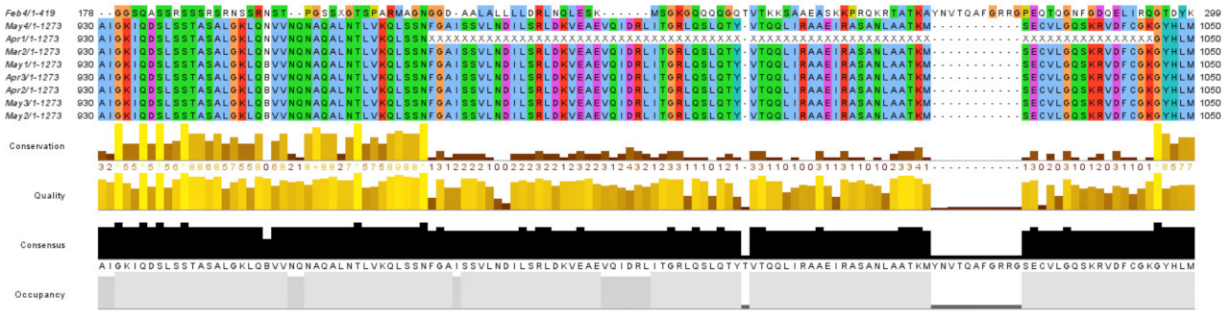


(**c**)

Figure 3. *Cont.*

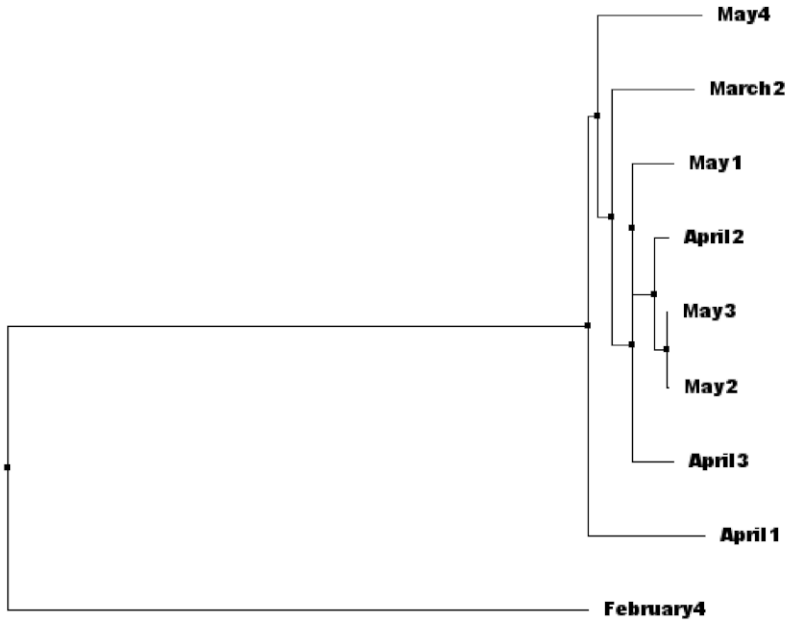


(**d**)

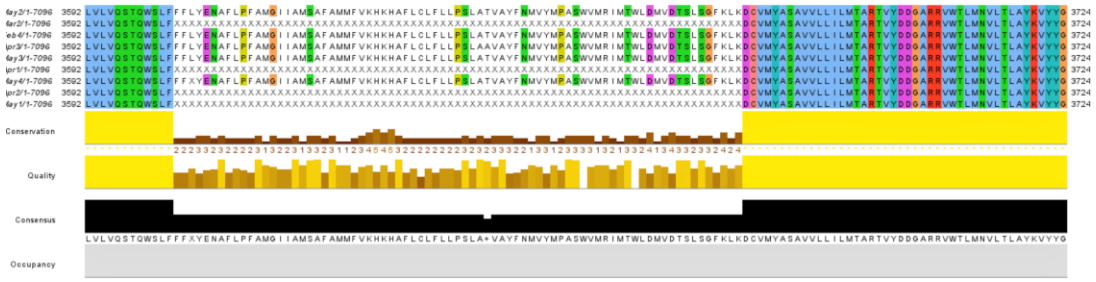


(**e**)

