A 30-day follow-up study on the prevalence of SARS-COV-2 genetic markers in wastewater from the residence of COVID-19 patient and comparison with clinical positivity

Md. Aminul Islam[1,2]*, Md. Arifur Rahman[1], Md. Jakariya[3]*, Newaz Mohammed Bahadur[4], Foysal Hossen[1], Sanjoy Kumar Mukharjee[1], Mohammad Salim Hossain[5], Atkeeya Tasneem[6], Md Atiqul Haque[7,8], Francesco Sera[9,10], Iqbal Kabir Jahid[11], Tanvir Ahmed[12], Mohammad Nayeem Hasan[13], Md.Tahmidul Islam[14], Md.Amzad Hossain[1], Md. Ruhul Amin[1], Ananda Tiwari[15,16], Md Didar-Ul-Alam[1], Kuldeep Dhama[17], Prosun Bhattacharya[18]*, Firoz Ahmed[1]*

[1]*COVID-19 Diagnostic Lab,Department ofMicrobiology, Noakhali Science and Technology University, Noakhali-3814, Bangladesh.*

[2]*Advanced Molecular Lab, Department ofMicrobiology, PresidentAbdulHamid Medical College, Karimganj, Kishoreganj, Bangladesh.*

[3]*Department ofEnvironmental Science and Management, North South University, Bashundhara, Dhaka-1229, Bangladesh*

[4]*Department of Applied Chemistry and Chemical Engineering, Noakhali Science and Technology University, Noakhali-3814, Bangladesh*

[5]*Department of Pharmacy, Noakhali Science and Technology University, Noakhali-3814, Bangladesh*

[6]*Department ofEnvironmental Science andDisaster Management, Noakhali Science and Technology University, Noakhali-3814, Bangladesh.*

[7]*Key Lab ofAnimalEpidemiologyandZoonosesofMinistryofAgricultureandRural Affairs, College ofVeterinaryMedicine, China Agricultural University, Beijing, China.*

[8]*Department of Microbiology, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh Science and Technology University, Dinajpur-5200, Bangladesh.*

[9]*Department of Statistics, Informatics, Applications, University of Florence, Florence, Italy.*

[10]*Department of Public Health, Environments and Society, London School of Hygiene & Tropical Medicine, London, UK.*

[11]*Department of Microbiology, Jashore University of Science and Technology, Jashore 7408, Bangladesh*

[12]*Department ofCivil Engineering, Bangladesh University of Engineering and Technology, Dhaka-1000,Bangladesh.*

[13]*Department ofStatistics, Shahjalal University of Science and Technology, Sylhet 3114, Bangladesh.*

[14]*WaterAid Bangladesh, Dhaka 1213, Bangladesh.*

[15]*Department of Food Hygiene and Environmental Health, Faculty of Veterinary Medicine, University of Helsinki, Finland.*

[16]*Department of Health Security, Expert Microbiology Research Unit, Finnish Institute for Health and Welfare, Finland.*

[17]*Division of Pathology, ICAR-Indian Veterinary Research Institute, Bareilly, Izatnagar, Uttar Pradesh- 243122, India*

[18]*COVID-19 Research, Department ofSustainable Development, Environmental Science and Engineering, KTH Royal Institute of Technology,Teknikringen 10B, SE 10044 Stockholm, Sweden.*

*** Corresponding authors**

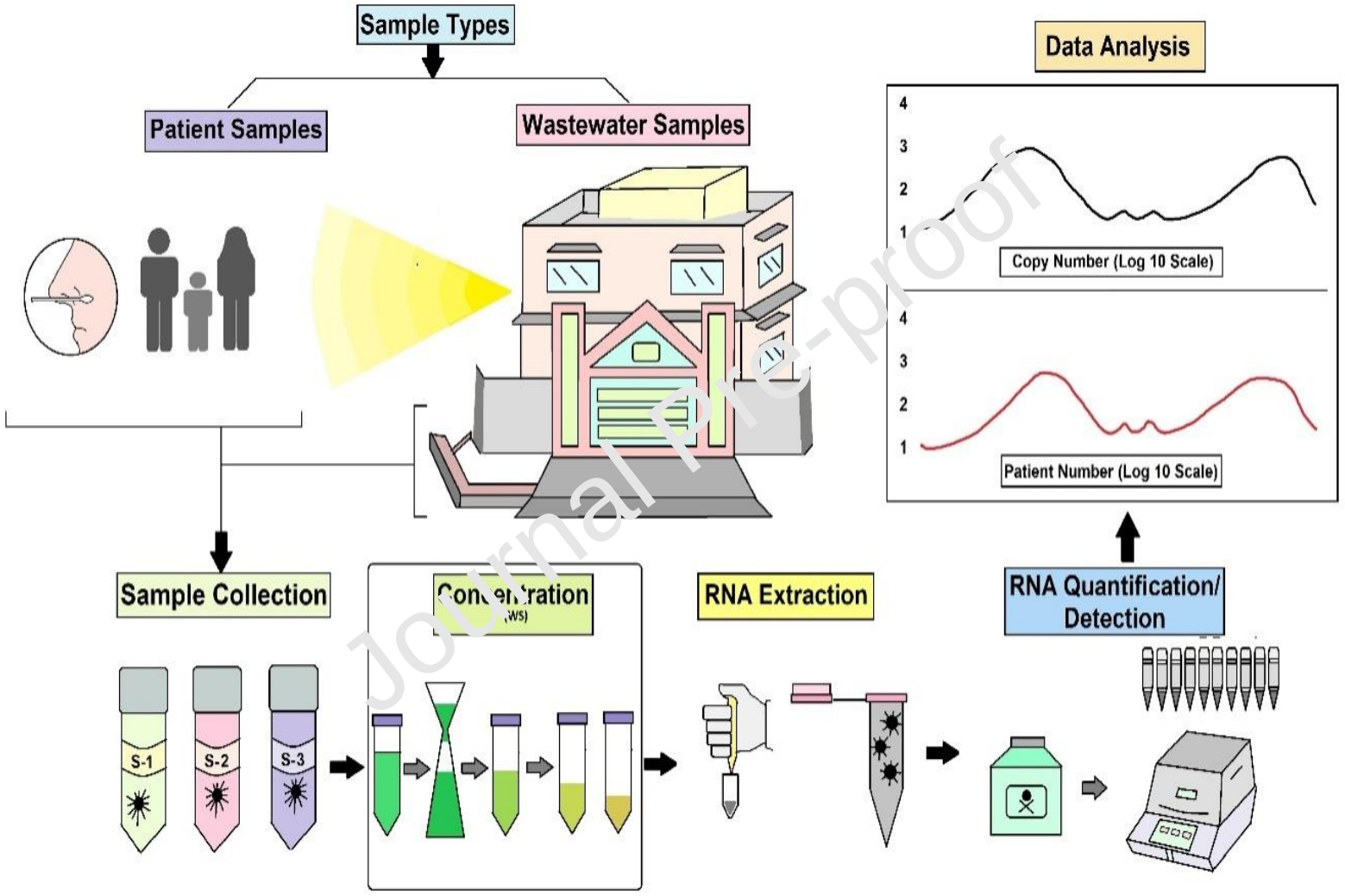
Prosun Bhattacharya, Ph.D. (E-mail: prosun@kth.se)

Firoz Ahmed, Ph.D. (E-mail: firoz@nstu.edu.bd, firoz19701016@gmail.com)

Md. Jakariya, Ph.D. (E-mail: md.jakariya@northsouth.edu, jakariya2009@gmail.com)

Md. Aminul Islam, MSc (E-mail: aminulmbg@gmail.com, aminul@pahmch.edu.bd)

Graphical Abstract



Highlights

- Wastewater SARS-CoV-2 RNA compared with COVID-19 clinical samples over a month.
- An approach to identify high-prevalence locations of COVID-19 infection.
- Positive correlation of patient number and SARS-CoV-2 genetic markers in wastewater.
- Temperature and pH affected SARS-CoV-2 RNA load in wastewater.
- Delta variant (B.1.617.2) was detected from both clinical and wastewater samples.

Abstract

Wastewater Based Epidemiology (WBE) is an important tool to fight against COVID-19 as it provides insights into the health status of the targeted population from a small single house to a large municipality level in acost-effective,rapid and non-invasive way. The implementation of WBE-based surveillance could reduce the burden on the public health system, management of pandemics, help make informed decisions and protect public health. In this study, a house of COVID-19 patients was targeted for monitoring the prevalence of SARS-CoV-2 genetic markers in wastewater samples (WS) with clinical specimens (CS) over a period of 30 days. RT-qPCR technique was employed to target nonstructural (ORF1ab) and structural-nucleocapsid (N) protein genes of SARS-CoV-2, according to the validated experimental protocol. Physiological, environmental, and biological parameters were also measured following the American Public Health Association (APHA) standard protocol. SARS-CoV-2 viral shedding in wastewater peaked when the highest number of COVID-19 cases were clinically diagnosed. Throughout the study period, 7450 to 23000 gene copies/1000 mL were detected, where we found 47 percent (57/120) positive samples from WS and 35 percent (128/360) from CS. When the COVID-19 patient number was the lowest (2), the highest CT value (39.4; i.e., lowest copy number) was identified from WS. On the other hand, when the COVID-19 patients were the highest (6), the lowest CT value (25.2 i.e., highest copy numbers) was obtained from WS. An increased signal of the SARS-CoV-2 viral load from the COVID-19 patient signal was found in the WS earlier than in CS. Using customized primer sets in a traditional PCR approach, we confirmed that all SARS-CoV-2 variants from CS and WS were Delta variants (B.1.617.2). To our knowledge, this is the first follow-up study to determine a relationship between COVID-19 patients and the discharge of SARS-CoV-2 RNA genetic markers in wastewater from a single house including all family

members for clinical sampling from a developing country (Bangladesh) without a proper sewage system. The findings of the study indicate that monitoring the genetic markers of the SARS-CoV-2 virus in wastewater could identifyCOVID-19 cases, which reduces the burden on the public health system in COVID-19 pandemics.

Keywords: Wastewater-based epidemiology (WBE), Clinical diagnosis, Wastewater samples, Clinical samples, SARS-CoV-2 variants, COVID-19 hotspots.

