

Comprehensive Surveillance of SARS-CoV-2 Spread Using Wastewater-based Epidemiology Studies

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Abstract

SARS-CoV-2 pandemic is having a devastating effect on human lives. Individuals who are symptomatic/asymptomatic or have recovered are reported to have/will have serious health complications in the future, which is going to be huge economic burden globally. Given the wide-spread transmission of SARS-CoV-2 it is almost impossible to test each and every individual for the same and isolate them. Recent reports have shown that sewage can be used as a holistic approach to estimate the epidemiology of the virus. Here we have estimated the spread of SARS-CoV-2 in the city of Hyderabad, India which is populated with 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 RT-PCR method. Based on the average viral particle shedding per individual, the total number of individuals exposed to SARS-CoV-2 (in a window of 35 days) is about 6.6% of the population, which clearly indicates the rate of community transmission and asymptomatic carriers is higher than the number of reported cases. It is important to note here that the samples collected from the inlet of STPs were positive for SARS-CoV-2, while the outlets were negative indicating the efficient treatment of sewage at STPs. These studies are going to be essential to manage the pandemic better and also to assess the effectiveness of control measure.

1. Introduction

The surveillance of disease prevalence during pandemic like Corona Virus 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 to any nation in the present scenario. Moreover, a majority of Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) infected population is asymptomatic. Emerging studies have shown the after effects of COVID-19, which is going to be huge economic burden globally and therefore pressing the importance of not only managing the infected individuals but also to keep the spread to minimum (McKibbin 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., 2020; Xiao et al., 2020; Zhang et al., 2020; Young et al., 2020; Woelfel et al., 2020). 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 (Holshue et al., 2020, Woelfel 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., 2020; Ahmed et al., 2020; Wu et al., 2020; Wurtzer et al., 2020; La Rosa et al., 2020b; Medema et al., 2020).

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, etc. (Lodder et al., 2012; Lodder et al., 2013; Ahmed et al., 2020). 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 (Ahmed et al., 2020; Wu et al., 2020; Wurtzer et al., 2020; La Rosa et al., 2020b; Medema 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 sign (Lodder et al., 2020; Medema et al., 2020; Mallapaty et al., 2020).

WBE approach will help to minimize the outbreak spread and also serve for future epidemic surveillance (Daughton 2018; Mao et al., 2020).

Here we have studied the spread of SARS-CoV-2 infection in Hyderabad, which is 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. Here we have simplified the sample processing method for viral detection by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and based on the RNA titer the number of infected individuals were estimated.

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 a capital for Telangana state occupies ~625 square kilometers. 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 712 million litres per day (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 7th July 2020 to 8th August 2020 taking all the safety measures as per the standard operating procedure (SOP) designed for this purpose (Supplementary Table 1). Sampling was performed in the day time, on the days with no report of rainfall during last 24 hours. A total of 30 samples were collected from 14 inlets and 14 treated outlets of 10 STPs and 2 samples from a gated community. A 10 MLD STP was selected for a time course study to understand the weekly variation in the viral load.