Figure 3. *Cont.*

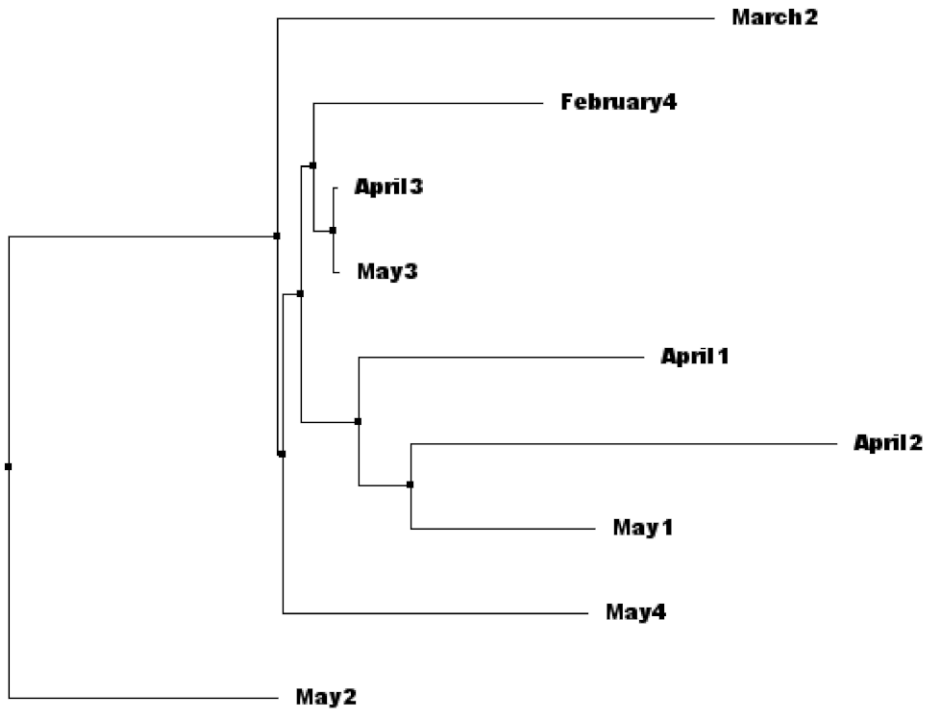


(f)



(g)

Figure 3. *Cont.*



(**h**)

Figure 3. Analysis of gene variations of the S (**a**,**b**), M (**c**,**d**), N (**e**,**f**), and ORF1ab genes (**g**,**h**). (**a**) Spike alignment between 704 and 835 bps, (**b**) Spike Phylogenetic tree, (**c**) M gene alignment at 1–222 bps, (**d**) Phylogenetic tree of the M gene, (**e**) N gene at 930–1050 bps, (**f**) Phylogenetic tree for the N gene, (**g**) ORF1ab polyprotein multiple sequence alignment at 3592–3724 bps, (**h**) Phylogenetic tree of ORF1ab.

