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Surveillance of SARS-CoV-2 spread using wastewater-based epidemiology: Comprehensive study

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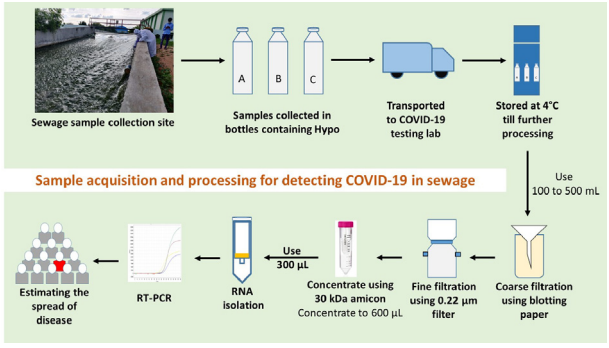
HIGHLIGHTS

- Study reports the least possible estimate of SARS-CoV-2 infected.
- This study estimates the infected and actively spreading population in Hyderabad, India during the study time course
- Our study is one of the first comprehensive report of wastewater-based SARS-CoV-2 surveillance in India
- The effluent from STPs were negative to SARS-CoV-2 indicating the efficient treatment

GRAPHICAL ABSTRACT



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ABSTRACT

SARS-CoV-2 pandemic is having a devastating effect on human lives. Recent reports have shown that majority of the individuals recovered from COVID-19 have serious health complications, which is going to be a huge economic burden globally. Given the wide-spread transmission of SARS-CoV-2 it is almost impossible to test every individual in densely populated countries. Recent reports have shown that sewage-based surveillance can be used as holistic approach to understand the spread of the pandemic within a population or area. Here we have estimated the spread of SARS-CoV-2 in the city of Hyderabad, India, which is a home for nearly 10 million people. The sewage samples were collected from all the major sewage treatment plants (STPs) and were processed for detecting the viral genome using the standard Reverse Transcription Polymerase Chain Reaction (RT-PCR) method. Interestingly, inlet samples of STPs were positive for SARS-CoV-2, while the outlets were negative, which indicates that the standard sewage treatment methods are efficient in eliminating the SARS-CoV-2 viral particles. Based on the detected viral gene copies per litre and viral particle shedding per individual, the total number of individuals exposed to SARS-CoV-2 was estimated. Through this study we suggest that sewage-based surveillance is an effective approach to study the infection dynamics, which helps in efficient management of the SARS-CoV-2 spread.

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1. Introduction

The surveillance of disease prevalence during pandemic like Coronavirus Disease-19 (COVID-19) is a crucial task considering the spreading rate and high population in different parts of the world. The massive testing of the population to contain the spread of the virus is a challenge. Moreover, the problem is further compounded because a majority of Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) infected individuals are asymptomatic. Emerging studies have shown the after effects of COVID-19 is going to be a huge economic burden globally and therefore pressing the importance of not only managing the infected individuals but also to keep a check on the spread (McKibbin and Fernando, 2020). Asymptomatic and symptomatic infections result in significant uncertainty in the estimated extent of SARS-CoV-2 infection (Li et al., 2020). Considering the present testing capacity and cost incurred, it is impractical to test all the individuals. Thus, there is a need for alternative strategies to assess the disease spread and therefore efficiently allocate resources for disease management.

Even though SARS-CoV-2 is majorly a respiratory pathogen, the persistence and replication of virus in the gastrointestinal (GI) tract and shedding through faeces is established (Wang et al., 2020a, 2020b; Xiao et al., 2020a, 2020b; Zhang et al., 2020; Young et al., 2020; Woelfel et al., 2020; Venkata Mohan et al., 2021). Different independent studies highlighted the presence and replication of SARS-CoV-2 in GI tract and the prolonged shedding of SARS-CoV-2 viral material through faeces during and after active infectious stage (Woelfel et al., 2020; Holshue et al., 2020; Kitajima et al., 2020; Cai et al., 2020; Ling et al., 2020; Wu et al., 2020; La Rosa et al., 2020a; Xiao et al., 2020a, 2020b; Ahmed et al., 2020a; Wurtzer et al., 2020; La Rosa et al., 2020b).

In this scenario, wastewater-based epidemiology (WBE) studies are suitable to understand and estimate the virus spread in a given population for effective disease surveillance. WBE was earlier used to detect and manage viral diseases such as polio, rotavirus, noroviruses etc. (Ahmed et al., 2020a; Usman et al., 2020; Murakami et al., 2020; Lodder and de RodaHusman, 2020; Hata and Honda, 2020; Venkata Mohan et al., 2021). Recent reports employed WBE-based approaches to detect SARS-CoV-2 in sewage water and estimated the percentage of infected individuals in a given population (Wu et al., 2020; Ahmed et al., 2020a; Wurtzer et al., 2020; La Rosa et al., 2020b; Medema et al., 2020; Usman et al., 2020). Wastewater offers an aggregate sample representing an entire community and is more easily accessible than pooled clinical samples (Murakami et al., 2020). The monitoring of SARS-CoV-2 in wastewater could quantify the scale of infection prevailing among the community with a benefit of detecting virus from symptomatic, asymptomatic, or pre-symptomatic cases which manifest as an early-warning signal (Medema et al., 2020; Lodder and de RodaHusman, 2020; Hata and Honda, 2020; Mallapaty, 2020; Naddeo and Liu, 2020; Qu et al., 2020). WBE approach has the potential to minimize the outbreak spread and also serve as an alarm for future outbreaks (Daughton, 2018; Mao et al., 2020; Zhou et al., 2020).

