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Research Article

# Sewage Analysis as a Tool for Environmental Surveillance of SARS-CoV-2: Experience from Delhi, India

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## I N F O

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## A B S T R A C T

*Background:* The COVID-19 pandemic caused by SARS-CoV-2 has resulted in more than 150 thousand deaths in India. SARS-CoV-2 is known to be excreted in stool samples in the range of $10^2$ to $10^8$ gene copies per gram of faeces. Waste-water Based Epidemiology has been advocated by researchers all over the world as a method for environmental surveillance of COVID-19.

*Objectives:* The present study was carried out to determine the presence of SARS-CoV-2, and to quantify it in sewage samples in Delhi, and to estimate the Sensitivity of WBE for COVID-19 Surveillance.

*Methods:* A total of 49 waste water samples from seven different sites in Delhi were each concentrated by three different methods, Ultrafiltration, PEG precipitation and Two-phase separation method. RT-qPCR was done for N1, N2, and E gene of SARS-CoV-2. The minimum number of infected individuals required to yield a positive result was calculated.

*Results:* Out of the 49 samples tested in triplicate, 35 (71.43%) were positive for at least one of the three primers for SARS-CoV-2 (N1, N2 or E) by Method A and 33(67.35%) by Method B. The average concentration calculated for Delhi, using data of the seven sites, was $1.25 \times 10^4$ g.c./L of sewage. Approximately 7.5 active cases per 1000 population are required to yield a positive result for Environmental Surveillance.

*Discussion:* SARS-CoV-2 RNA could be successfully isolated from sewage samples in Delhi using Ultrafiltration and PEG precipitation for concentration. The practical limit of detection of the virus in waste water is low enough to make this a highly sensitive method for Environmental Surveillance of COVID-19. The detection of SARS-CoV-2 in sewage is a very useful tool with immense public health significance. It can provide an early warning signal of the presence of disease, and facilitate preparedness for the same.

**Keywords:** SARS-CoV-2, Waste-water Based Surveillance, COVID-19 Surveillance, Environmental Surveillance, Sensitivity of Waste-water testing

## Introduction

The ongoing COVID-19 pandemic is the third major outbreak in humans caused by Coronaviruses (CoV).[32] Considering the rapid spread of the virus and associated severity of illness, WHO declared a global health emergency on 30 January 2020. Subsequently, on 11 March 2020, this was declared to be a pandemic.[47] As of 31 March 2021, the pandemic has resulted in 12,149,335 confirmed cases of this disease, and 1,62,468 deaths in India.[34] Efforts are being made all over the world, to prevent any further spread of this potentially deadly virus by implementing prevention and control measures.

SARS-CoV-2 is primarily a respiratory virus that is transmitted through aerosol/ droplets or contact with contaminated surfaces, and predominantly affects the respiratory system.[46] However, gastrointestinal symptoms are seen in a considerable number (16% to 33%) of COVID-19 patients.[24] Approximately 50% of patients with COVID-19 have detectable virus in their stool.[12] In a study evaluating virus dynamics, it was observed that the median duration of virus in stool was 22 days (interquartile range 17–31 days), which was significantly longer than in respiratory airways (18 days) and in serum (16 days).[57] The magnitude of viral presence in the intestinal tract varies from $10^2$ to $10^8$ RNA copies per gram of faeces.[25,35,50,38] Thus, it can be seen from the reported literature that the respiratory virus SARS-CoV-2 has a significant association with the gastrointestinal tract.

The presence of SARS-CoV-2 RNA in wastewater has been reported worldwide.[49,7] Waste-water Based Epidemiology (WBE) has been identified as an important tool for surveillance of infectious diseases with a proven track record for Polio[44] and Hepatitis A.[17] WBE, therefore, holds considerable promise for COVID-19 surveillance. This is a non-invasive and an early-warning tool for monitoring the status and trend of COVID-19 infection, and is an instrument for fine-tuning public health response and directing policy.[38]

The present study was carried out in Delhi, India with the following objectives: (i) to detect the presence of SARS-CoV-2 in sewage samples, (ii) to study the utility of ultrafiltration, PEG precipitation, and two-phase separation method for concentration of SARS-CoV-2 RNA from sewage, (iii) to quantify the viral RNA in sewage samples in Delhi, and (iv) to determine the minimum number of infected individuals required in a catchment area for a positive test result.

## Methods

### Detection of SARS-CoV-2 in Sewage Samples

#### Waste-water Sampling

This cross-sectional study was carried out in June-July 2020. Sewage samples were collected, on a weekly basis, from each of the seven identified sites in Delhi (Sewage Pumping Station Batla House, Bhalaswa Lake Drain, Nangloi, Red Cross Hospital Shahdara, Sonia Vihar, Swarn Cinema, and Wazirpur JJ Colony). A total of 49 samples were collected for seven weeks during the above mentioned period (from seven sites for seven weeks).