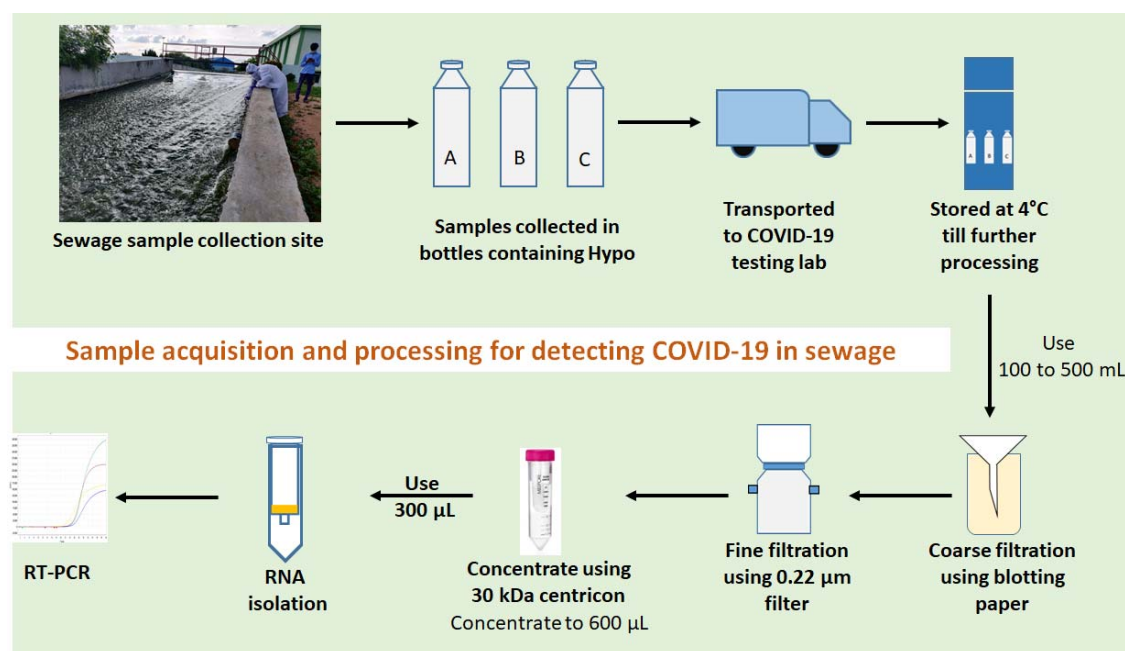


Figure 1: Schematic of sample collection and processing: The sewage samples are collected from STPs in bottles containing Hypo (0.1-4%) followed by shipping to the COVID-19 testing laboratory and stored at 4 °C until further processing. The samples are initially filtered using blotting paper followed by 0.22 µm filter. These filtered samples are further concentrated using 30 kDa centricon filters. The concentrates are used for RNA isolation, the isolated RNA is subjected to RT-PCR using COVID-19 specific primers and probes.

2.3 Optimization of disinfectant concentration

Optimum concentration of sodium hypochlorite (5%; Qualigens) addition during sampling was optimized by collecting samples in various initial concentrations of hypochlorite (0.1%, 0.5%, 1%, 2%, 3% and 4%) using samples collected from 10 MLD STP. Grab samples were collected from the inlet point of 10 MLD STP in disposable plastic bottles containing 20 ml of the above mentioned concentrations of sodium hypochlorite. Collected samples were sealed to avoid leakage and wrapped in plastic covers in two layers and transferred to the laboratory immediately and stored at 4°C. Samples were processed within 24 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 larger particles followed by filtration using 0.2 µm filtration units (Nalgene® vacuum filtration system; Thermo Fisher Scientific, USA) to remove bacteria and other debris. The filtrate was collected in 1000 mL sterile wide-mouth bottles (Borosil, India). 100 mL of the total filtrate was concentrated to ~600 µl using 30 kDa Amicon® Ultra-15 (Merck Millipore, USA) by centrifuging at 4000 rpm at 4 °C. The concentrated samples were further processed for RNA isolation. Sample filtration, concentration and processing till detection were performed in Biosafety level 2 (BSL-2). All the materials after use were discarded in biosafety bags followed by decontamination.

2.5. RNA extraction and RT-PCR

The RNA extraction from 300 µl of concentrate was performed 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 Fosun COVID-19 RT-PCR Detection Kit (Shanghai Fosun Long March Medical Science Co., Ltd, China). The kit detects Envelope (E; ROX labelled), Nucleocapsid (N; JOE labelled) and open reading frame1ab (ORF1ab; FAM labelled) genes of SARS-CoV-2 and the RT-PCR was performed as per manufacturer recommendation. A C_T value of 32 corresponds to 300 copies per mL when using this kit (<https://www.fda.gov/media/137120/download>). The samples were tested either in duplicates or triplicates.

2.6. Calculation of number of infected people in a population

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., 2020)

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

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

One positive person sheds 10⁷ RNA copies/g of faeces (maximum estimate, Foladori et al., 2020)

Method 2: (Hellmér et al., 2014)

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

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 1.07g/mL, (Foladori et al., 2020)). Total RNA copies excreted per person = 1.2×10^9 copies.

3. Results and Discussion

3.1 Optimization of Disinfection Concentration

Sodium hypochlorite was used to disinfect the wastewater collected from the STPs. 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 wastewater in the presence of 20 mL of six different initial concentrations of sodium hypochlorite (Table 1) from the 10 MLD STP. 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 $\geq 0.04\%$ (initial concentration of $\geq 2\%$) did not yield any result for the same set of samples, indicating a possible complete inactivation of viral materials by hypo (Table 1; Figure 2). Hence, we used 20 mL of 0.1% sodium hypochlorite per litre of wastewater for all further sample collections.

3.2 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 Sewage Treatment Plants (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 (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 (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 in trace amounts (Table 3). As a testimony of efficient wastewater treatment, no viral RNA copies were detected in the outlet of the STPs that we sampled (Table 2).