Here we have studied the spread of SARS-CoV-2 infection in Hyderabad, one of the major and densely populated metropolitan cities of India. The wastewater infrastructure of the city was used as an effective resource to access and estimate the spread of SARS-CoV-2 across the city of Hyderabad. We have simplified the sample processing method for viral detection by RT-PCR. A minimum estimate of the number of infected individuals was calculated based on the concentration of SARS-CoV-2 RNA in wastewater.

2. Materials and methods

2.1. Sampling sites

The detection of SARS-CoV-2 genetic material in domestic sewage was performed by collecting samples from different sewage treatment plants (STPs) in Hyderabad Metropolitan City, India. Hyderabad (17.37°N 78.48°E) is fifth-largest urban economy in India and is the capital of Telangana state that is spread over ~625 km^2. It is the fourth-most populous city in India with 10 million residents in the metropolitan region. The raw sewage samples were collected from inlet and outlet points from the STPs with a total coverage of 603.5 million litres per day (MLD) out of 735 MLD, that receive wastewater from all parts of the city (80% coverage of the existing STPs).

2.2. Sampling procedure

The sewage samples were collected from 8th July 2020 to 6th August 2020 (Table 1) taking all the safety measures as per the standard operating procedure (SOP) designed for this purpose. A total of 30 samples were collected from 14 inlets (equalization tanks outlet (ET)) and 14 treated wastewaters (outlets of secondary clarifiers (SC)) of 10 STPs and 2 samples from a gated community (outlet of collection tank prior to disposing to drains). A 10 MLD STP was selected for a time course study to understand the weekly variation in the viral load, where weekly samples were collected and analysed. The basis for sample collection from ET/SC of STP is that it would provide a composite sample accounting for a period of retention time (1–5 h). Grab sampling protocol (Rimoldi et al., 2020) was employed for sampling 1 L of sewage in a disposable bottle (plastic) of 1 L capacity with 20 mL of 0.1% of sodium hypochlorite (NaOCl)/L to inactivate the pathogens. After sampling the surface of the sample container was disinfected with 90% ethanol and sealed in multi-layered plastic covers, labelled and transported (2–4 °C) immediately to lab prior to storing at 4 °C until further processing. All the samples were processed within 12 h of the sampling event unless mentioned otherwise. During sampling, care was taken to follow all biosafety protocols. All the sampling activities were performed during the daytime when peak load was available (8 am to 4 pm), on the days with no report of rainfall events during last 24 h.

2.3. Optimization of disinfectant concentration

Optimum concentration of sodium hypochlorite (NaOCl) (Qualigens) addition during the sampling was optimized by various initial concentrations of sodium hypochlorite (0.1%, 0.5%, 1%, 2%, 3% and 4%) using samples collected from 10 MLD STP. Grab samples were collected from the ET outlet point of 10 MLD STP in disposable plastic bottles containing 20 ml of the above-mentioned concentrations of sodium hypochlorite. Collected samples were sealed and wrapped in plastic covers in two layers and transferred to the laboratory immediately and stored at 4 °C.

Table 1
Sampling information: Sewage samples collected from various STP of Hyderabad Metropolitan City. ET=equalization tank; SC=secondary clarifier.

Date of Collection	Capacity of STP	Sample Collection Point	Sample ID
08-07-2020	10 MLD (W1)	ET	ET-1
		SC	SC-1
	20 MLD	ET	ET-2
		SC	SC-2
14-07-2020	30 MLD	ET	ET-3
		SC	SC-3
	10 MLD (W2)	ET	ET-4
		SC	SC-4
29-07-2020	10 MLD (W3)	ET	ET-5
		SC	SC-5
	339 MLD	ET	ET-6
		SC	SC-6
06-08-2020	2.5 MLD	ET	ET-7
		SC	SC-7
	172 MLD	ET	ET-8
		SC	SC-8
	30 MLD	ET	ET-9
		SC	SC-9

Samples were processed within 12 h of sampling for the detection of SARS-CoV-2 RNA.

2.4. Processing of samples

Collected samples were subjected to gravity filtration with 1 mm thick blotting sheets to remove the debris or larger particles followed by filtration using 0.2 μm filtration units (Nalgene® vacuum filtration system; Thermofisher Scientific) to remove bacteria and other particles/debris. The filtrate was collected in 1000 mL sterile wide-mouth bottles (Borosil). 100 mL of the total filtrate was concentrated to ~600 μL using 15 mL 30 kDa Amicon® Ultra-15 (Merck Millipore) by centrifugation at 4000 rpm (4 °C; 10 min). The concentrated samples were further processed for RNA isolation. Sample filtration, concentration and processing till detection were performed in a Biosafety level 2 (BSL-2) facility. All the materials after use were discarded in biosafety bags followed by decontamination.