1. Introduction

The (COVID-19) pandemic, caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), has stemmed about 596.29 million confirmed cases and about 6.45 million deaths globally as of August 16, 2022 (WHO 2022). Clinical diagnostic tests such as real-time polymerase chain reaction (q-PCR), quantitative reverse transcription PCR (RT-qPCR), rapid antigen and antibody test (RAT), and traditional serological testsareaccepted as gold standard methods for detecting causative agents of many diseases including COVID-19. Unfortunately, clinically diagnosis of COVID-19 can detect viral genetic materials from 7–14 days following the exposure of SARS-CoV-2 and is unable to detect symptomatic individuals (silent spreader of COVID-19) within the communities (Figure 1) (Biggerstaff et al., 2014; Garg et al., 2020). Following the infection, the SARS-CoV-2 virus and the various genetic components are shed through feces, urine, saliva, and other respiratory discharges from infected patients (Tang et al., 2020; Wang et al., 2020). Human wastes and biological fluids containing the viral loads are discharged through wastewater outletsduring brushing, washing, sneezing, coughing, bathing or showering, washing clothes or hands, and wipes (Zheng et al., 2020).

WBE of SARS-CoV-2 has been reported as a useful and complementary approach for tracking the pandemic through the identification of COVID-19 hotspots and monitoring of the infection trends (Ahmed et.al, 2021, Barceló, 2020a; D'Aoust et al., 2021; Bivins et al., 2020; Kumar et al., 2021a, b, c; Haramoto et al., 2020; Weidhaas et al., 2021; Wu et al., 2020; Jakariya et al., 2021). On the other hand, WBE can also unravel the genetic markers of the viral RNA contributed by mild or asymptomatic patients and provides clinically unreported transmission episodes (Tang et al., 2020) (**Figure 1**). Moreover, new mutations with genetic variants Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), and Omicron (B.1.1.529) also can be tracked through WBE (Aleem et al., 2022). There are limited studies that link the SARS-CoV-2 viral concentration in wastewater with the identification of clinical cases in a specific residential area lacking wastewater treatment plants from developing countries (Randazzo et al., 2020; Rimoldi et al., 2020; Wurtzer et al., 2021; Zhang et al., 2021). However, the recovery of the SARS-CoV-2 viral RNA from wastewater is very challenging due to differential stability in wastewater streams (Kumar et al., 2021), various environmental factors such as rainfall and temperature, as well as the presence of inhibitory substances (Ribonuclease Enzyme-RNase) (Farkas et al., 2018; Polo et al., 2020).

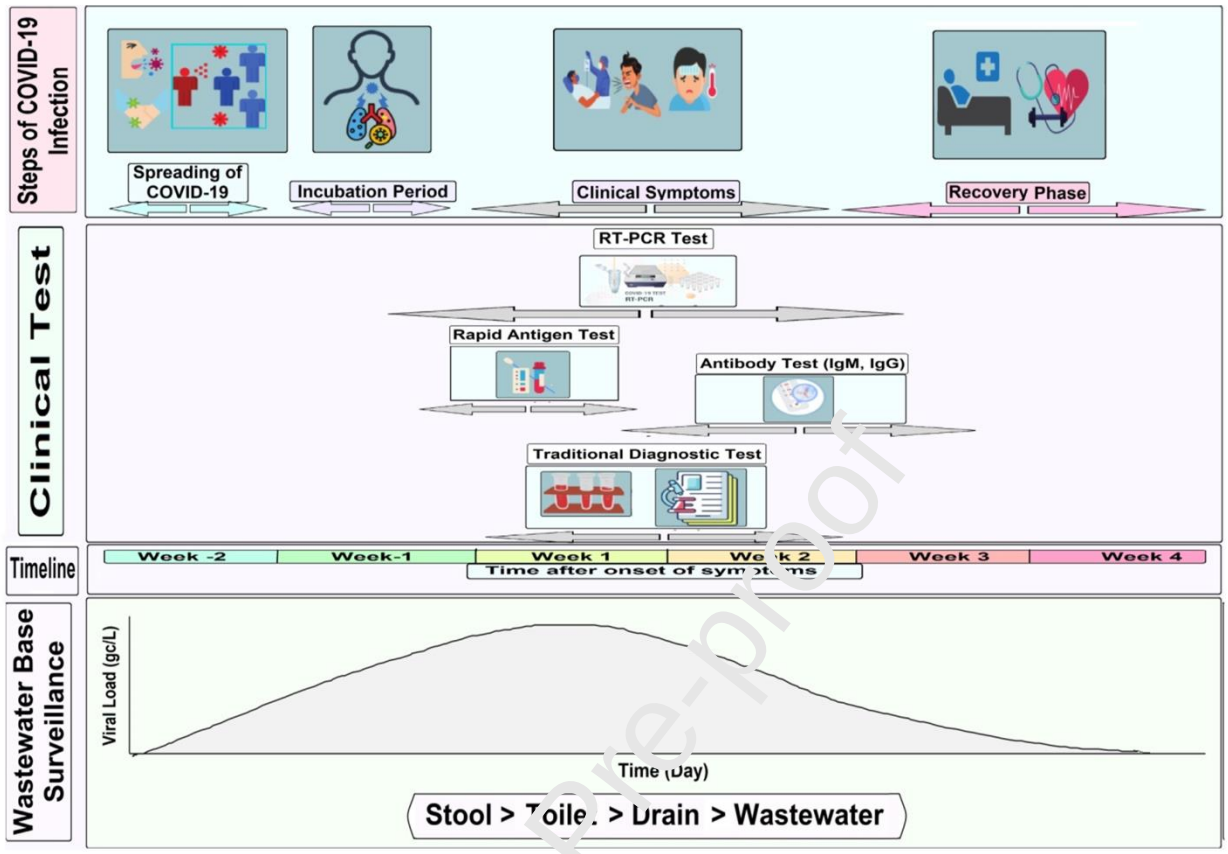


Figure 1. Schematic diagram showing the timeline of COVID-19 diagnosis using clinical tests and wastewater-based epidemiology (WBE). The WBE can detect individuals before clinical testing as stool contains viral RNA. Clinical diagnostic testsmostly detect symptomatic patients seeking testing.

The present study aims to link the number of clinically confirmed COVID-19 cases with the prevalence of SARS-CoV-2 RNA markers in wastewater samplesbased on a pilot investigation in a single household with clinically diagnosed patients and variants investigation. We tracked the main sources of SARS-CoV-2 RNA markers in wastewater from the patient's house and predicted COVID-19 cases with SARS-CoV-2 RNA in wastewater. To the best of our

knowledge, this is the first follow-up study of COVID-19 wastewater and clinical samples from a single residence which will be useful for other developing countries like Bangladesh to detect COVID-19 cases earlier than the clinical diagnosis.

2. Material and methods

2.1 Wastewater and clinical samples collection

Wastewater samples were collected daily for one month from the house of symptomatic COVID-19-positive patients located in the Noakhali district of southern Bangladesh (**Figure2**, Supplementary Table S1), which had previously been confirmed by the Directorate General of Health Services, DGSH report (Supplementary Figure SF1, SF2). The sampling area is less than 1,000 square feet with five toilets, two bathrooms, four sinks, four basins, and three kitchens with ~5 gal/day average wastewater flow. Among the twelve family personnel, three are babies (< 2 years), two are children (2-12 years), four are adults (12-70 years), and three are aged (>70) in the selected house.

Wastewater includes all the water from toilets, showers, baths, basins and sinks, kitchens, and laundries. Four sampling sites covered all the drains of the selected house, where S1 was the main drain connected with feces and urines; S2 was linked with bathing outlets; S3 came from basin sand sinks; S4 was associated with household wastewater and kitchen outlets (Supplementary Table S1). Composite samples were collected daily from sampling sites of the house wastewater drain system from 22nd October to 20th November 2021. 100 mL of wastewater samples from 10 PM to 10 AM were collected and transferred to the NSTU laboratory in a sample transportation box. The samples were processed without refrigeration within 1hour of collection time without any refrigeration. Following standards and guidelines for

wastewater,relevant Physico-chemical, environmental, and bacteriological data were obtained using a sample collection form (Supplementary Table S2, S3). To prevent cross-contamination during transportation, autoclaved sample collection bottles wer eused. All the experiments and analyses were conducted at the COVID-19 Diagnostic Laboratory in the Department of Microbiology, NSTU, Bangladesh (A government laboratory supported by GoB-Government of Bangladesh and quality controlled by the WHO). To assess the internal quality of the laboratory, testing methods, and results, 5% of samples were analyzed using RT-PCR in other laboratories (North-South Genomic Research Center (NSU) and Jashore University of Science and Technology (JUST) Genome Center). During the wastewater sample collection period, clinical samples from 12 persons in the selected house were collected daily by nasal and oral swab using viral transport media (VTM) (Cat: NSTF90184; Invitrogen, UK) following the CDC COVID-19 clinical sample collection protocol (CDC, 2020).

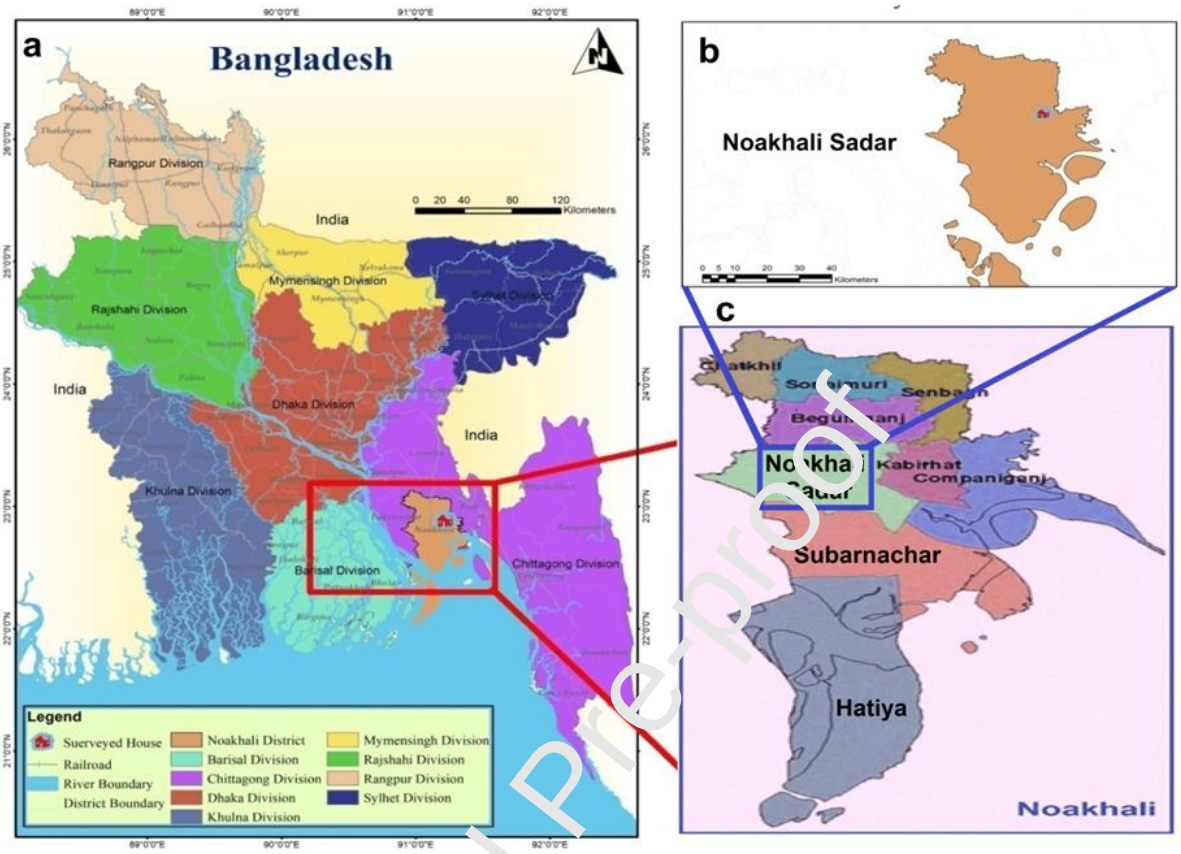


Figure 2. a). Map of Bangladesh showing the study area in Noakhali district (marked red box); b) The selected house for sampling with SARS-CoV-2 positive patient; and c) The location of the study house in the Noakhali.