Samples were collected in the morning hours during peak sewage flow. The personnel collecting sewage were fully equipped with Personal Protective Equipment (heavy-duty gloves, shoe covers, masks, gowns, goggles, and face shields) before approaching the sample collection site. The samples were collected by the Grab Method. After straining through a sterile muslin cloth, they were transferred to a 1000 ml sterile, screw-capped, labelled, polypropylene plastic bottle. Collected samples were transported on ice to the laboratory within 60-90 minutes. In the laboratory, the samples were kept at -20ºC till further processing for Method A (Ultra-filtration) and Method B (PEG precipitation), and were concentrated on the same day for Method C (Two-phase separation PEG-dextran method).

#### Sample Inactivation

The sewage samples were inactivated for 30 minutes at 56ºC to increase the safety of the analytical protocol, both for the laboratory personnel and for the environment. After heat inactivation, samples were further processed in Class II biological safety cabinets following standard practices.

#### Waste-water Concentration and RNA Extraction

Collected samples were concentrated by three different methods with some modifications. These methods are referred to as Method A (Ultra-filtration),[18,1] Method B (PEG precipitation),[55] and Method C (Two-phase separation PEG-dextran method).[44]

#### Method A (Ultra-filtration using Centricon Plus-70 filter)

200 ml of sewage sample was centrifuged at 4700g for 30 minutes. The supernatant was collected carefully without disturbing the pellet and further centrifuged through Centricon Plus-70 centrifugal filter (Merck Millipore Ltd. Catalogue No. UFC701008) with a molecular cut off of 10 kDa, at 3220g for 15 minutes. The concentrate cup was inverted and placed on top of the sample filter cup. It was again centrifuged at 1500g for 2 minutes. The final concentrate was collected from the collection cup with the help of a pipette.

### Method B (PEG precipitation)

The sample was centrifuged at 5000 rpm for 30 minutes. The resultant supernatant was decanted and the pellet was washed with 3 ml Phosphate Buffered Saline (PBS). The washed pellet was transferred to a 50 ml centrifuge

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tube, in which 20% (v/v) chloroform and 5-6 glass beads were added, and homogenised on a vortex shaker for 6-8 minutes. After homogenisation, the product was centrifuged on a swing bucket rotor at 5000 rpm for 20 minutes. The product was then transferred to a fresh 50 ml centrifuge tube and made up to a volume of 40 ml with supernatant obtained from the initial centrifugation step. To these contents, 4 grams of PEG 8000 and 0.9 gram of NaCl were added and mixed thoroughly. This was then centrifuged at 13000 rpm for 2 hours at 4ºC on a fixed rotor to obtain a pellet. Finally, the pellet was re-suspended in Rnase-free water for RNA extraction.

**Method C (Two-phase Separation PEG-dextran Method)**

The sewage was concentrated using the two-phase PEG-dextran separation method.[44] In brief, 550 ml of sewage sample was processed with 39.5 ml of 22% dextran, 287 ml of 29% PEG 6000, and 35 ml of 5N NaCl on the first day, and left overnight at 4ºC. On the second day, the lower phase along with the interphase was collected, and treated with chloroform and antibiotics to get the final concentrate. The final concentrate was used for RNA extraction.

**RNA Extraction**

The concentrates obtained from all the three methods were processed for RNA extraction using RNeasy Power Microbiome Kit (Qiagen), according to the kit literature.

**RT-qPCR and Quality Control**

RT-qPCR assays for SARS-CoV-2 were performed with ABI 7500 Real-Time PCR Instrument. As shown in Table 1, two recently published assays, CDC N1 and N2 assays[6] (IDT Catalogue No. 10006606, Lot No. 0000513132), and E_Sarbeco assay[10] (IDT Catalogue No. 10006804, Lot No. 0000517078), were used for the detection of SARS-CoV-2 in wastewater.

A series of positive controls of known concentration ($2x10^5$ to 2x10 gene copies per µL) were included for each target (N1 and N2, IDT Catalogue No. 10006625, Lot No. 0000527879 and E gene IDT Catalogue No. 10006896, Lot No. 0000518062). A human specimen extraction control, (IDT Catalogue No. 10006626, Lot No. 0000512968) was also included in the PCR run to serve as an extraction control to validate extraction reagents, thus implying successful RNA extraction. CDC recommended master mix for RT-qPCR (Quantabio qScript XLT One-Step RT-qPCR ToughMix) was used in this study (Catalogue No. 95134-500, Lot No. 66171348).

RNA extraction, master mix preparation, and RT-qPCR were performed in separate laboratories to minimise contamination. A negative extraction control (molecular grade water) was included to ensure that all extraction reagents are free of contamination. Five positive controls with known concentrations ($2x10^5$ to 2x10 gene copies/µL), human extraction control, and negative controls were included in each PCR run. The PCR run was considered valid only if all the controls worked accurately.