2.5. RNA extraction and RT-PCR

To quantify SARS-CoV-2 RNA in sewage samples, a total of 300 μl concentrate was used for RNA extraction using QIAamp Viral RNA isolation kit (Qiagen, Germany) by following manufacturer's protocol. The isolated RNA samples were tested for presence of SARS-CoV-2 RNA using FDA (Food and Drug Administration, USA Government) approved Fosun COVID-19 RT-PCR Detection Kit (Shanghai Fosun Long March Medical Science Co., Ltd., China) (https://www.fda.gov/media/137120/download, n.d). It contains primers and probes which targets the envelope protein coding gene (*E*-gene; ROX labelled), nucleocapsid gene (N-gene; JOE labelled) and open reading frame1ab (ORF1ab; FAM labelled) of SARS-CoV-2 and the RT-PCR was performed as per manufacturer recommendation on QuantStudio™5. Reaction conditions include two initial cycles, one at 50 °C for 15 min (Reverse transcription) and the other at 95 °C for 3 min (Initial denaturation) followed by 45 cycles at 95 °C for 5 s and 60 °C for 40 s (Initial 5 cycles without data acquisition and 40 with data collection). The signals of FAM (ORF1ab), JOE (N gene), ROX (E gene), and CY5 (Internal reference) fluorescence channels were collected at 60 °C. Positive and negative controls provided in the Fosun RT-PCR kit were also included in the amplification plates, and the C_T values were in accordance with the manufacturer protocol proving to be efficient and devoid of contamination. All the samples were tested in triplicates.

2.6. Estimation of RT-PCR kit efficiency

To assess the performance and efficiency of the qRT-PCR kit used in this work, $2.14*10^7$ pfu/mL viral culture was inactivated at 55 °C for 30 min and provided to us by Dr. H H Krishnan, CSIR-CCMB. RNA was isolated from the heat-inactivated SARS-CoV-2 which was followed by the preparation of $\log_{10}$ dilutions of the RNA. RT-PCR was performed in triplicates for each dilution. The R^2 values obtained from linear regression and efficiency was calculated as described (Ginzinger, 2002).

2.7. Standard curve for copy number calculation

To calculate the number of RNA copies, present in the wastewater samples, the E gene amplified from the SARS-CoV-2 RNA was cloned into the vector pcDNA3.1 between *Kpn*I and *Hin*dIII restriction sites. The cloned plasmid was then quantified using Qubit™ dsDNA HS Assay Kit (Invitrogen, USA) and Qubit™ 4 Fluorometer (Invitrogen, USA). The copy number per nanogram was calculated using the E gene and vector sequences retrieved from https://www.ncbi.nlm.nih.gov/nuccore/NC_045512.2?report=fasta&from=26245&to=26472 and https://www.addgene.org/browse/sequence_vdb/2093/, respectively. The plasmid was serially diluted from 9.01 $\log_{10}$ copies to 0.01 $\log_{10}$ copies and the RT-PCR reaction was performed as mentioned in Section 2.5 in triplicates. The C_T values were plotted against the log copy number and a linear fit equation was obtained (Supplementary Table 3; Supplementary Fig. 1).

2.8. Virus recovery from sewage

To find out the recovery of SARS-CoV-2 from sewage samples, 1 mL of 2.14×10^7 pfu/mL SARS-CoV-2 virus (heat-inactivated) was added to 100 mL of sewage water and four $\log_{10}$ dilutions were prepared (with sewage water). As a control, similar dilutions were made using milliQ water (MQ). The RNA was isolated from all the samples and RT-PCR was performed in triplicates.

2.9. Calculation of number of infected people in a population

To identify the viral copy number present in the wastewater samples, the linear fit equation obtained from the standard curve for E-gene was used (Supplementary Fig. 1). We followed two different methods to calculate the number of infected individuals in a given population based on the average number of RNA copies present in the sewage water.

Method 1 (Ahmed et al., 2020a):

$$No.of\ infected\ individuals = \frac{\left(\frac{RNA\ copies}{L\ water}\right) * \left(L\frac{water}{day}\right)}{\left(\frac{g\ faeces}{day}\right) * \left(\frac{RNA\ copies}{g\ faeces}\right)}$$

Faeces excreted/person/day = 128 g. (Rose et al., 2015).

One positive person sheds 10^7 RNA copies/g of faeces (maximum estimate, Foladori et al., 2020, Bivins et al., 2020).

Method 2 (Hellmér et al., 2014).

$$No.of\ infected\ individuals = \frac{No.of\ RNA\ copies\ per\ liter\ of\ waste\ water}{Contribution\ of\ RNA\ copies\ per\ person\ to\ total\ sewage\ water}$$

Number of RNA copies excreted per mL of faeces = 10^7

Volume of faeces excreted = 120 mL (calculated by considering the density of human faeces is 1.07 g/mL (Foladori et al., 2020).