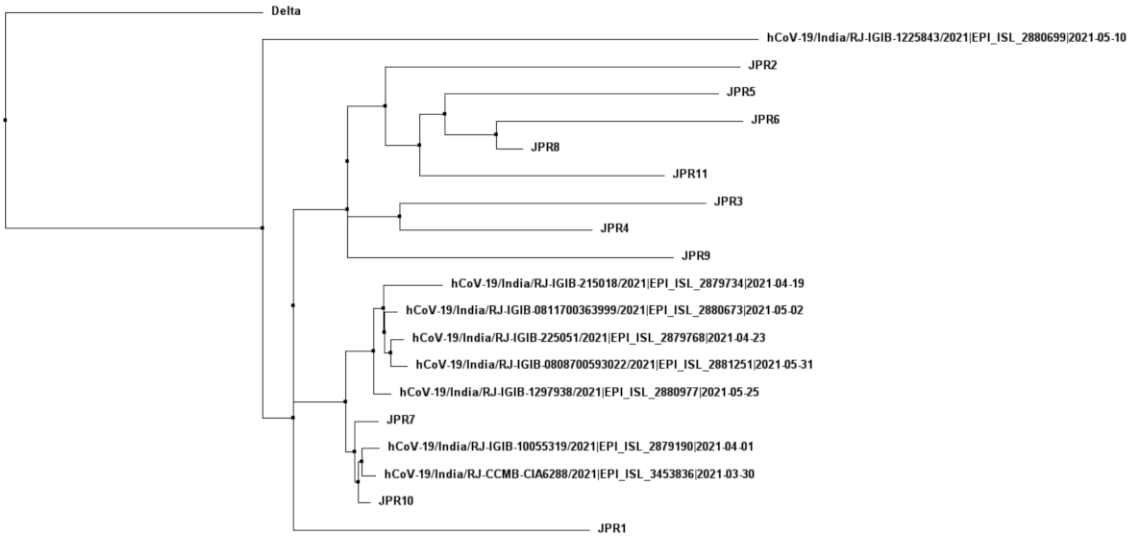


Figure 4. Phylogenetic tree of wastewater sample with clinical patient sample.

3.7. Sequences on Public Databases

The obtained sequences have already been submitted to GISAID (EPI_ISL_4106468 EPI_ISL_4198506, EPI_ISL_4198392, EPI_ISL_4198393, EPI_ISL_4198461, EPI_ISL_4197532, EPI_ISL_4198503, EPI_ISL_4198504, EPI_ISL_4198119, EPI_ISL_4198513, EPI_ISL_4198502)

and the NCBI gene database (OK090988 and OK342100-OK342107) to further strengthen the research and advancements in combating this pandemic, and are available for various analyses [31–33].

4. Discussion

It has been established that investigating the spread of SARS-CoV-2 and identifying variations in wastewater may reliably determine viral strain prevalence [7]. The method uses pooled samples and can only detect mutations per place in the genome, not specific strains. Nonetheless, it has several advantages over human clinical sampling, primarily in terms of ethical concerns and population capture, as only a few samples of wastewater can provide a comprehensive picture of viral variety as compared to thousands of samples from humans. From February to June 2021, we used a well-established system of wastewater sampling and viral concentration techniques to conduct a large-scale surveillance of SARS-CoV-2 variants using next-generation sequencing of SARS-CoV-2 genomes, which were sampled monthly in 11 different WWTPs across Jaipur.

Attempts have been made across the world to keep track of the pandemic and its causal agents by keeping a constant eye on new varieties. This monitoring is carried out through many national and international programs, such as INSACOG and GISAID [30,33,34], which are connected to the daily samples taken and sequenced from patients. When compared to the viral load circulating in community wastewater samples, which also contains many organic and inorganic contaminants, the viral load seen in a single patient sample proves to be extremely concentrated. The study's first goal was to collect RNA samples of sufficient quantity and quality in order to be processed on next-generation sequencing (NGS) technologies. The Nanodrop quality checks were passed by all eleven samples prepared for sequencing.

Despite the high Ct values that are typical of such materials, the sequencing of SARS-CoV-2 from wastewater was effective, with good coverages of the genome acquired for most samples and sufficient depth to call it a mutation. These findings made it possible to keep a close eye on variations all throughout Jaipur, even in areas where clinical sampling revealed little morbidity [28]. The increased proportions of sequenced viruses belonging to the B.1.617.1 and B.1.617.2 lineages submitted to the GISAID EpiCoV database have been linked to an increase in case numbers in India.