2.2 Ethics statement

The study was reviewed and approved by the ethics committee of the Directorate General of Health Sciences (DGHS), Bangladesh, and by the National Research Ethics Committee (NREC) (Ref: BMRCAIREC/2019-2022I708) of the Bangladesh Medical Research Council (BMRC). Noakhali Science and Technology University (NSTU) Review Board for Human Subjects Protection looked over the work based on some criteria (NSTU 0165). NSTU COVID-19

Diagnostic Laboratory is a Bangladesh Government approved national COVID-19 testing center. Relevant demographic, clinical, and laboratory data were retrieved from the clinical records of the patient and signed written informed consents were obtained from participants and/or their legal guardians (Supplementary Figure SF5).

2.3 Environmental and physiochemical data

Environmental data were obtained from the database reported in the open information system on Worldmeter (Worldmeters.info, 2022), the official national data on WHO Coronavirus (COVID-19) Dashboard (WHO, 2022), and Bangladeshi public news reports. The analytical tests of the collected wastewater samples were performed as described in APHA (Ayaliew Werkneh, 2015) for different parameters such as temperature, pH, chemical oxygen demand (COD), dissolved oxygen (DO), conductivity, total suspended solids (TSS), total dissolved solids (TDS), and *E. Coli* counted as a standard microbiological procedure (Haque et al., 2022; Hossain et al., 2021).

2.4 Wastewater sample preparation and viral RNA extraction

All the wastewater samples (120mL from each of the four sites) were filtered using a 0.22µL syringe filter (Cat. No.231GE; Merck, USA Sartorius Products) and concentrated using Polyethylene Glycol (PEG-8000) (**Figure 3**) following the standard procedure published elsewhere (Chen et al., 2020; Ahmed et al., 2021; Kumar et al.2021b).Total RNA was extracted from both concentrated wastewater samples and clinical samples using the QIAGEN Viral RNA extraction Mini Kit (CAT NO./ID 52940) according to the kit protocol. RNA quantity of all extracted wastewater samples checked by NanoDrop (Thermo Scientific TM Nanodrop 2000 and 2000c, BioRad). RT-qPCR (BioRad-CFX 96; CFX Maestro Software 2.2) was used to identify

SRS-CoV-2 positive samples. To check internal laboratory quality, another RT-PCR equipment (Applied Biosystems™ Quant Studio 5 package-Thermo Fisher-Scientific; Software- Quant 5 Studio) used in this study. For detecting SARS-CoV-2 positive patients, Bangladesh DGHS selected two genes (ORF1ab, N), with the human RNase P gene serving as the internal control (IC) for RT-PCR diagnostics. Hence, SARS-CoV-2 RNA from sewage samples and clinical samples were determined using the same genes. The Human RNase P gene was used as a endogenous control indicating the validation of RNA extraction and the presence of inhibitors in the wastewater samples, which is a standard gene fragment with all of the RT-PCR runs to detect the human Ribonuclease P gene.

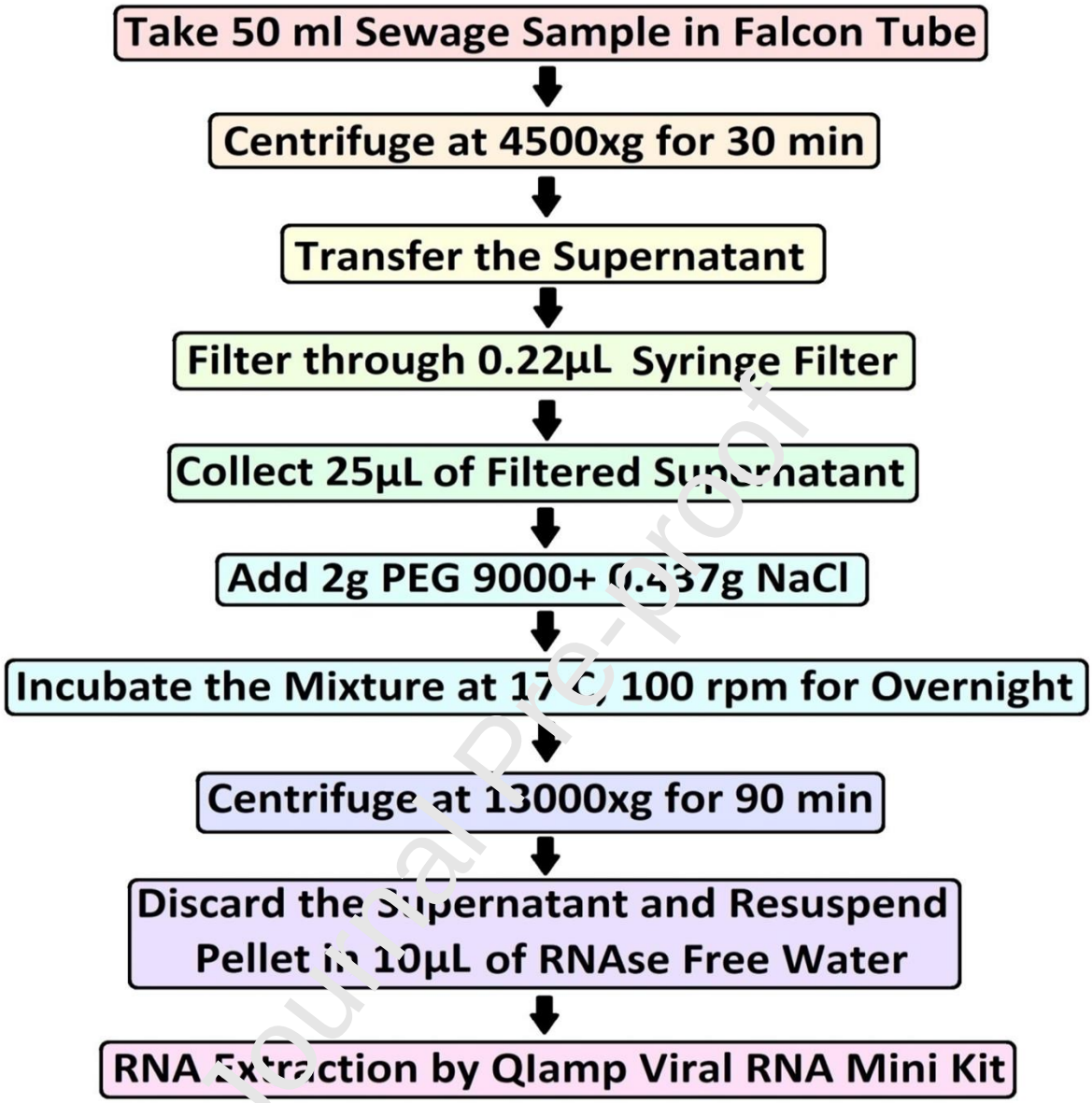


Figure 3. Optimized methodfor the concentration of the SARS-CoV-2 RNA using PEG following the methods described earlier (Chen et al., 2020; Ahmed et al., 2021; Kumar et al., 2021b).

Predominantly, SARS-CoV-2 genetic components were detectedusinga commercial RT-PCR kit (Sansure Biotech Inc., China) (Cat: 034BF234),and the results were interpreted according to the kit protocol (Doc. #: 2019-nCoV IFU). In summary, we used a 45-cycle RT-PCR technique to

detect fluorescence FAM dye for ORF1ab, ROX dye for the N gene, and CY5 for human RNase-p (Supplementary Table S4). RT-PCR reactions were run on a CFX96 Touch Real-Time PCR Detection System at 50 °C for 30 minutes, then 47 cycles of 95 °C for 10 seconds and 60 °C for 30 seconds. The sample was selected as positive for both WS and CS if the cycle threshold was below or equal to 40 cycles. To validate the RT-PCR kit, we used another commercial kit (BGI; 2019-nCoV RT-PCR kit) to test 5% positive and negative samples at random and found similar results. SARS-CoV-2 genes were quantified using 10-fold dilutions (range of 1.0E+02 to 1.0E+05 copies per assay) with the 2019-nCoV N plasmid DNA as known positive control from IDT (Leuven, Belgium).

2.5 Estimation of SARS-CoV-2 cases from wastewater

To estimate the prevalence of COVID-19 cases by analyzing wastewater samples, we followed a formula based on the average number of RNA copies in four sampling sites (Ahmed et al., 2020). Equation 1 was used to estimate COVID-19 patients where feces excreted/person/day = 128 g was assigned (Rose et al., 2015). The Monte Carlo method was employed to determine the number of SARS-CoV-2 RNA copies shed in stool by infected people using Oracle Crystal Ball (Release 11.1.2.4.600, Redwood City, CA). The daily wastewater was assumed to be an average of 22.5 L /day (Okoffo et al., 2019).

$$\text{infected number} = \frac{\left(\frac{RNAcopies}{literwastewater}\right)\times\left(\frac{literswastewater}{day}\right)}{\left(\frac{gfeces}{\frac{person}{day}}\right)\times\left(\frac{RNAcopies}{gfeces}\right)} \quad \text{Equation .....(1)}$$

2.6 Identification of SARS-CoV-2 variants

The cDNA was synthesized from viral RNA using the SuperScriptTM III First-Strand Synthesis System (InvitrogenTM, Thermo Fisher Scientific, USA) at the COVID-19 Diagnostic Laboratory, NSTU and Genome Center, JUST. The cDNA concentrations were measured using the dsDNA HS Assay Kit combined with Qubit 4 Fluorometer (Thermo Fisher Scientific, USA). In this study, New England Biolab 2X master mix and designed primer sets were used (Table 1), and 50 µL of PCR reaction volume was performed in a T100 Thermal cycler (Bio-Rad, United States) using a validated annealing temperature (Supplementary Table S4), then confirmed by 2% gel electrophoresis using Bio-Rad Gel Documentation system. Primers for SARS-CoV-2 variants were designed using previously submitted data sets from GISAID (https://www.gisaid.org/). Samples sequenced by whole-genome sequence (GISAID Accession ID- EPI_ISL_1626483 to EPI_ISL_1664527, EPI_ISL_2036272, EPI_ISL_2350142, EPI_ISL_234980 submitted by Noakhali COVID-19 Diagnostic Lab, NSTU with NSU Genome Research Center and JUST Genome Center were used for primer validation (Hossain et al., 2021).