3. Results and discussion

3.1. Determining the RT-PCR kit efficiency

Earlier studies have shown that the regular SARS-CoV-2 kits which are used for testing individuals can be employed for the detection and quantification of the viral RNA from sewage (Wu et al., 2020; Rimoldi et al., 2020; Or et al., 2020). To independently examine the efficiency of the RT-PCR kit used in this study, we isolated RNA from 150 μl of 2.14×10^7 pfu/mL virus culture and subjected the RNA to $\log_{10}$ serial dilutions. The C_T values and standard deviations (from triplicates) obtained from dilutions up to 10^{-7} are presented in Fig. 1. The observed average C_T difference of 3.2, between $\log_{10}$ dilutions for all three genes provides further proof for the performance of the kit and its usefulness to estimate the number of viral RNA molecules (Table 2). Based on the C_T values a linear curve was plotted and subjected to regression analysis, where R^2 0.9987, 0.9993, and 0.9976 and the calculated efficiency of 106.55%, 105.59%, and 103.01% for *E*-gene, N-gene and ORF1ab were noted respectively. Overall, the average slope of −3.2070 and R^2 of 0.9985 obtained from linear regression with the calculated efficiency of 105.05% suggests the standard performance (nearly 100%) of the kit.

Fig. 1d, with C_T values ($\log_{10}$ dilutions) of all three genes plotted together indicates the acceptable efficiency of all primer sets used in the RT-PCR. The R^2 value 0.998 and slope 1.018 represents (Fig. 1d) a

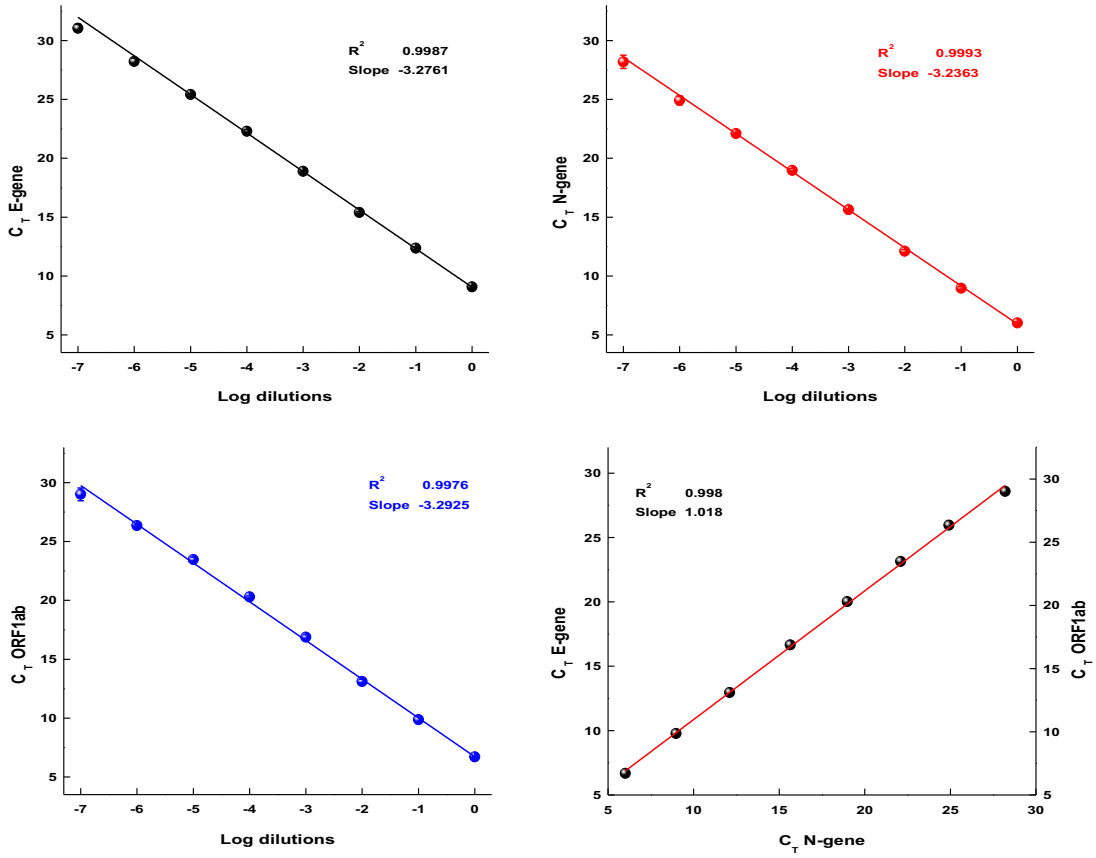

Fig. 1. The standard curves of a) *E*- gene, b) N-gene and c) ORF1ab gene, plotted with C_T values from $\log_{10}$ dilutions of SARS-CoV-2 RNA. d) Plot representing the linear fit of all three genes C_T values.

good linear regression fit and concordance among the primers performance in the given experimental conditions.

3.2. Recovery of SARS-CoV-2 RNA from sewage

One of the major bottlenecks in analysing sewage samples is to estimate the recovery of the samples compared to the actual presence. Earlier studies have shown that processing sewage samples by ultrafiltration followed by concentration lead to 70% loss of samples and therefore making the recovery to be only around 30%. (Ahmed et al., 2020b). To check the virus recovery and efficiency of the method implemented in this work, we performed the $\log_{10}$ dilutions from 100 ml of sewage water which was spiked with 1 ml of 2.14×10^7 pfu/mL SARS-CoV-2 virus. For comparison, similar dilutions were performed with MQ water to check the effect of sewage on virus recovery. The RNA was isolated from independently processed samples and RT-PCR was performed. The C_T values from different dilutions and samples are presented (Table 3). The average C_T value differences (all three genes) of 3.72 and 3.61 for sewage and MQ water dilutions, respectively, suggests efficient recovery from log dilution samples. A C_T value difference of 3.74 between identical $\log_{10}$ dilutions of spiked sewage and MQ samples were observed, which is possible due to inhibitors in sewage and this difference largely depends on heterogeneity among different samples. To eliminate the unwanted large particles from sewage samples, which could damage the filter membrane we implemented prior filtration of samples using a blotting sheet. To rule out the effect of this, we processed two spiked samples with and without the extra filtration step. The average C_T difference of 0.02 indicates the absence of unwanted effect due to blotting sheet filtration.