In December 2020, B.1.617.1 was discovered for the first time in India. It first rose in proportion in India, peaking at roughly 50% of weekly reported sequences in the GISAID EpiCoV database in late March 2021, before declining in April 2021 [35]. It has been discovered in India (290), the United Kingdom (247), the United States (137), Singapore (64), Germany (28), Australia (21), Denmark (21), Bahrain (9), Japan (9), Angola (8), Switzerland (8), Hong Kong (7), Ireland (7), Portugal (7), Belgium (5), Luxembourg (5), South Korea (5), Canada (4), the Netherlands (4), New Zealand (4), Sweden (4), France (3), Jordan (3), Czechia (2), Guadeloupe (2), Russia (2), Sint Maarten (2), Spain(2), Cambodia (1), Greece (1), Italy (1), Malaysia (1), and Mexico (1).There are signs that the variant is becoming more common in the EU/EEA, although it is still only found in a small percentage of sequences, with the greatest proportions reported in Luxembourg (0.6%) and Spain (0.3%) [36].

Within B.1.617, there are three separate lineages, each with its own mutation profile, necessitating evaluation at the lineage level rather than as a whole. The spike protein amino acid alterations L452R, E484Q, D614G, P681R, and Q1071H characterize B.1.617.1 (some viruses also carry V382L). The spike protein changes T19R, 157–158, L452R, E484Q, D614G, P681R, and D950N have allowed it to be classified as a VOI by ECDC and WHO, and as a VOC by the UK, indicating that its transmissibility is at least as high as that of VOC B.1.1.7. B.1.617.3 is defined by the spike protein changes T19R, 157–158, L452R, E484. The ECDC and WHO have categorized this lineage as a VOI, but the UK has classified it as a VUI [37]. Below are some of the specific spike protein modifications associated with these lineages that have been described as having an impact on viral properties.

E484Q (only B.1.617.1 and B.1.617.3)—changes at this site are associated with reduced neutralization by convalescent sera and specific therapeutic antibodies. L452R—changes at this site are associated with increased transmissibility and reduced neutralization by convalescent plasma and specific therapeutic antibodies. Although this has not been proven in practice, for the P681R—alteration is positioned exactly near the furin cleave site and might potentially influence S1/S2 cleavage, cell entrance, and infectivity. D614G—this alteration is linked to increased transmissibility with a high degree of certainty. The great majority of currently circulating viruses, however, carry it [38].

To summarize, this study demonstrates the benefits of wastewater sample sequencing as a reliable method of monitoring the diversity of SARS-CoV-2 strains circulating in a community, warning against the emergence of epidemiologically or clinically relevant mutations or variants, and assisting in public health decision making.

The detection of existing circulating variants and dominant mutations in populations using SARS-CoV-2 genome sequencing in wastewater explains the link between dominant variants and the pandemic situation in Jaipur, India. It demonstrates the potential of SARS-CoV-2 genomic surveillance in wastewater as an early warning indicator system and its ability to detect rapidly emerging new variants in any significant epidemic.

5. Significance of the Work and Conclusions

The usefulness of wastewater surveillance in COVID-19 trend tracking in several areas has been demonstrated in this study. The COVID-19 cases were substantially linked to SARS-CoV-2 gene quantities in wastewater. In early March 2021, the B.1.617.2 variant was first detected in Jaipur as part of the national SARS-CoV-2 sequencing program, which was newly established to track VOC in clinical samples. This demonstrates that surveillance by wastewater is a robust strategy that may cover wide areas with few samples, providing early notification as regards the penetrance and spread of VOC in a region where clinical sampling is insufficient. Thus, integrating WBE surveillance with genome sequencing and pathogen variant surveillance might be an effective and efficient step towards monitoring, detecting, and managing new infection waves similar to those shown by infectious SARS-CoV-2 variants early on, and therefore protect the communities from loss of life and economic resources in any future catastrophes similar to this this pandemic.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w14030297/s1, Table S1: Details of Samples sent for Sequencing & RT-qPCR Detection; Table S2: Mapping and Consensus Statistics; Table S3: RT qPCR Detection.