Table 1. Primer sets used for SARS-CoV-2 variants in this study

Name	Sequence	Product size (bp)	Tm (◦C)	Reference
WV	F:CTCCAGGGCAAACTGGAAAG R:CAGTTGCTGGTGCATGTAGAA	338	54^0C	This study
IV-1	F: GCACACCTTGTAATGGTGTTC R:GGGACTTCTGTGCAGTTAACAC	390	51^0C	
IV-2	F:GGTTGGTGGTAATTATAATTACCG	78	51^0C	

	R:CCTTCAACACCATTACAAGGTT		
SAV-1	F: CTCCAGGGCAAACTGGAAAT R:GGACTTCTGTGCAGTTAACAC	629	53^0C
SAV-2	F: GCACACCTTGTAATGGTGTTA R: GGTTGGTAACCAACACCATA	90	48^0C
UKV-1	F: GCACACCTTGTAATGGTGTTA R: GGACTTCTGTGCAGTTAACAC	392	48^0C
UKV-2	F: CATATGGTTTCCAACCCACTT R:GGACTTCTGTGCAGTTAACAC	341	46^0C

Note: WV=Wuhan/Conventional Variants; IV=Indian Variant; SA=South African Variant; UKV=United Kingdom Variant.

2.7 Results validation and quality control

To avoid cross-contamination during sample collection and transportation, one sample collection bottle was filled with normal saline and checked during the RT-PCR run; positive, negative, and no-template controls were also used as per the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009; Huggett et al., 2013). An extraction control was used with a positive and negative COVID-19 patient sample. To check and verify RT-PCR inhibition, a known sample (Positive Standard Sample-Confirmed by RT-PCR from three different labs) was used in each run, if the results were similar (acceptable 5% deviation) with the previously determined positive and negative samples by RT-PCR, all samples were used for further analysis. As described in the standard protocol, for viral seeding, 100 mL wastewater samples were concentrated using the precipitation method. Then, a positive clinical sample for SARS-CoV-2, with 7.3×10^6 gene copies/L (GC/L) was employed. The recovery efficiency of SARS-CoV-2 was assessed in this study using bovine coronavirus

(BCoV) based on gene copies quantified by RT-qPCR (Joshi et al., 2022). To verify primer-primer dimer and false-positive results, a melt curve was used before the analysis and avoided all false-positive results.

2.8 Data analysis and cost calculation

Quantitative variables were summarized using mean and standard deviation (SD). The association between quantitative variables was calculated using the Pearson correlation coefficient and linear regression. Statistically, a significant difference was judged as *p<0.05 and **p<0.01. The statistical analysis was done using the R programming tool and SPSSv.25. Wastewater and clinical sampling, experiments, and analysis costs were calculated using standard prices of reagents.

3. Results

3.1. Detection of SARS-CoV-2 genetic marker in the wastewater and clinical samples

Overall, 47% (57/120 of the total samples) of wastewater samples and 35% (128/360 of the total samples) of clinical samples were positive for two SARS-CoV-2 genetic RNA markers of (ORF1ab or N). The results indicate that the SARS-CoV-2 positive rate is found higher (p = 0.020) in wastewater rather than in clinical samples. Both ORF1ab and N regions of SARS-CoV-2 were detected in 8 samples (7%) of wastewater and 65 (18%) clinical samples. On the contrary, the observation showed that SARS-CoV-2 RNA genetic markers in the clinical samples were consistently stable (p = 0.002) than in wastewater samples, which could be due to the availability of RNase enzymes and other cofactors in the environment (Jakariya et al., 2022). In the wastewater samples, only nucleocapsid (N-gene) region was found in 31 (65%), the Nonstructural region (ORF1ab gene) in 21 (44%), and the internal control human genes (RNase-

P gene) in 8 samples (17 %) (**Figure 4**). In addition that, 100% IC-internal control gene (RNase-p gene), 85% N genes, and 30% ORF1ab genes were determined from clinical samples of the positive patient's house (**Figure 4**). The RNase-p gene detected the presence of human genes in collected wastewater samples, indicating that the Ribonuclease P gene was common in all the clinical samples, where 17% were identified in wastewater samples.

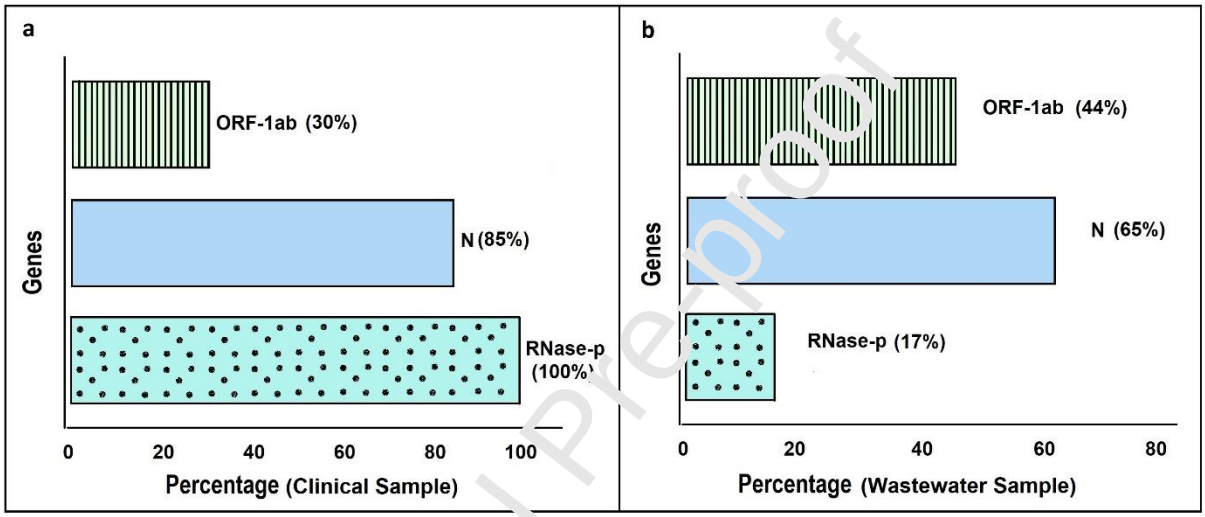


Figure 4. Percentage of SARS-CoV-2 genes (ORF1ab, N, and RNase-P) in wastewater and clinical samples (a) Percentage of genes in Clinical samples (b) Percentage of genes in wastewater samples.

From the clinical samples, the highest CT values detected for the ORF1ab gene (39.37), N gene (39.00), and RNase-P gene (39.48), while the lowest ORF1ab and N genes CT values were correspondingly observed at 26.14 and 26.28), with RNase-P value 25.45. When analyzing the day-wise prediction, the lowest SARS-CoV-2 RNA was observed during the last week of sample collection as patient numbers declined. In the wastewater samples, the highest CT values were observed for the ORF1ab gene (CT =39.4) and N gene (CT =39.65), with the RNase-P gene (CT =39.7), while the lowest CT values for ORF1ab, N and RNase-P genes were 32.11, 31 and 25.2 respectively during the third week of sampling.

Prior to the data analysis, positive control, negative control, extraction control, and no template control (NTC) are checked. From four sampling sites, maximum positive samples were found in site 1 which was obtained from urine and feces connected with toilets (Jones et al., 2020) (Supplementary Table S6**)**. We identified the potential sources of SARS-CoV-2 RNA captured from four sampling points of the selected house (Supplementary Table ST6). Compared to the other three points, we found the highest percentage of SARS-CoV-2 positive samples in S1 (50%) and the lowest in S3 (30%) (Li et al., 2022). Our study findings matched with previous similar studies carried out for the detection of SARS-CoV-2 RNA in the patient's bodily fluids such as blood, feces, urine, saliva, and sputum (Peng et al., 2020).

Table 2: SARS-CoV-2 recovery from sewage samples concentrated by PEG precipitation

SARS-CoV-2 seeded (GC/L)	SARS-CoV-2 recovered genes		Mean Concentration (GC/L) ± SD[a]	Mean Recovery %
	ORF1ab	N		
7.3×10^3	(2/3)	(2/3)	3.6×10^3 -6.1×10^3	66.12
7.3×10^2	(3/3)	(2/3)	4.4×10^2 -5.9×10^2	70.32
7.3×10^1	(1/3)	(3/3)	3.3×10^3-5.2×10^3	58.43

Note: SD[a] = Standard Deviation

3.2. Early detection and higher SARS-CoV-2 RNA from wastewater

We observed a high positivity rate in wastewater samples, with 75% positive samples being reported between November 3rd and November 9th, 2021, followed by a decline in SARS-CoV-2 positive samples. The SARS-CoV-2 positive rate in clinical samples ranged from 16% (2/12) in the first week of monitoring to a maximum of 50% (6/12) in the 3rd and early half of the 4th

week, before reverting to 25% at the end of the study (Table 3). Notably, the rising trend of the positive rate in wastewater samples is similar to the positive rate observed in clinical samples. This result suggests that wastewater can be used for the early detection of COVID-19 infection and hotspots. Wastewater tracking can indicate whether the number of people infected with COVID-19 in a community is going up or down.

Table 3. Comparative evaluation of the percentage of positive SARS-CoV-2 genes in wastewater samples (WS) and clinical samples (CS).

Sampling Date	October 2021										November 2021																				
	22	23	24	25	26	27	28	29	30	31	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Positive Rate in CS	16%						25%				41%				50%									33%				25%		
Positive Rate in WS	25%						50%								75%						50%							25%		

The results indicate that Athe positive rate ofthe abundance of both the generic markers ofSARS-CoV-2 RNA (ORF1ab, N) detected in WS was higher than in clinical samples (Wu et al., 2020).