To further understand the efficiency of viral recovery from both sewage and MQ dilutions, we performed a standard curve from C_T values obtained. The percentage efficiency (94.85), slope (−3.45), and R^2 values (0.96) achieved (from sewage) indicates a good recovery efficiency of the viral particles (Table 4).

Table 2
The C_T values of viral-specific genes from $\log_{10}$ dilution samples.

Dilution	E Gene		N gene		ORF1ab	
	Average Ct	SD[a]	Average Ct	SD[a]	Average Ct	SD[a]
Undiluted	9.08	0.10	6.01	0.10	6.71	0.03
10^{-1}	12.36	0.10	8.97	0.12	9.86	0.31
10^{-2}	15.40	0.08	12.10	0.16	13.10	0.20
10^{-3}	18.89	0.03	15.64	0.03	16.87	0.22
10^{-4}	22.30	0.14	18.97	0.16	20.29	0.11
10^{-5}	25.43	0.01	22.10	0.03	23.48	0.21
10^{-6}	28.23	0.28	24.92	0.38	26.35	0.07
10^{-7}	31.06	0.33	28.20	0.57	29.01	0.54
Efficiency (%)	**106.55**		**105.59**		**103.01**	
Slope	**−3.1744**		**−3.1947**		**−3.2519**	
R^2	**0.9987**		**0.9993**		**0.9976**	

[a] Standard deviation.

3.3. Optimization of disinfection concentration and storage

Sodium hypochlorite was used to disinfect the sewage samples collected from the STPs in order to reduce the possible pathogenicity of virus/bacteria during transport and processing as the scope of the study is only to evaluate the SARS-CoV-2 RNA. In order to find the least concentration of sodium hypochlorite that result in identifying maximum number of RNA copies using RT-PCR, we performed an optimisation step. We collected 1 L of real-field wastewater (from the 10 MLD STP) in the presence of 20 mL of six different initial concentrations

Table 3
The C_T values obtained from log_{10} dilutions of spiked sewage and MQ water samples.

Sample	E gene				N gene				ORF1ab			
	Sewage		MQ		Sewage		MQ		Sewage		MQ	
	Average C_T	SD*	Average C_T	SD*	Average C_T	SD*	Average C_T	SD*	Average C_T	SD*	Average C_T	SD*
UD+	11.29	0.41	8.50	0.04	8.13	0.44	5.44	0.03	9.74	0.49	6.32	0.08
1/10	17.02	0.35	12.96	0.04	14.02	0.34	9.61	0.18	16.25	0.37	11.48	0.08
1/100	19.73	0.30	15.06	0.08	16.33	0.45	11.85	0.20	18.73	0.39	13.55	0.09
1/1000	22.87	0.11	19.99	0.15	19.65	0.20	16.43	0.25	21.75	0.28	18.48	0.17
1/10000	25.88	0.47	22.79	0.13	22.95	0.48	19.48	0.04	24.97	0.50	21.35	0.13
Filtered (0.22 μm)	11.15	0.14			8.36	0.18			9.58	0.51		

* Standard deviation; + Undiluted

Table 4
Calculated values obtained from C_T values of log_{10} dilutions of spiked sewage and MQ water samples.

Gene	Efficiency (%)		Slope		R^2 value	
	Sewage	MQ	Sewage	MQ	Sewage	MQ
E gene	94.54	89.47	−3.4601	−3.6030	0.9750	0.9685
N gene	95.87	92.71	−3.4251	−3.5099	0.9657	0.9995
ORF1ab	94.13	87.11	−3.4710	−3.6752	0.9465	0.9711
Average	94.85	89.76	−3.45	−3.60	0.96	0.98

of sodium hypochlorite (Supplementary Table 1). From the results it was observed that three concentrations of hypochlorite (0.1%, 0.5% and 1%) were positive for all the three genes *E*-gene, N-gene and ORF1ab of SARS-CoV-2 with a minimum difference of ~1 C_T. Among the tested concentrations, samples collected with 20 ml of 0.1% hypochlorite resulted in better detection of SARS-CoV-2 RNA. It was also observed that the final concentration of ≥0.04% (initial concentration of ≥2%) did not yield any result for the same set of samples, indicating a possible complete inactivation of viral genetic material by sodium hypochlorite (Supplementary Table 1; Fig. 2). The results suggest that addition of 0.1% sodium hypochlorite did not affected the presence of viral RNA. Hence, we used 20 mL of 0.1% sodium hypochlorite/L of wastewater for all further samples collected due to the safety during transport. The earlier reports on SARS reported the complete inactivation of virus with ≥0.5 mg/L of free chlorine (FC) within 30 min of contact time at 22 ± 3 °C (Wang et al., 2005). Hospital based wastewater was detected to be positive to SARS-CoV-2 RNA even after the addition of sodium hypochlorite (Wang et al., 2020a, 2020b; Kataki et al., 2020).