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References

1. Jin, H.; Wang, H.; Li, X.; Zheng, W.; Ye, S.; Zhang, S.; Zhou, J.; Pennington, M. Economic burden of COVID-19, China, January–March, 2020: A cost-of-illness study. *Bull. World Health Organ.* **2021**, *99*, 112. [CrossRef]
2. Available online: https://www.who.int/news/item/28-11-2021-update-on-omicron (accessed on 14 December 2021).
3. Bakhshandeh, B.; Jahanafrooz, Z.; Abbasi, A.; Goli, M.B.; Sadeghi, M.; Mottaqi, M.S.; Zamani, M. Mutations in SARS-CoV-2; Consequences in structure, function, and pathogenicity of the virus. *Microb. Pathog.* **2021**, *154*, 104831. [CrossRef]
4. Harvey, W.T.; Carabelli, A.M.; Jackson, B.; Gupta, R.K.; Thomson, E.C.; Harrison, E.M.; Ludden, C.; Reeve, R.; Rambaut, A.; Peacock, S.J.; et al. SARS-CoV-2 variants, spike mutations and immune escape. *Nat. Rev. Microbiol.* **2021**, *19*, 409–424. [CrossRef]
5. Amereh, F.; Negahban-Azar, M.; Isazadeh, S.; Dabiri, H.; Masihi, N.; Jahangiri-Rad, M.; Rafiee, M. Sewage systems surveillance for SARS-CoV-2: Identification of knowledge gaps, emerging threats, and future research needs. *Pathogens* **2021**, *10*, 946. [CrossRef] [PubMed]
6. Available online: https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports (accessed on 14 December 2021).
7. Nemudryi, A.; Nemudraia, A.; Wiegand, T.; Surya, K.; Buyukyoruk, M.; Cicha, C.; Vanderwood, K.K.; Wilkinson, R.; Wiedenheft, B. Temporal detection and phylogenetic assessment of SARS-CoV-2 in municipal wastewater. *Cell Rep. Med.* **2020**, *1*, 100098. [CrossRef] [PubMed]
8. Di Marcantonio, C.; Chiavola, A.; Gioia, V.; Frugis, A.; Cecchini, G.; Ceci, C.; Spizzirri, M.; Boni, M.R. Impact of COVID19 restrictions on organic micropollutants in wastewater treatment plants and human consumption rates. *Sci. Total Environ.* **2021**, *811*, 152327. [CrossRef] [PubMed]
9. Available online: https://www.who.int/en/activities/tracking-SARS-CoV-2-variants/ (accessed on 14 December 2021).
10. Altmann, D.M.; Boyton, R.J.; Beale, R. Immunity to SARS-CoV-2 variants of concern. *Science* **2021**, *371*, 1103–1104. [CrossRef]
11. Challen, R.; Brooks-Pollock, E.; Read, J.M.; Dyson, L.; Tsaneva-Atanasova, K.; Danon, L. Risk of mortality in patients infected with SARS-CoV-2 variant of concern 202012/1: Matched cohort study. *BMJ* **2021**, *372*, n579. [CrossRef]
12. Grint, D.J.; Wing, K.; Williamson, E.; McDonald, H.I.; Bhaskaran, K.; Evans, D.; Evans, S.J.; Walker, A.J.; Hickman, G.; Nightingale, E.; et al. Case fatality risk of the SARS-CoV-2 variant of concern B. 1.1. 7 in England, 16 November to 5 February. *Eurosurveillance* **2021**, *26*, 2100256. [CrossRef]
13. Davies, N.G.; Jarvis, C.I.; Edmunds, W.J.; Jewell, N.P.; Diaz-Ordaz, K.; Keogh, R.H. Increased mortality in community-tested cases of SARS-CoV-2 lineage B. 1.1. 7. *Nature* **2021**, *593*, 270–274. [CrossRef]