3.3 Environmental factors

SARS-CoV-2 viral copy numbers correlated with temperature and pH in wastewater showed in the Figure 5. An increase in temperature was linked to a decrease in cycle threshold values (p = 0.001), especially for the ORF1ab and N genes. Most of the SARS-CoV-2 genes were found at a temperature ranging from 27 to 35°C (Table 4). Similarly, there was a larger presence of SARS-CoV-2 RNA in the neutral to alkaline pH (6.23-11.89) ranges, greater than the tolerance level. The temperature of wastewater strongly correlated with CT values (**Figure 5**). This study was conducted during the summer season when the lowest temperature detected was 27.3^0C. Hence, winter season or cold days are not linked with COVID-19 cases during the study period.

Notably, another study evaluated the effect of temperature on SARS-CoV-2 RNA in wastewater, where most of the findings are similar to our study results (Weidhaas et al., 2021).

Table 4. Physiochemical and biological parameters of wastewater

Parameter		Maximum	Minimum	Mean± SD	P-Value
Physicochemical	pH	11.89	6.23	7.64 ± 1.37	0.04*
	Temperature(^{0}C)	35.6	27.01	30.4±61.66	0.03*
	TDS (ppt)	7.62	2.16	5.149±1.74	0.67
	TSS (mg/L)	14	[illegible]	6.6 ±8	0.28
	EC (mS/m)	10.84	[illegible]	6.27±1.4	0.33
	Salinity (ppt)	5.17	[illegible]	3.62±1.2	0.54
	COD (mg/L)	[illegible]	15	55.33±15	0.12
	DO (mg/L)	[illegible]	1.4	3.07±1.4	0.65
Biological	*E. Coli*/100mL	[illegible]	120	292.16±120	0.12
Environmental (Air)	Temperature(^{0}C)	38	18	25± 2.12	0.21
	Rainfall (mm/h)	6.1	0.1	2.4 ± 0.76	0.53
	Humidity (g.kg^{-1})	96	49	63 ± 1.5	0.65

Note: SD= Standard deviation; TDS= Total dissolved solid; TSS= Total suspended solid; EC=Electric Conductivity; COD= Chemical oxygen demand; DO= Dissolved oxygen; *=Significant at 5% level.

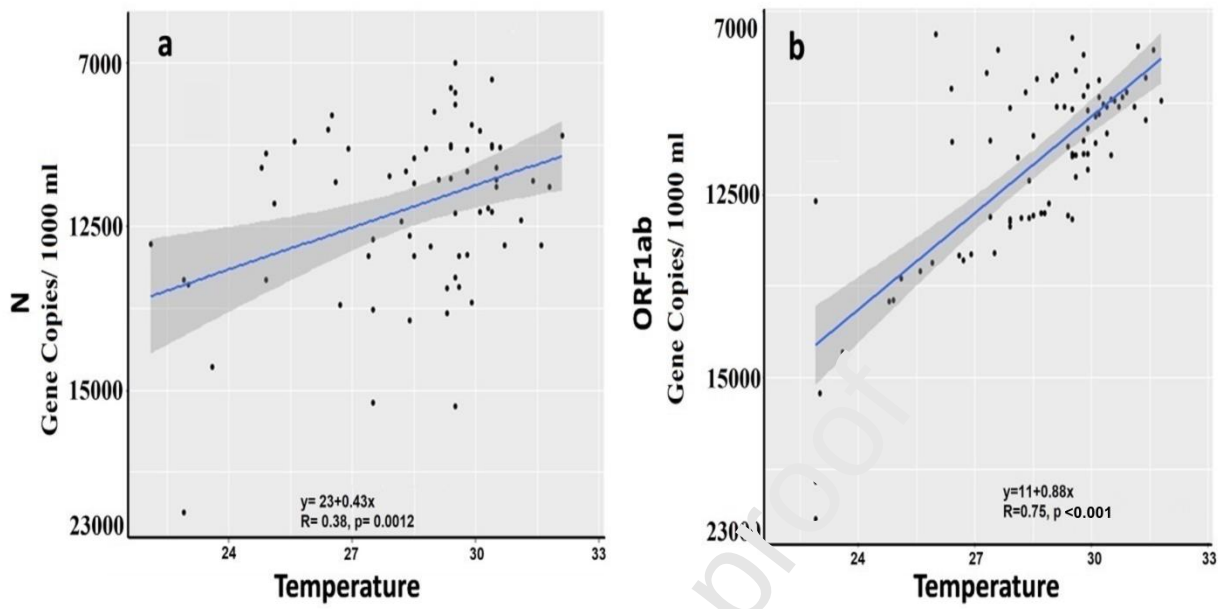


Figure 5. Correlation of physicochemical parameters with the abundances of the genetic markers of SARS-CoV-2 in wastewater. a) correlation of temperature (Y-axis) with N gene (X-axis);b) correlation of temperature (Y-axis) with ORF-1ab gene (X-axis).

3.4 SARS-CoV-2 CT value versus confirmed cases

The number of positive cases washighly linked with viral gene copy number based on the result of wastewater (**Figure 6**). When the number of SARS-CoV-2 positive patients was lowest (two positive subjects), the highest CT value of 39.4 was found in wastewater samples with an average copy number of 7450/1000 mL of wastewater. In contrast, the lowest CT value of 25.2 was found when the number of patients was the highest (six positive subjects) with copy number 23000/1000mL (**Figure6**). Further, we found four positive patients from wastewater on the first day, with the number increasing until the 15th day, then decreasing. We also noticed that the number of calculated positive cases from wastewater increased higher than the number of

positive cases from the clinical sample and decreased more slowly. We can deduce that wastewater exhibited a patient's positive number before the clinical tests based on our COVID-19 case estimation result.

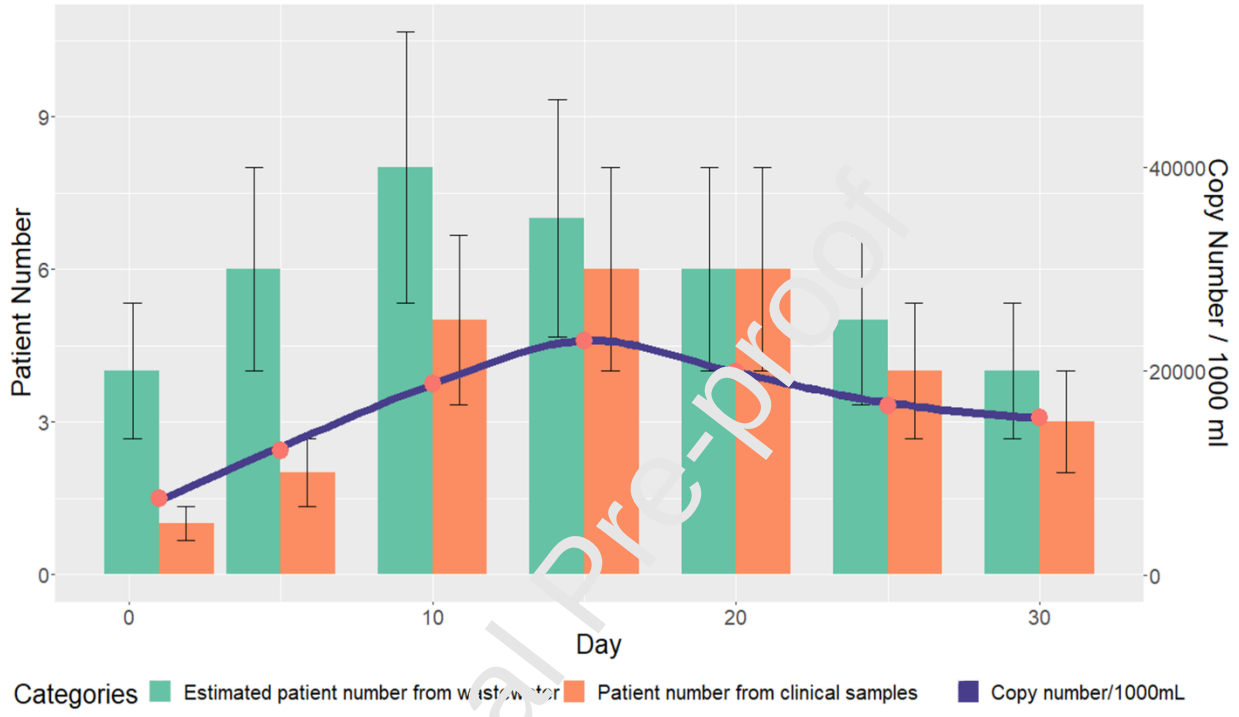


Figure 6. Gene copy number of SARS-CoV-2 in wastewater correlated with COVID-19 patient number. The trend line shows the average gene copy number/1000mL in wastewater, and bar charts show the COVID-19 patients detected concurrently with wastewater sampling.

3.5 Detection of the genetic markers of SARS-CoV-2 variants

The Delta variant was shown to be compatible with clinical samples in the wastewater study. We looked into other variants of concern (VoC), but six wastewater samples (WS-12, WS-20, WS-23, WS-26, WS-43, WS-64) tested positive for the Delta variant (Supplementary Figure S4) ensuring positive control (The whole-genome sequenced samples). This study indicated that

surveillance of wastewater is an approach that allows monitoring the diversity of SARS-CoV-2 variants circulating in the community (Nag et al., 2022). We designed primer sets for variants of interest (VoI) of COVID-19 targeting mutation points which were confirmed by conventional PCR test (Supplementary Fig S3). Our findings indicated that L452R and T478K mutations are available in Delta (B.1.617.2) variants. It was also observed that the L452R mutation of COVID-19 enhanced the infectivity and evaded the cellular immunity of patients (Suchard et al., 2018). Additionally, L452R helped in decreasing the binding of specific monoclonal antibodies (mAbs) with neutralization. Another common mutation of the Delta variant, T478K, was possibly associated with increasing ACE2 binding sites, which helps to increase transmissibility (Zhang et al., 2022). This study also demonstrated that the Delta variant of SARS-CoV-2 was found as the dominant variant in the clinical samples and found to be more transmissible (60%) during the study tenure (Joshi et al., 2022).

3.6 Cost implications of WBE than clinical surveillance

WBE surveillance is more affordable (60 $/day) than clinical sample surveillance (419 $/day) systems for predicting the COVID-19 pandemic (Supplementary Table ST5) (Abdalhamid et al., 2020; Esbin et al., 2020; Won et al., 2020). Although the sample preparation and RNA concentrationneed extended time, the overall procedure was more accessible and completed within 5-6 hours. The pilot cost calculation report of this study indicated that WBEcould be employed for monitoring of COVID-19 pandemic in low-income developing countries (Zhang et al., 2022).