We also looked for the effect of sample storage on the detection of SARS-CoV-2 RNA from wastewater samples. For this, part of the sample collected on 7th July 2020 was filtered and processed within 24 h of collection and the rest (filtered) was stored at 4 °C. The stored sample was processed on 31st July 2020 (after 24 days of initial collection). RT-PCR showed a difference of approximately 4 C_T values between the samples with the stored sample showing higher C_T (Fig. 3). This indicates the presence of viral genome even after 3 weeks when the samples are stored at 4 °C, however, it is best to analysed the samples before 24 h for all practical purpose.

3.4. Detection of SARS-CoV-2 in various STPs and residential community

RT-PCR based detection of SARS-CoV-2 RNA was used for screening the inlet water in the STPs that cover about 80% of Hyderabad's STP capacity i.e. 603.5 MLD. SARS-CoV-2 RNA was detected in the inlets of all the tested STPs (Supplementary Table 2), indicating that the infection is widespread. We observed that the level of viral RNA in the STPs was dynamic, as implied by the changes in C_T values of the samples collected on different days. As a testimony of efficient wastewater treatment, no viral RNA copies were detected in the outlet of the STPs that we sampled (Fig. 4; Supplementary Table 2). We also surveyed samples collected from a gated residential community where confirmed positive cases were reported during the sample collection period and observed the presence of SARS-CoV-2 RNA in the samples (Table 5).

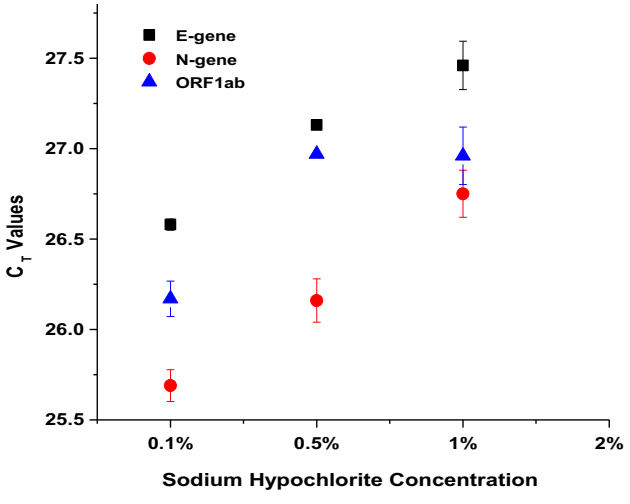

Fig. 2. Concentration of sodium hypochlorite affects the detection ability of SARS-CoV-2 RNA. Scatter plot showing the effect of different concentrations of sodium hypochlorite on the C_T values of viral targets. Each dot represents average C_T values obtained from two replicates and the bar represents the standard error of mean.

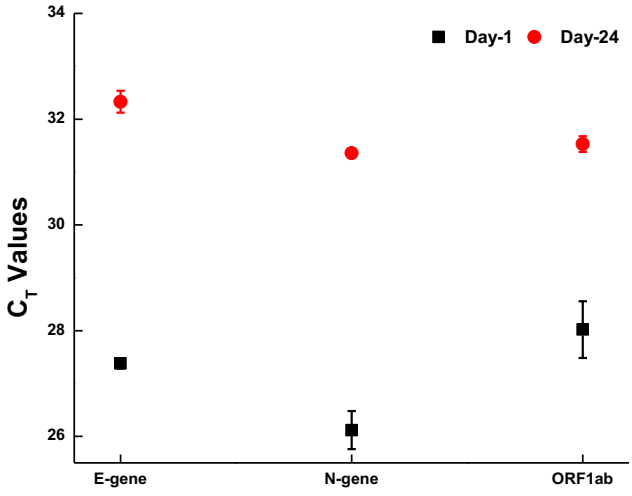

Fig. 3. Degradation of Viral genome with time. Scatter plot showing the degradation of viral genome with the span of 24 days. Each dot represents average CT values obtained from two replicates and the bar represents the standard error of mean.