14. Oran, D.P.; Topol, E.J. The Proportion of SARS-CoV-2 Infections That Are Asymptomatic: A Systematic Review. *Ann. Intern. Med.* **2021**, *174*, 655–662. [CrossRef]
15. Crits-Christoph, A.; Kantor, R.S.; Olm, M.R.; Whitney, O.N.; Al-Shayeb, B.; Lou, Y.C.; Flamholz, A.; Kennedy, L.C.; Greenwald, H.; Hinkle, A.; et al. Genome sequencing of sewage detects regionally prevalent SARS-CoV-2 variants. *MBio* **2021**, *12*, e02703-20. [CrossRef]
16. Otero, M.C.B.; Murao, L.A.E.; Limen, M.A.G.; Gaite, P.L.A.; Bacus, M.G.; Acaso, J.T.; Corazo, K.; Knot, I.E.; Sajonia, H.; Francis, L.; et al. Wastewater-Based Epidemiology and Whole-Genome Sequencing for Community-Level Surveillance of SARS-CoV-2 in Selected Urban Communities of Davao City, Philippines: A Pilot Study. *medRxiv* **2021**. Available online: https://www.medrxiv.org/content/10.1101/2021.08.27.21262450v1 (accessed on 14 December 2021).
17. Rothman, J.A.; Loveless, T.B.; Kapcia, J., III.; Adams, E.D.; Steele, J.A.; Zimmer-Faust, A.G.; Langlois, K.; Wanless, D.; Griffith, M.; Mao, L.; et al. RNA Viromics of Southern California Wastewater and Detection of SARS-CoV-2 Single-Nucleotide Variants. *Appl. Environ. Microbiol.* **2021**, *87*, e01448-21. [CrossRef]
18. Jahn, K.; Dreifuss, D.; Topolsky, I.; Kull, A.; Ganesanandamoorthy, P.; Fernandez-Cassi, X.; Bänziger, C.; Stachler, E.; Fuhrmann, L.; Jablonski, K.P.; et al. Detection of SARS-CoV-2 variants in Switzerland by genomic analysis of wastewater samples. *medRxiv* **2021**. Available online: https://www.medrxiv.org/content/10.1101/2021.01.08.21249379v1.full.pdf (accessed on 14 December 2021).
19. Wilton, T.; Bujaki, E.; Klapsa, D.; Majumdar, M.; Zambon, M.; Fritzsche, M.; Mate, R.; Martin, J. Rapid increase of SARS-CoV-2 variant B. 1.1. 7 detected in sewage samples from England between October 2020 and January 2021. *Msystems* **2021**, *6*, e00353-21. [CrossRef]
20. Landgraff, C.; Wang, L.Y.; Buchanan, C.; Wells, M.; Schonfeld, J.; Bessonov, K.; Ali, J.; Robert, E.; Nadon, C. Metagenomic sequencing of municipal wastewater provides a near-complete SARS-CoV-2 genome sequence identified as the B. 1.1. 7 variant of concern from a Canadian municipality concurrent with an outbreak. *medRxiv* **2021**. Available online: https://www.medrxiv.org/content/10.1101/2021.03.11.21253409v1.full.pdf (accessed on 14 December 2021).
21. Dharmadhikari, T.; Rajput, V.; Yadav, R.; Boargaonkar, R.; Panse, D.; Kamble, S.; Dastager, S. High throughput sequencing based detection of SARS-CoV-2 prevailing in wastewater of Pune, West India. *medRxiv* **2021**. Available online: https://www.medrxiv.org/content/10.1101/2021.06.08.21258563v1.full.pdf (accessed on 14 December 2021).
22. Medema, G.; Been, F.; Heijnen, L.; Petterson, S. Implementation of environmental surveillance for SARS-CoV-2 virus to support public health decisions: Opportunities and challenges. *Curr. Opin. Environ. Sci. Health* **2020**, *17*, 49–71. [CrossRef]