Table: 1 SARS-CoV-2 RNA C_T values (and standard error of mean) detected by real-time RT-PCR of raw sewage samples collected from 10 MLD STP with varied sodium hypochlorite concentrations. n.d.=not detected; PC=Positive Control; NTC=No Template Control

Sodium Hypochlorite (%)	C_T Values		
	E-gene	N-gene	ORF1ab
0.1%	26.58±0.031	25.69±0.088	26.17±0.098
0.5%	27.13	26.16±0.120	26.970
1%	27.46±0.134	26.75±0.130	26.96±0.159
2%	n.d.	n.d.	n.d.
3%	n.d.	n.d.	n.d.
4%	n.d.	n.d.	n.d.
PC	25.94	25.47	23.13
NTC	n.d.	n.d.	n.d.

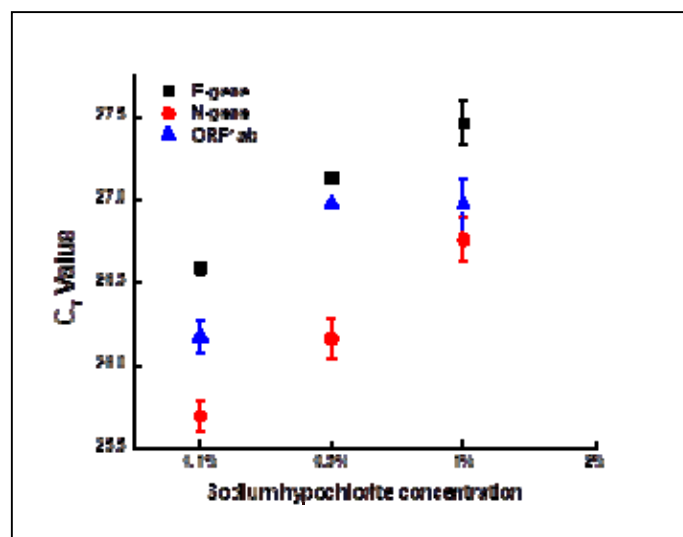


Figure 2: Concentration of sodium hypochlorite affects the detection ability of SARS-CoV-2 RNA. Scatter plot showing the effect of different concentrations of sodium hypochlorite on the C_T values of viral targets. Each dot represents average C_T values obtained from two replicates and the bar represents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

Table 2: SARS-CoV-2 RNA C_T values detected by real-time RT-PCR of raw sewage samples collected from various STP of Hyderabad Metropolitan City. IN=inlet; OU=outlet; W=week; n.d.=not detected; PC=positive control.

Date of Collection	Capacity of STP	Sample ID	C _T Values		
			E-gene	N-gene	ORF1ab
08-07-2020	10 MLD (W1)	IN-1	27.38±0.098	26.12±0.360	28.02±0.537
		OU-1	n.d.	n.d.	n.d.
		PC	23.00	22.55	23.10
14-07-2020	20 MLD	IN-2	30.83±0.243	31.58±0.302	31.33±0.005
		OU-2	n.d.	n.d.	n.d.
	30 MLD	IN-3	28.94±0.270	29.32±0.083	30.33±0.467
		OU-3	n.d.	n.d.	n.d.
	10 MLD (W2)	IN-4	31.73±0.388	32.08	ND
		OU-4	n.d.	n.d.	n.d.
29-07-2020	10 MLD (W3)	PC	23.98	25.20	23.80
		IN-5	26.58	25.69±0.120	26.17
		OU-5	n.d.	n.d.	n.d.
06-08-2020	339 MLD	PC	25.94	25.47	23.13
		IN-6	26.39±0.107	25.88±0.040	26.15±0.090
	2.5 MLD	OU-6	n.d.	n.d.	n.d.
		IN-7	26.63±0.313	26.28±0.247	26.70±0.371
	172 MLD	OU-7	n.d.	n.d.	n.d.
		IN-8	27.51±0.205	27.31±0.144	27.71±0.045
	30 MLD	OU-8	n.d.	n.d.	n.d.
		IN-9	26.11±0.062	25.93±0.132	27.25±0.270
		OU-9	n.d.	n.d.	n.d.
		PC	23.78	24.90	23.45

Table 3: SARS-CoV-2 RNA C_T values detected by real-time RT-PCR of raw sewage samples of selected gated community. n.d.=not detected; PC=positive control; NTC=no template control. IN=inlet; OU=outlet; W=week; 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.

3.3 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 30818 copies per L wastewater to as high as 266360 copies per L wastewater (Table 4). 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.