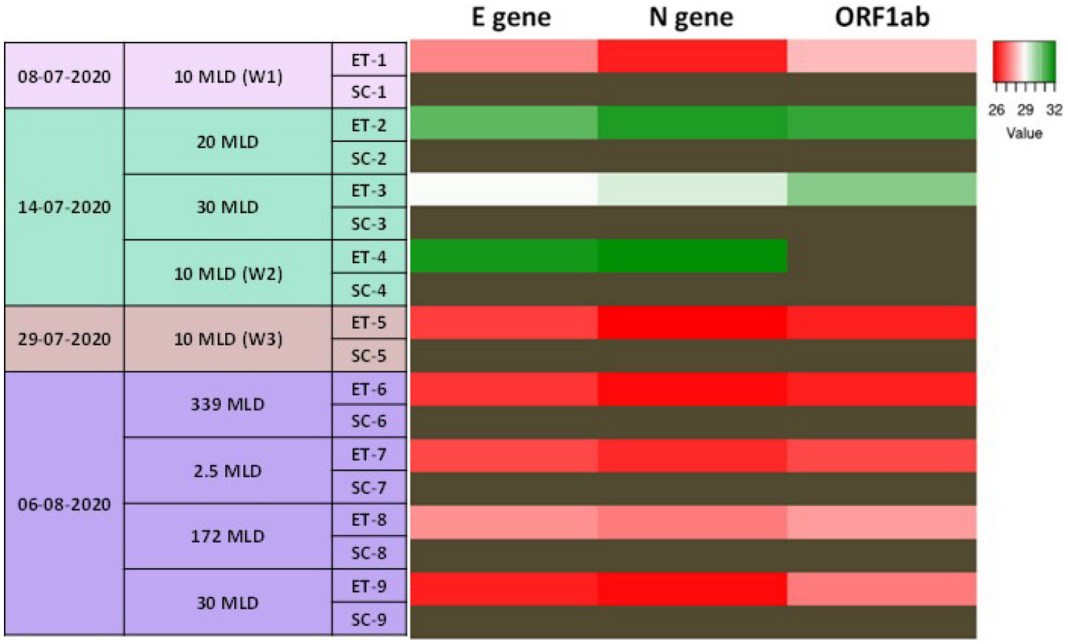

Fig. 4. SARS-CoV-2 RNA is present in Hyderabad's sewage water: Heat map showing the C_T values of E gene, N gene, and ORF1ab in the wastewater samples collected from various STPs in the city of Hyderabad on different days during the pandemic. The experiments were performed in duplicates or triplicates. Dark brown cells correspond to samples with no amplification. MLD-Million Litres per Day; ET-Equalization Tank; SC-Secondary Clarifier.

3.5. Long term viral load monitoring

One of the STPs (10 MLD), was sampled at different time to assess the dynamics of disease spread with time. We observed a highly fluctuating pattern of viral RNA presence with time, from as low as 661 copies/L wastewater (on 14-07-2020) to as high as 24,469 copies/L wastewater (on 29-07-2020) (Table 6; Supplementary Table 3; Supplementary Fig. 1). The reason for variations could be sampling time, number of actually infected people and the amount of viral shedding by infected individuals, and temporal presence of other compounds (such as surfactants) that could affect the viral material stability in the domestic sewage.

3.6. Calculation of number of infected people

We used two previously published methods for calculating the number of infected people from the number of RNA copies in the wastewater samples (Ahmed et al., 2020a; Hellmér et al., 2014). These methods take into account the number of RNA copies present in the wastewater and the number of RNA copies present in the faecal matter of infected individuals (Section 2.8). Previous studies have established these numbers and we used them for calculating the number of infected individuals. Existing reports suggest that an infected individual shed viral material in faeces for up to 47 days since the symptom onset and remains infectious till 14 days since symptom onset (Wu et al., 2020; Foladori et al., 2020). This suggests that for approximately 35 days, a person sheds viral material while not being infectious. This indicates that 2 in 5 infected people are infectious at any given point of time during the 30 days window. We used this fact to calculate the number of infected people in the active phase of infection (Tables 7 and 8). Owing to the uncertainty and difference in the number of viral particles excreted by infected individuals, we calculated the possible number of infected people for three different shedding rates within the reported range (10^5, 10^6, and 10^7 copies/mL faeces) (Table 8). Results indicate that the number of infected people might be anywhere between thirty thousand and three million during the study period (Table 8). Studies have reported the loss of 0.02 to 3000 viral RNA copies/mL during the transit of faeces from the point of excretion to the STP (Foladori et al., 2020). This could further influence the correct estimation of the number of infected individuals. Resampling from these sites periodically would give a better estimate to understand where the disease spread rate is decreasing or increasing with time. In addition, this study puts forth the necessity for large-scale studies on the excretion dynamics of viral particles by infected individuals which could help in

Table 5
Community surveillance: SARS-CoV-2 RNA C_T values detected by real-time RT-PCR from raw sewage samples of selected gated community. n.d.=not detected; PC=positive control; NTC=no template control.

Date of Collection	Capacity of STP	Sample ID	C_T Values		
			E gene	N gene	ORF1ab
14-07-2020	72 Houses	C1	31.87±0.317	31.93±0.453	31.65±0.570
		C2	31.06±1.047	32.56	33.32
		PC	23.98	25.20	23.80
		NTC	n.d.	n.d.	n.d.

Table 6
Temporal SARS-CoV-2 monitoring: SARS-CoV-2 RNA C_T values detected by real-time RT-PCR of raw sewage samples of selected STP (10MLD) for weekly monitoring. ET=equalization tank; SC=secondary clarifier; PC=positive control; NTC=no template control; n.d.=not detected; RNA copies were calculated based on the equation obtained from the standard curve (Supplementary Figure 1).

Date of Collection	Week	Sample ID	C_T Values – E gene	Average number of RNA copies/L water
08-07-2020	W1	ET-1	27.38±0.098	13,964
		SC-1	n.d.	
		PC	23.00	
		NTC	n.d.	
14-07-2020	W2	ET-4	31.73±0.388	661
		SC-4	n.d.	
		PC	23.98	
		NTC	n.d.	
29-07-2020	W3	ET-5	26.58	24,469
		SC-5	n.d.	
		PC	25.94	
		NTC	n.d.	