23. Medema, G.; Heijnen, L.; Elsinga, G.; Italiaander, R.; Brouwer, A. Presence of SARS-Coronavirus-2 RNA in sewage and correlation with reported COVID-19 prevalence in the early stage of the epidemic in the Netherlands. *Environ. Sci. Technol. Lett.* **2020**, *7*, 511–516. [CrossRef]
24. Bivins, A.; North, D.; Ahmad, A.; Ahmed, W.; Alm, E.; Been, F.; Bhattacharya, P.; Bijlsma, L.; Boehm, A.B.; Brown, J.; et al. Wastewater-based epidemiology: Global collaborative to maximize contributions in the fight against COVID-19. *Environ. Sci. Technol.* **2020**, *54*, 7754–7757. [CrossRef]
25. Arora, S.; Nag, A.; Sethi, J.; Rajvanshi, J.; Saxena, S.; Shrivastava, S.K.; Gupta, A.B. Sewage surveillance for the presence of SARS-CoV-2 genome as a useful wastewater based epidemiology (WBE) tracking tool in India. *Water Sci. Technol.* **2020**, *82*, 2823–2836. [CrossRef]
26. Arora, S.; Nag, A.; Rajpal, A.; Tyagi, V.K.; Tiwari, S.B.; Sethi, J.; Sutaria, D.; Rajvanshi, J.; Saxena, S.; Shrivastava, S.K.; et al. Imprints of lockdown and treatment processes on the wastewater surveillance of SARS-CoV-2: A curious case of fourteen plants in northern India. *Water* **2021**, *13*, 2265. [CrossRef]
27. Arora, S.; Nag, A.; Kalra, A.; Sinha, V.; Meena, E.; Saxena, S.; Sutaria, D.; Kaur, M.; Pamnani, T.; Sharma, K.; et al. Successful Application of Wastewater-Based Epidemiology in Prediction and Monitoring of the Second Wave of COVID-19 in India with Fragmented Sewerage Systems-A Case Study of Jaipur (India). *medRxiv* **2021**. Available online: https://www.medrxiv.org/content/10.1101/2021.09.11.21263417v1.full.pdf (accessed on 14 December 2021).
28. Available online: https://www.cdc.gov/ (accessed on 8 January 2022).
29. Available online: www.covid19india.org (accessed on 8 January 2022).
30. Sievers, F.; Higgins, D.G. Clustal Omega, accurate alignment of very large numbers of sequences. In *Multiple Sequence Alignment Methods*; Humana Press: Totowa, NJ, USA, 2014; pp. 105–116.
31. Available online: https://www.ncbi.nlm.nih.gov/nuccore/OK090988 (accessed on 14 December 2021).
32. Available online: https://www.ncbi.nlm.nih.gov/nuccore/OK342100 (accessed on 14 December 2021).
33. Available online: https://www.ncbi.nlm.nih.gov/nuccore/OK342107 (accessed on 14 December 2021).
34. Kannan, S.R.; Spratt, A.N.; Cohen, A.R.; Naqvi, S.H.; Chand, H.S.; Quinn, T.P.; Lorson, C.L.; Byrareddy, S.N.; Singh, K. Evolutionary analysis of the Delta and Delta Plus variants of the SARS-CoV-2 viruses. *J. Autoimmun.* **2021**, *124*, 102715. [CrossRef]
35. Emergence of SARS-CoV-2 B.1.617 Variants in India and Situation in the EU/EEA. Available online: https://www.ecdc.europa.eu/sites/default/files/documents/Emergence-of-SARS-CoV-2-B.1.617-variants-in-India-and-situation-in-the-EUEEA_0 (accessed on 14 December 2021).
36. Assessment, R.R. *Coronavirus Disease 2019 (COVID-19) in the EU/EEA and the UK–Ninth Update*; European Centre for Disease Prevention and Control: Stockholm, Sweden, 2020.
37. Available online: https://indianexpress.com/article/explained/explained-who-classifies-india-variant-as-being-of-global-concern-what-does-it-mean-7310999/ (accessed on 14 December 2021).
38. Volz, E.; Hill, V.; McCrone, J.T.; Price, A.; Jorgensen, D.; O'Toole, Á.; Southgate, J.; Johnson, R.; Jackson, B.; Nascimento, F.F.; et al. Evaluating the effects of SARS-CoV-2 spike mutation D614G on transmissibility and pathogenicity. *Cell* **2021**, *184*, 64–75. [CrossRef]