4. Discussion

According to our results, CT value and SARS-CoV-2 gene copy numbers can be used to evaluate COVID-19 trends, which will eventually aid in determining the number of COVID-19 patients (Daughton, 2020). Based onthe RT-qPCR CT (25-39) value of WS, it can be concluded that the

range was varied,which supports previous data (Mlejnkova et al., 2020). One previous study in Massachusetts between March and May 2020 found that the amount of SARS-CoV-2 RNA in WS fluctuates in a similar way toCOVID-19 patients' trends (Wanget al., 2007). Another study in Utah used 9-week WS sampling and found a link between a community outbreak and an increase in SARS-CoV-2 RNA (Weidhaas et al., 2021). As there is no wastewater treatment systemin the selected house and no interlinkage with other drain systems, all the SARS-CoV-2 RNA in the wastewater samples represented the shedding from 12 persons in the house. We also observed an increasing trend of SARS-CoV-2 RNA in WS with the increased number of COVID-19 patients in the household as well as time-dependent decreasein the copies of SARS-CoV-2 RNA genetic markers with the recovery of patients. We also noticed that the trend of ups and downs was first documented from the WBE surveillance of the WS.

The study also reveals that SARS-CoV-2 RNA appears to be damaged or destroyed more frequently in WS than in CS (Ahmed et.al, 2021). In CS, Nucleocapsid protein genes (N gene) were less damaged than nonstructural protein genes (ORF-1ab), and the percentage ofthe internal control gene, RNase-P, was higher. The exact causes for these findingsare still unclear and further research is needed. The viral percentage in WS was found to be higher than in CS (47%>35%), and an increasing trend was first observed in WS than in CS. These results support WBE as a robust and reliable tool for anearly warning system. Some previous WBE studies around the world also support the early detection of SARS-CoV-2 RNA from WS. Madema et al., (2020) reported the SARS-CoV-2 genetic material in WS in February, which was beforethe official confirmation of the first clinical case in the Netherlands. La Rosa et al. (2020) detected SARS-CoV-2 genes in WS before the first official report for two cities in Italy. Environmental parameters are also linkedto SARS-CoV-2 RNA, as evidenced byan increase in wastewater

temperature resulting from a decrease in viral gene copy numbers (Bardiet.al., 2021; Hart et al., 2020). In aprevious study,virus concentrations in wastewater samples in Canada (Ottawa)surged by >400% just 48 hours after a >300% risein recognizedcases (D'Aoust et al., 2021), and Utah showed a strong linkbetween communityoutbreaks and increases in SARS-CoV-2 RNA in wastewater (Weidhaas et al., 2021). From the four sampling sites, we have observed larger two drains carried maximum viral gene copies of 17000/1000 mL (Ahmed et al., 2021).

COVID-19 can be diagnosed using a variety of clinical laboratory tests (Alkhateeb et al., 2021), however implementing these could be difficult for various factors, such as lack of consumables, and shortage of reagents with high cost, ambiguity, and a lack of monitoring experts. Another major problem is that viral particles are exhibited later in feces than in a clinical sample. These obstacles prompted the development of different epidemiological methods (Foladori et al., 2020). WBE can be used to detect asymptomatic (Individuals without any symptoms) and pre-symptomatic (Individuals before showing any symptoms) persons (Rosa et al., 2020) because they both excrete the virus with feces.The current study result also supports WBE as a complementary tool because we were also able to detect SARS-CoV-2 variants from WS, where all detected variants were delta variants. We observed that WBE is less time consumable and needs low cost than CS as this study required 3X CS (Number of Clinical Sample =360, Wastewater Sample =120) than WS and 7X cost higher for CS than WS (Esbin et al., 2020).

We found SARS-CoV-2 RNA in all four sampling sites, with urine and feces accounting for 35% of positive samples,and this sampling site connected directly with toilets indicating that toilet wastewater can be used for sampling. Other sampling sites linked with bathing outlets, basins, sinks, and kitchen outlets can also be used as secondary sources. The lowest percentages of positive samples were found in basins and sink wastewater when soap, hand wash, and other

disinfectants were used. Positive samples were found only from toilet wastewater for the first three daysto strengthen toilets wastewater for the SARS-CoV-2 surveillance system. SARS-CoV-2 genetic material and its propensity for dissemination are not uniformly distributed across the country. To determine the pandemic trend, wastewater samples must be analyzed at regular intervals over a longer period. The seasonal variation must also be considered, as it significantly impacts the propagation of harmful viruses and bacteria. To our knowledge, this follow-up study from non point waste water sources without wastewater treatment plants showed significant results that will help developing countries like Bangladesh to identify COVID-19 cases earlier than the clinical test.

This current study suggest that wastewater surveillance could be useful for monitoring the COVID-19 pandemic. However, more research is required to determine the link between COVID-19 symptom severity and SARS-CoV-2 RNA shedding in fecal samples and eventually wastewater discharges. The concentration of viral RNA in wastewater can be affected by environmental variables such as temperature and pH, as these factors can affect the decay rate of SARS-CoV-2 RNA (Tiwari et al., 2022). The study also illustrates that the WBE can cover all four aspects of SARS-CoV-2 surveillance, including early warning, monitoring of propensity trends, genetic diversity, and prevalence of the SARS-CoV-2 variants in rural areas lacking proper sewage or drainage system.

5. Conclusion

This study exclusively compared the prevalence of SARS-CoV-2 RNA (ORF1ab and N genes) in wastewater and the clinical samples from COVID-19 patients for one month in a house where COVID-19 was confirmed. The COVID-19 patient numbers in the house affected the prevalence of SARS-CoV-2 RNA in their wastewater. However, the temporal variations in SARS-CoV-2

RNA concentrations need to be further investigated from multiple perspectives. WBE can be used as a useful tool to estimate COVID-19 patients at the community level, especially indeveloping and under-developing countries with limited clinical diagnostic facilities. WBE needs to be integrated with other public health services, namely campaign-based and randomized testing of individuals (presence of COVID-19), clinical testing,web contact tracing, and self-diagnostic reporting systems. Therefore, it is necessary to develop a validated method for predicting COVID-19 patient numbers from SARS-CoV-2 viral shredding in wastewater compared with clinically confirmed positive cases. The COVID-19 pandemic in Bangladesh is now in its third stage but based on the experiences of the other countries, further waves of infection may be foreseen; therefore WBE system involving community-level health management would help to control the future outbreaks of the pandemic at scale.

6. Ethical Statement

This study was approved and reviewed by Ethical Reviewing Board at Noakhali Scienceand Technology University. Directorate General of Health Sciences (DGHS), Bangladesh, approved clinical sample collection based on some conditions (BMRCAIREC/2021/ I 708).

7. Declaration of Competing Interest

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

.

8. Acknowledgments

This study was supported by the North South University, Noakhali Science and Technology University, Water Aid Bangladesh (Grant number JP20H02284), and the International Training Network of Bangladesh University of Engineering and Technology (ITN-BUET) - Centre for Water Supply and Waste Management. PB and MTI would gratefully acknowledge the Life Science Technology Platform, Science for Life Laboratory for the seed funding to initiate the wastewater-based epidemiological studies for SARS-CoV-2 in Bangladesh.

9. Funding Sources

This study was supported by the North-South University, Noakhali Science and Technology University, Water Aid Bangladesh (Grant number JP20H02284), and the International Training Network of Bangladesh University of Engineering and Technology (ITN-BUET) - Centre for Water Supply and Waste Management. PB and MTI would gratefully acknowledge the Life Science Technology Platform, Science for Life Laboratory for the seed funding to initiate the wastewater-based epidemiological studies for SARS-CoV-2 in Bangladesh.

10. References

Abdalhamid, B., Bilder, C. R., McCutchen, E. L., Hinrichs, S. H., Koepsell, S. A., & Iwen, P. C. (2020). Assessment of Specimen Pooling to Conserve SARS CoV-2 Testing Resources. *American Journal of Clinical Pathology*, *153*(6), 715–718. https://doi.org/10.1093/ajcp/aqaa064

Ahmed, F., Islam, M. A., Kumar, M., Hossain, M., Bhattacharya, P., Islam, M. T., Hossen, F., Hossain, M. S., Islam, M. S., Uddin, M. M., Islam, M. N., Bahadur, N. M., Didar-ul-Alam, M., Reza, H. M., & Jakariya, M. (2021). First detection of SARS-CoV-2 genetic material in the vicinity of COVID-19 isolation Centre in Bangladesh: Variation along the sewer

network. *Science of The Total Environment*, *776*, 145724. https://doi.org/10.1016/j.scitotenv.2021.145724

Ahmed, W., Angel, N., Edson, J., Bibby, K., Bivins, A., O'Brien, J. W., Choi, P. M., Kitajima, M., Simpson, S. L., Li, J., Tscharke, B., Verhagen, R., Smith, W. J. M., Zaugg, J., Dierens, L., Hugenholtz, P., Thomas, K. V., & Mueller, J. F. (2020). First confirmed detection of SARS-CoV-2 in untreated wastewater in Australia: A proof of concept for the wastewater surveillance of COVID-19 in the community. *Science of the Total Environment*, *728*. https://doi.org/10.1016/j.scitotenv.2020.138764

Ahmed, W., Bivins, A., Smith, W. J. M., Metcalfe, S., Stephens, M., Jennison, A. V., Moore, F. A. J., Bourke, J., Schlebusch, S., McMahon, J., Hewitson, G., Nguyen, S., Barcelon, J., Jackson, G., Mueller, J. F., Ehret, J., Hosegood, I., Tian, W., Wang, H., … Simpson, S. L. (2022). Detection of the Omicron (B.1.1.529) variant of SARS-CoV-2 in aircraft wastewater. *Science of The Total Environment*, *820*, 153171. https://doi.org/10.1016/j.scitotenv.2022.153171

Aleem, A., Akbar Samad, A. B., & Slenker, A. K. (2022). Emerging Variants of SARS-CoV-2 And Novel Therapeutics Against Coronavirus (COVID-19). In *StatPearls*.