Table 7
Estimated number of RNA copies per litre of wastewater processed in each of the STPs; RNA copies were calculated based on the equation obtained from the standard curve (Supplementary Figure 1).

Date of Collection	Capacity of the STP (MLD)	E Gene	
		Average C_T	RNA copies/1 L
08-07-2020	10	27.38	13,964
	20	30.83	1243
	30	28.94	4677
14-07-2020	10	31.73	661
29-07-2020	10	26.58	24,469
	339	26.4	27,760
	2.5	26.64	23,461
	172	27.52	12,658
06-08-2020	30	26.12	33,782

estimating the near-precise number of infected individuals in a given locality.

Based on the recent learning from SARS-CoV-2, it is evident that screening large population to contain the spread is an inconceivable task and it is more complex in urban areas with high population density. As the SARS-CoV-2 colonise the GI tract and is released through faeces the WBE studies provide an effective edge for mass surveillance to prevent the spread of virus. The work presented here covered nearly 80% of STPs capacity (603.5 MLD) in the metropolitan city Hyderabad, India, for the detection and estimation of SARS-CoV-2 infected individuals in a window of 30 days. Based on number of viral RNA copies present in the sewage samples collected from different locations, here, we clearly estimated a range of the number of infected individuals and the actively spreading population during the given time window. The estimations were done based on published independent studies and WHO guidelines (https://www.who.int/news-room/commentaries/detail/criteria-for-releasing-covid-19-patients-from-isolation, n.d). pertaining to SARS-CoV-2 infected individuals. The wastewater infrastructure has been previously shown to function as a surveillance system for poliovirus (Lodder et al., 2012). and Aichi virus (Lodder et al., 2013). WBE approach, apart from helping to minimize the existing outbreak spread, can also serve as a tool for future epidemics surveillance (Lodder and de RodaHusman, 2020; Mallapaty, 2020; Daughton, 2018). Considering the present and previous reports on SARS-CoV-2 WBE studies, we recommend collection of sewage samples with a window of 15 days from same localities to get a better estimation of cases.

This study provides a concrete evidence for the application of WBE as a potential method for disease as well as environmental surveillance. These results will be an immense resource for the healthcare and associated departments to vigilantly allocate the necessary resources to manage existing cases as well as to carefully contain the disease spread. Along with clinical data, WBE could provide critical monitoring of SARS-CoV-2 transmission within a community including the beginning, tapering, or reemergence of an epidemic (Bivins et al., 2020. Hence, sewage-based surveillance provides a holistic approach to manage the pandemic and also to monitor for future outbreaks, if any. The current study also offers a framework to monitor other pathogens to avoid future epidemic. Overall, this study provides a simplistic framework for WBE studies with basic resources available in majority of the labs towards sustainable environmental Surveillance. We strongly recommend the scientific community and the healthcare agencies to pursue similar studies periodically, for allocating resources appropriately to fight the pandemic.

CRediT authorship contribution statement

Manupati Hemalatha: Methodology, Investigation, Data curation, Writing – original draft. **Uday Kiran:** Methodology, Investigation, Data curation, Writing – original draft. **Santosh Kumar Kuncha:** Conceptualization, Investigation, Writing – original draft. **Harishankar Kopperi:** Methodology, Investigation, Writing – original draft. **C.G. Gokulan:** Methodology, Investigation, Formal analysis, Writing – original draft. **S. Venkata Mohan:** Conceptualization, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing. **Rakesh K. Mishra:** Conceptualization, Supervision, Funding acquisition, Validation, Writing – review & editing.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.144704.

Table 8
Disease dynamics: Estimate of the number of people infected during the sampling window, which includes individuals who are symptomatic, asymptomatic, and recovered.

Capacity of the STP (MLD)	Per person contribution to STP (10^7 copies/mL faeces)	Method 1	Method 2	Per person contribution to STP (10^6 copies/mL faeces)	Method 1	Method 2	Per person contribution to STP (10^5 copies/mL faeces)	Method 1	Method 2
10	120	109	116	12	1091	1164	1.20	10,909	11,636
20	60	19	21	6	194	207	0.60	1942	2071
30	40	110	117	4	1096	1169	0.40	10,961	11,692
10	120	5	6	12	52	55	1.20	517	551
10	120	191	204	12	1912	2039	1.20	19,116	20,391
339	4	7352	7842	0.35	73,521	78,423	0.04	735,213	784,227
2.5	480	46	49	48	458	489	4.80	4582	4888
172	7	1701	1814	0.7	17,009	18,143	0.07	170,092	181,432
30	40	792	845	4	7918	8446	0.40	79,177	84,456
Total (603.5 MLD)		10,325	11,013	Total (603.5 MLD)	103,251	110,134	Total (603.5 MLD)	1,032,510	1,101,344
Total (1800 MLD)		30,796	32,849	Total (1800 MLD)	307,956	328,487	Total (1800 MLD)	3,079,565	3,284,869
Average estimate of infected individuals		31,822			318,222			3,182,217	
Estimate of the population in active phase of the infection		12,729			127,289			1,272,887	

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