Table 4: SARS-CoV-2 RNA C_T values detected by real-time RT-PCR of raw sewage samples of selected STP (10 MLD) for weekly monitoring

Date of Collection	Week	Sample ID	C _T Values			Average number of RNA copies/L water
			E-gene	N-gene	ORF1ab	
08-07-2020	W1	IN-1	27.38±0.098	26.12±0.360	28.02±0.537	219540
		OU-1	n.d.	n.d.	n.d.	
		PC	23.00	22.55	23.10	
14-07-2020	W2	IN-4	31.73±0.388	32.08	n.d.	30818
		OU-4	n.d.	n.d.	n.d.	
		PC	23.98	25.20	23.80	
29-07-2020	W3	IN-5	26.58	25.69±0.120	26.17	266360
		OU-5	n.d.	n.d.	n.d.	
		PC	25.94	25.47	23.13	

3.5. 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., 2020, 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 2.6). Previous studies have established these numbers and we used them for calculating the number of infected individuals (Table 5). Our data indicates that approximately 6.57% of Hyderabad's population is infected during the window of our study period (Table 6). Existing reports suggest that an infected person 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 35 days window. We used this fact to calculate the number of infected people in the active phase of infection (Table 5). Our calculation indicates that approximately 2.6% of the total population of Hyderabad are potentially infectious during the study period.

Table 5: Estimated number of people infected in the areas surveyed through WBE

Date of Sample Collection	Sample ID	Average RNA Copies/L	Contribution per person (copies)	Number of infected individuals - Method 1	Number of infected individuals - Method 2
08-07-2020	10 MLD (W1)	219394	120	1714	1828
	20 MLD	34242	60	535	571
14-07-2020	30 MLD	112272	40	2631	2807
	10 MLD (W2)	30864	120	241	257
29-07-2020	10 MLD (W3)	192151	120	1501	1601
	339 MLD	532121	4	140929	150324
07-08-2020	2.5 MLD	496364	480	969	1034
	172 MLD	407879	7	54809	58463
	30 MLD	506061	40	11861	12652

Table 6: Extrapolation of our data shows the total number of infected and potentially active people in the city of Hyderabad.

Capacity of STP covered (MLD)	603.5
Average no. of infected people during the window (07-07-2020 to 08-08-2020)	220343
Total STP capacity of Hyderabad (MLD)	772
Number of infected (extrapolating to total STP capacity)	281864
Total wastewater generated in Hyderabad (MLD)	1800
Number of infected (extrapolating to entire population)	657197
Population of Hyderabad	10004144
% Virus shedding individuals	6.57%
Fraction of active cases in the population	0.4
Number of individuals in potentially active phase of infection	262878

4. Conclusion

Our study provides a concrete evidence for the application of WBE as a potential method for disease surveillance. Here we performed an extensive survey of the STPs in Hyderabad for the presence of SARS-CoV-2 genome traces in wastewater. As a result, we observed the presence of SARS-CoV-2 genetic material in all the studied STPs and calculated the extent of spread by estimating the number of infected people and possible number of active cases. These results will be of 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. 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 which may be useful managing future epidemics.

Author's contribution

RKM, SVM and SKK conceptualized the idea. MH, UK, KH and CGG performed the experiments. MH, KH, SVM, UK, SKK, and CGG wrote the manuscript. All the authors have read and approved the manuscript.

Declaration

The authors declare no conflict of interest.

Acknowledgments

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Supplementary Table 1: Standard Operating Procedure (SOP) for sampling of wastewater for the detection of viral genome and wastewater-based epidemiological studies

- The actual sampling site should be visited and accessed in the presence of the local responsible personnel.
- Proper training and written protocols for sampling should be clearly provided to the personnel collecting the samples. Complete care should be taken by wearing PPE, shoes and using disinfectants. Personnel must be aware of hazards that exist during sampling and the associated risks, safe working procedures, and emergency preparedness.
- Grab method is preferred for sampling water/wastewater wherein raw wastewater/water will be collected at a selected sampling site, either at one point in time or at different time intervals. Grab sample volume of one litre is generally recommended at one time. Three replicates of grab sample are preferred in flowing water for a specified period.
- The sample should be collected either in clean glass (borosilicate) or plastic 1 litre bottle containing disinfectant (20 ml of 0.1% hypochlorite/lit) to inactivate the pathogens. The bottle should be sealed in secondary sealing bags (multi-layered) that are suitable for cold transportation of the samples.
- The surface of the sample container should be disinfected with hypochlorite/alcohol/hydrogen peroxide. Before packaging, the samples should be properly labelled with collection site information and date. The properly labelled samples should be transported and stored at 2-8°C until further processing.
- After the collection, inactivation, and packaging of the samples, the personnel can remove PPE by following standard procedure safely. The personnel involved in collection and transportation of the samples should follow recommended hygienic practices to ensure bio-safety.
- All utilities should be placed in a sealed bio-hazard bag for later sterilization and disposal activities. The unused sample and materials should be properly discarded after inactivation/disinfection.
- The laboratory should be notified in advance about the sample collection and be made aware of the sample storage and processing protocol.