Alkhateeb, K. J., Cahill, M. N., Ross, A. S., Arnold, F. W., & Snyder, J. W. (2021). The reliability of saliva for the detection of SARS-CoV-2 in symptomatic and asymptomatic patients: Insights on the diagnostic performance and utility for COVID-19 screening. *Diagnostic Microbiology and Infectious Disease*, *101*(3), 115450. https://doi.org/10.1016/j.diagmicrobio.2021.115450

Ayaliew Werkneh, A. (2015). Physico-Chemical Analysis of Drinking Water Quality at Jigjiga City, Ethiopia. *American Journal of Environmental Protection*, *4*(1), 29.

https://doi.org/10.11648/j.ajep.20150401.14

Barceló, D. (2020). Wastewater-Based Epidemiology to monitor COVID-19 outbreak: Present and future diagnostic methods to be in your radar. *Case Studies in Chemical and Environmental Engineering*, *2*, 100042. https://doi.org/10.1016/j.cscee.2020.100042

Bardi, M. J., & Oliaee, M. A. (2021). Impacts of different operational temperatures and organic loads in anaerobic co-digestion of food waste and sewage sludge on the fate of SARS-CoV-2. *Process Safety and Environmental Protection*, *146*, 464–472. https://doi.org/10.1016/j.psep.2020.11.035

Bi, P., Wang, J., & Hiller, J. E. (2007). Weather: driving force behind the transmission of severe acute respiratory syndrome in China? *Internal Medicine Journal*, *37*(8), 550–554. https://doi.org/10.1111/j.1445-5994.2007.01358.x

Biggerstaff, M., Jhung, M. A., Reed, C., Fry, A. M., Balluz, L., & Finelli, L. (2014). Influenza-like illness, the time to seek healthcare, and influenza antiviral receipt during the 2010-2011 influenza season - United States. *Journal of Infectious Diseases*, *210*(4), 535–544. https://doi.org/10.1093/infdis/jiu224

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., & Wittwer, C. T. (2009). The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry*, *55*(4), 611–622. https://doi.org/10.1373/clinchem.2008.112797

Chen, N., Zhou, M., Dong, X., Qu, J., Gong, F., Han, Y., Qiu, Y., Wang, J., Liu, Y., Wei, Y., Xia, J., Yu, T., Zhang, X., & Zhang, L. (2020). Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *The*

Lancet, *395*(10223), 507–513. https://doi.org/10.1016/S0140-6736(20)30211-7

D'Aoust, P. M., Graber, T. E., Mercier, E., Montpetit, D., Alexandrov, I., Neault, N., Baig, A. T., Mayne, J., Zhang, X., Alain, T., Servos, M. R., Srikanthan, N., MacKenzie, M., Figeys, D., Manuel, D., Jüni, P., MacKenzie, A. E., & Delatolla, R. (2021). Catching a resurgence: Increase in SARS-CoV-2 viral RNA identified in wastewater 48 h before COVID-19 clinical tests and 96 h before hospitalizations. *Science of The Total Environment*, *770*, 145319. https://doi.org/10.1016/j.scitotenv.2021.145319

Daughton, C. G. (2020). Wastewater surveillance for population-wide Covid-19: The present and future. *Science of The Total Environment*, *736*, 139631. https://doi.org/10.1016/j.scitotenv.2020.139631

Esbin, M. N., Whitney, O. N., Chong, S., Maurer, A., Darzacq, X., & Tjian, R. (2020). Overcoming the bottleneck to widespread testing: a rapid review of nucleic acid testing approaches for COVID-19 detection. *RNA*, *26*(7), 771–783. https://doi.org/10.1261/rna.076232.120

Farkas, A., Pap, B., Kondorosi, É., & Maróti, G. (2018). Antimicrobial Activity of NCR Plant Peptides Strongly Depends on the Test Assays. *Frontiers in Microbiology*, *9*. https://doi.org/10.3389/fmicb.2018.02600

Foladori, P., Cutrupi, F., Segata, N., Manara, S., Pinto, F., Malpei, F., Bruni, L., & La Rosa, G. (2020). SARS-CoV-2 from faeces to wastewater treatment: What do we know? A review. *Science of The Total Environment*, *743*, 140444. https://doi.org/10.1016/j.scitotenv.2020.140444

Garg, S., Kim, L., Whitaker, M., O'Halloran, A., Cummings, C., Holstein, R., Prill, M., Chai, S. J., Kirley, P. D., Alden, N. B., Kawasaki, B., Yousey-Hindes, K., Niccolai, L., Anderson, E.

J., Openo, K. P., Weigel, A., Monroe, M. L., Ryan, P., Henderson, J., … Fry, A. (2020). Hospitalization Rates and Characteristics of Patients Hospitalized with Laboratory-Confirmed Coronavirus Disease 2019 — COVID-NET, 14 States, March 1–30, 2020. *MMWR. Morbidity and Mortality Weekly Report*, *69*(15), 458–464. https://doi.org/10.15585/mmwr.mm6915e3

Haque, M. A., Wang, F., Chen, Y., Hossen, F., Islam, M. A., Hossain, M. A., Siddique, N., He, C., & Ahmed, F. (2022). Bacillus spp. Contamination: A Novel Risk Originated From Animal Feed to Human Food Chains in South-Eastern Bangladesh. *Frontiers in Microbiology*, *12*. https://doi.org/10.3389/fmicb.2021.783103

Haramoto, E., Malla, B., Thakali, O., & Kitajima, M. (2020). First environmental surveillance for the presence of SARS-CoV-2 RNA in wastewater and river water in Japan. *Science of the Total Environment*, *737*. https://doi.org/10.1016/j.scitotenv.2020.140405

Hart, O. E., & Halden, R. U. (2020). Computational analysis of SARS-CoV-2/COVID-19 surveillance by wastewater-based epidemiology locally and globally: Feasibility, economy, opportunities and challenges. *Science of The Total Environment*, *730*, 138875. https://doi.org/10.1016/j.scitotenv.2020.138875

Hossain, F. E., Islam, S., Islam, M. A., Islam, S., & Ahmed, F. (2021). Detection of virulence genes of APEC (avian pathogenic Escherichia coli) isolated from poultry in Noakhali, Bangladesh. *Bioresearch Communications*, *7*(1), 967–972. https://doi.org/10.3329/brc.v7i1.54253

Hossain, M., Saiha Huq, T., Rahman, A., Aminul Islam, M., Naushin Tabassum, S., Nadim Hasan, K., Khaleque, A., Sadique, A., Salim Hossain, M., Mohammed Bahadur, N., Ahmed, F., & Mahmud Reza, H. (2021). *Novel mutations identified from whole-genome*

sequencing of SARS-CoV-2 isolated from Noakhali, Bangladesh.

Huggett, J. F., Foy, C. A., Benes, V., Emslie, K., Garson, J. A., Haynes, R., Hellemans, J., Kubista, M., Mueller, R. D., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., Wittwer, C. T., & Bustin, S. A. (2013). The Digital MIQE Guidelines: Minimum Information for Publication of Quantitative Digital PCR Experiments. *Clinical Chemistry*, *59*(6), 892–902. https://doi.org/10.1373/clinchem.2013.206375

Jakariya, M., Ahmed, F., Islam, M. A., Ahmed, T., Marzan, A. Al, Hossain, M., Reza, H. M., Bhattacharya, P., Hossain, A., Nahla, T., Bahadur, N. M., Hasan, M. N., Islam, M. T., Hossen, M. F., Alam, M. D. ul, Mou, N., & Jahan, H. (2021). Wastewater based surveillance system to detect SARS-CoV-2 genetic material for countries with on-site sanitation facilities: an experience from Bangladesh. *MedRxiv*, *8852000*, 2021.07.30.21261347.

Jakariya, M., Ahmed, F., Islam, M.A., Al Marzan, A., Hasan, M.N., Hossain, M., Ahmed, T., Hossain, A., Reza, H.M., Hossen, M.F., Nahla, T., Rahman, M.M., Bahadur, N.M., Islam, M.T., Didar-ul-Alam, M., Mow, N., Jahan, H., Barceló, D., Bibby, K. & Bhattacharya, P. (2022) Wastewater-based epidemiological surveillance to monitor the prevalence of SARS-CoV-2 in developing countries with onsite sanitation facilities. *Environmental Pollution* xx:119679. https://doi.org/10.1016/j.envpol.2022.119679 (In Press)

Jones, D. L., Baluja, M. Q., Graham, D. W., Corbishley, A., McDonald, J. E., Malham, S. K., Hillary, L. S., Connor, T. R., Gaze, W. H., Moura, I. B., Wilcox, M. H., & Farkas, K. (2020). Shedding of SARS-CoV-2 in feces and urine and its potential role in person-to-person transmission and the environment-based spread of COVID-19. *Science of The Total Environment*, *749*, 141364. https://doi.org/10.1016/j.scitotenv.2020.141364

Joshi, M., Kumar, M., Srivastava, V., Kumar, D., Rathore, D., Pandit, R., Graham, D. W., & Joshi, C. G. (2022). Genetic sequencing detected the SARS-CoV-2 delta variant in wastewater a month prior to the first COVID-19 case in Ahmedabad (India). *Environmental Pollution*, 119757. https://doi.org/10.1016/j.envpol.2022.119757

Kumar, M., Joshi, M., Patel, A. K., & Joshi, C. G. (2021). Unravelling the early warning capability of wastewater surveillance for COVID-19: A temporal study on SARS-CoV-2 RNA detection and need for the escalation. *Environmental Research*, *196*. https://doi.org/10.1016/j.envres.2021.110946

Kumar, M., Joshi, M., Shah, A. V., Srivastava, V., & Dave, S. (2021). Wastewater surveillance-based city zonation for effective COVID-19 pandemic preparedness powered by early warning: A perspectives of temporal variations in SARS-CoV-2-RNA in Ahmedabad, India. *Science of the Total Environment*, *793*. https://doi.org/10.1016/j.scitotenv.2021.148367

Li, X., Kulandaivelu, J., Guo, Y., Zhang, S., Shi, J., O'Brien, J., Arora, S., Kumar, M., Sherchan, S. P., Honda, R., Jackson, G., Luby, S. P., & Jiang, G. (2022). SARS-CoV-2 shedding sources in wastewater and implications for wastewater-based epidemiology. *Journal of Hazardous Materials*, *432*, 128667. https://doi.org/10.1016/j.jhazmat.2022.128667

Miura, F., Kitajima, M., & Omori, R. (2021). Duration of SARS-CoV-2 viral shedding in faeces as a parameter for wastewater-based epidemiology: Re-analysis of patient data using a shedding dynamics model. *Science of The Total Environment*, *769*, 144549. https://doi.org/10.1016/j.scitotenv.2020.144549

Mlejnkova, H., Sovova, K., Vasickova, P., Ocenaskova, V., Jasikova, L., & Juranova, E. (2020). Preliminary Study of Sars-Cov-2 Occurrence in Wastewater in the Czech Republic. *International Journal of Environmental Research and Public Health*, *17*(15), 5508.

https://doi.org/10.3390/ijerph17155508

Nag, A., Arora, S., Sinha, V., Meena, E., Sutaria, D., Gupta, A. B., & Medicherla, K. M. (2022). Monitoring of SARS-CoV-2 Variants by Wastewater-Based Surveillance as a Sustainable and Pragmatic Approach—A Case Study of Jaipur (India). *Water (Switzerland)*, *14*(3). https://doi.org/10.3390/w14030297

Nsubuga, P., White, M. E., Thacker, S. B., Anderson, M. A., Blount, S. B., Broome, C. V., Chiller, T. M., Espitia, V., Imtiaz, R., Sosin, D., Stroup, D. F., Tauxe, R. V., Vijayaraghavan, M., & Trostle, M. (2006). Public Health Surveillance: A Tool for Targeting and Monitoring Interventions. *Disease Control Priorities in Developing Countries*, 997–1015.

Okoffo, E. D., O'Brien, S., O'Brien, J. W., Tscharke, B. J., & Thomas, K. V. (2019). Wastewater treatment plants as a source of plastic in the environment: a review of occurrence, methods for identification, quantification and fate. *Environmental Science: Water Research & Technology*, *5*(11), 1908–1931. https://doi.org/10.1039/C9EW00428A

Peng, X., Xu, X., Li, Y., Cheng, L., Zhou, X., & Ren, B. (2020). Transmission routes of 2019-nCoV and controls in dental practice. *International Journal of Oral Science*, *12*(1), 9. https://doi.org/10.1038/s41368-020-0075-9

Polo, D., Quintela-Baluja, M., Corbishley, A., Jones, D. L., Singer, A. C., Graham, D. W., & Romalde, J. L. (2020). Making waves: Wastewater-based epidemiology for COVID-19 – approaches and challenges for surveillance and prediction. *Water Research*, *186*, 116404. https://doi.org/10.1016/j.watres.2020.116404

Prevost, B., Lucas, F. S., Goncalves, A., Richard, F., Moulin, L., & Wurtzer, S. (2015). Large scale survey of enteric viruses in river and waste water underlines the health status of the

local population. *Environment International*, *79*, 42–50. https://doi.org/10.1016/j.envint.2015.03.004

Randazzo, W., Truchado, P., Cuevas-Ferrando, E., Simón, P., Allende, A., & Sánchez, G. (2020). SARS-CoV-2 RNA in wastewater anticipated COVID-19 occurrence in a low prevalence area. *Water Research*, *181*, 115942. https://doi.org/10.1016/j.watres.2020.115942

Rezaei, M., Razavi Bazaz, S., Zhand, S., Sayyadi, N., Jin, D., Stewart, M. P., & Ebrahimi Warkiani, M. (2020). Point of Care Diagnostics in the Age of COVID-19. *Diagnostics*, *11*(1), 9. https://doi.org/10.3390/diagnostics11010009

Rimoldi, S. G., Stefani, F., Gigantiello, A., Polesello, S., Comandatore, F., Mileto, D., Maresca, M., Longobardi, C., Mancon, A., Romeri, F., Pagani, C., Cappelli, F., Roscioli, C., Moja, L., Gismondo, M. R., & Salerno, F. (2020). Presence and infectivity of SARS-CoV-2 virus in wastewaters and rivers. *Science of the Total Environment*, *744*. https://doi.org/10.1016/j.scitotenv.2020.140911

Rose, C., Parker, A., Jefferson, B., & Cartmell, E. (2015). The Characterization of Feces and Urine: A Review of the Literature to Inform Advanced Treatment Technology. *Critical Reviews in Environmental Science and Technology*, *45*(17), 1827–1879. https://doi.org/10.1080/10643389.2014.1000761

Shereen, M. A., Khan, S., Kazmi, A., Bashir, N., & Siddique, R. (2020). COVID-19 infection: Origin, transmission, and characteristics of human coronaviruses. *Journal of Advanced Research*, *24*, 91–98. https://doi.org/10.1016/j.jare.2020.03.005

Sons, H. U., Grams, G., Danneberg, A., & Dellmann, A. (n.d.). [Sternocostoclavicular hyperostosis]. *Beitrage Zur Orthopadie Und Traumatologie*, *37*(11–12), 661–667.

https://doi.org/(https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8307577/).

Suchard, M. A., Lemey, P., Baele, G., Ayres, D. L., Drummond, A. J., & Rambaut, A. (2018). Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. *Virus Evolution*, *4*(1). https://doi.org/10.1093/ve/vey016

Tang, A., Tong, Z., Wang, H., Dai, Y., Li, K., Liu, J., Wu, W., Yuan, C., Yu, M., Li, P., & Yan, J. (2020). Detection of Novel Coronavirus by RT-PCR in Stool Specimen from Asymptomatic Child, China. *Emerging Infectious Diseases*, *26*(6), 1337–1339. https://doi.org/10.3201/eid2606.200301

Tang, C. F., Ding, H., Jiao, R. Q., Wu, X. X., & Kong, L. D. (2020). Possibility of magnesium supplementation for supportive treatment in patients with COVID-19. *European Journal of Pharmacology*, *886*, 173546. https://doi.org/10.1016/j.ejphar.2020.173546

Tiwari, A., Lipponen, A., Hokajärvi, A.-M., Luomala, O., Sarekoski, A., Rytkönen, A., Österlund, P., Al-Hello, H., Juutinen, A., Miettinen, I. T., Savolainen-Kopra, C., & Pitkänen, T. (2022). Detection and quantification of SARS-CoV-2 RNA in wastewater influent in relation to reported COVID-19 incidence in Finland. *Water Research*, *215*, 118220. https://doi.org/10.1016/j.watres.2022.118220

Wang, M., Cao, R., Zhang, L., Yang, X., Liu, J., Xu, M., Shi, Z., Hu, Z., Zhong, W., & Xiao, G. (2020). Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro. *Cell Research*, *30*(3), 269–271. https://doi.org/10.1038/s41422-020-0282-0

Weidhaas, J., Aanderud, Z. T., Roper, D. K., VanDerslice, J., Gaddis, E. B., Ostermiller, J., Hoffman, K., Jamal, R., Heck, P., Zhang, Y., Torgersen, K., Laan, J. Vander, & LaCross, N. (2021). Correlation of SARS-CoV-2 RNA in wastewater with COVID-19 disease burden in

sewersheds. *Science of the Total Environment*, *775*. https://doi.org/10.1016/j.scitotenv.2021.145790

Won, J., Lee, S., Park, M., Kim, T. Y., Park, M. G., Choi, B. Y., Kim, D., Chang, H., Kim, V. N., & Lee, C. J. (2020). Development of a Laboratory-safe and Low-cost Detection Protocol for SARS-CoV-2 of the Coronavirus Disease 2019 (COVID-19). *Experimental Neurobiology*, *29*(2), 107–119. https://doi.org/10.5607/en20009

Wu, Y., Guo, C., Tang, L., Hong, Z., Zhou, J., Dong, X., Yin, H., Xiao, Q., Tang, Y., Qu, X., Kuang, L., Fang, X., Mishra, N., Lu, J., Shan, H., Jiang, G., & Huang, X. (2020). Prolonged presence of SARS-CoV-2 viral RNA in faecal samples. *The Lancet Gastroenterology & Hepatology*, *5*(5), 434–435. https://doi.org/10.1016/S2468-1253(20)30083-2

Wurtzer, S., Marechal, V., Mouchel, J. M., Maday, Y., Teyssou, R., Richard, E., Almayrac, J. L., & Moulin, L. (2020). Evaluation of lockdown impact on SARS-CoV-2 dynamics through viral genome quantification in Paris wastewaters. *MedRxiv*. https://doi.org/10.1101/2020.04.12.20062679

Younes, N., Al-Sadeq, D. W., Al-Jighefee, H., Younes, S., Al-Jamal, O., Daas, H. I., Yassine, H. M., & Nasrallah, G. K. (2020). Challenges in Laboratory Diagnosis of the Novel Coronavirus SARS-CoV-2. *Viruses*, *12*(6), 582. https://doi.org/10.3390/v12060582

Zhang, N., Jack Chan, P. T., Jia, W., Dung, C. H., Zhao, P., Lei, H., Su, B., Xue, P., Zhang, W., Xie, J., & Li, Y. (2021). Analysis of efficacy of intervention strategies for COVID-19 transmission: A case study of Hong Kong. *Environment International*, *156*, 106723. https://doi.org/10.1016/j.envint.2021.106723

Zhang, Y., Zhang, T., Fang, Y., Liu, J., Ye, Q., & Ding, L. (2022). SARS-CoV-2 spike L452R mutation increases Omicron variant fusogenicity and infectivity as well as host glycolysis.

Signal Transduction and Targeted Therapy, *7*(1), 76. https://doi.org/10.1038/s41392-022-00941-z

Zheng, L., Shu, W.-J., Li, Y.-M., Mari, M., Yan, C., Wang, D., Yin, Z.-H., Jiang, W., Zhou, Y., Okamoto, K., Reggiori, F., Klionsky, D. J., Song, Z., & Du, H.-N. (2020). The Paf1 complex transcriptionally regulates the mitochondrial-anchored protein Atg32 leading to activation of mitophagy. *Autophagy*, *16*(8), 1366–1379. https://doi.org/10.1080/15548627.2019.1668228

Author Contributions

Md. Aminul Islam: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, computer simulation, Validation, Roles/Writing - original draft, Writing - review & editing

Md. Arifur Rahman: Data curation, Investigation, Visualization, Writing - review & editing

Md. Jakariya: Conceptualization, Funding, Project administration, Supervision

Newaz Mohammed Bahadur: Writing - review & editing

Foysal Hossen: Data curation, Investigation, Visualization

Sanjoy Kumar Mukharjee: Writing - review & editing

Mohammad Salim Hossain: Writing - review & editing

Atkeeya Tasneem: Data curation, Investigation, Visualization

Md Atiqul Haque: Data curation, Investigation, Visualization

Francesco Sera: Writing - review & editing

Iqbal Kabir Jahid: Writing - review & editing

Tanvir Ahmed: Funding,Writing - review & editing

Mohammad Nayeem Hasan: Writing - review & editing

Md. Tahmidul Islam: Project administration**,**Funding

Md. Amzad Hossain: Writing - review & editing

Md. Ruhul Amin: Writing - review & editing

Ananda Tiwari : Writing - review & editing

Md. Didar-ul-Alam: Writing - review & editing

Kuldeep Dhama: Writing - review & editing

Prosun Bhattacharya: Conceptualization, Funding, Project administration, Roles/Writing - original draft, Writing - review & editing, Supervision Project administration

Firoz Ahmed: Conceptualization, Funding, Project administration, Roles/Writing - original draft, Writing - review & editing, Supervision Project administration

All authors critically scrutinisedand approved the final version of the manuscript. The corresponding authors are responsible for confirming that the descriptions are accurate and agreed by all authors.