For N1 N2 assay: The master mix was prepared by using 3.5 µL nuclease-free water, 1.5 µL combined primer/ probe mix, and 10 µL qScript XLT One-Step ToughMix (2X). Finally, 5 µL of template (extracted RNA) was added to the prepared master mix. The RT-qPCR run conditions used were as follows: holding stage 1 (for 10 minutes at 50ºC), and holding stage 2 (for 3 minutes at 95ºC). This was followed by cycles of 3 seconds at 95ºC and 30 seconds at 55ºC, for 45 cycles.

For E_Sarbeco assay: The master mix was prepared by using 5 µL nuclease-free water, 2.5 µL combined primer/ probe mix, and 12.5µL qScript XLT One-Step ToughMix (2X). The RT-qPCR run conditions used were as follows: holding stage 1 (for 10 minutes at 55ºC), and holding stage 2 (for 3 minutes at 95ºC). This was followed by cycles of 15 seconds at 95ºC and 30 seconds at 58ºC, for 45 cycles.

A sample was considered positive for the target gene if amplification was seen at a cycle threshold (CT) of less than 40.

**Statistical Analysis**

Statistical analysis was performed using Graph Pad Instat Version 3.10. Chi-squared test (or Fisher's exact test, if the value in any of the cells was less than 5) was used for the analysis of nominal data. P value of $< 0.05$ was considered as significant.

## Quantification of SARS-CoV-2

**Viral Load Calculation**

For quantification, the number of gene copies per µL (g.c./µL) of the tested elute was calculated using the standard curve obtained (ABI 7500 Software version 2.3) by testing five positive controls with known concentrations (runs with $R^2$ values more than 0.98 were considered). As analysis on environmental matrices may occasionally display background fluorescence or non-exponential amplification,[24] the RT-qPCR runs were manually checked for exponential amplification.

**Statistical Analysis**

Statistical analysis was performed using Graph Pad Instat Version 3.10. The data were checked for normal distribution by Kolmogorov-Smirnov Normality Test. As the data were not normally distributed, for comparison of quantities, Mann-Whitney test (non-parametric) was used. Only positive samples were considered for quantitative analysis. The quantity of virus (gene copies per µL) for each of the primers N1, N2, and E by Method A and Method B were compared with each other in pairs (AN1 vs AN2, AN1 vs

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AE, AN2 vs AE, BN1 vs BN2, BN1 vs BE and BN2 vs BE). P value of < 0.05 was considered as significant.

### Estimation of Sensitivity of WBE for COVID-19 Surveillance

How many minimum number of infected persons should be present in the population of interest in order to pick up COVID-19 in wastewater? To answer this question mathematically, following calculations were done.

Minimum number of infected persons per 1000 population $= \frac{A \times B \times 1000}{C \times D}$ where.

- A is the lowest detectable sewage concentration (RNA copies per litre)
- B is the average amount of wastewater produced per person per day in litres
- C is the average amount of faeces produced by a person per day in grams
- D is the average amount of RNA copies per gram of faeces in an infected person

The average amount of RNA copies per gram of faeces in a COVID-19 positive patient was considered to be $10^8$ gene copies per gram of faeces.[50] The per capita waste generated was considered to be 121 litres per day.[8] The amount of stool generated per person was calculated to be 334 grams per day based on a study from North India.[40,9] The limit of detection of RT-qPCR was determined as explained below. The recovery for the method of concentration of sewage for Method A was taken to be 28%.[2] The lowest detectable concentration of RNA in the sewage sample was thus calculated. The minimum number of infected persons required to yield a positive result per 1000 population was calculated using the above equation.

#### Sensitivity of the RT-qPCR Assay

The standards with known concentrations for N1 and N2 genes were used to prepare serial dilutions of 1, 0.9, 0.8, 0.7, 0.6, 0.5, and 0.3 gene copies per µL. Each dilution was tested in five replicates under the same RT-qPCR run conditions. $LOD_{50}$ was calculated according to Wilrich and Wilrich, 2009, using the tools available at http://www.wiwiss.fuberlin.de/fachbereich/vwl/iso/ehemalige/wilrich/index.html.[43,24]

## Results

### Detection of SARS-CoV-2 in Sewage Samples

Sewage samples were collected from each of the seven identified sites in Delhi, on a weekly basis for seven weeks. Thus a total of 49 samples were collected (from seven sites for seven weeks).

For the initial 30 samples, each sewage sample was concentrated by three different methods (Method A, B, and C) followed by RNA extraction and RT-PCR. An interim analysis was made with the results of these 90 tests (30 samples each tested by all three methods).

For this qualitative analysis, the positivity rates by the three methods were compared. Samples considered negative were those which did not show amplification or which had a CT value of more than or equal to 40. On comparison between the results of Methods A, B, and C, it was found that 17/30 (56.67%) were positive by Method A, 14/30 (46.67%) were positive by Method B, however only 1/30 (3.33%) samples was positive by Method C (P < 0.001). It was therefore decided to reject Method C, and continue the study further with only Methods A and B. A comparison between Method A and Method B was therefore done for a total of 49 samples.

#### Method A (Ultra-filtration using Centricon Plus-70 Filter)

Out of the 49 samples tested, 35 (71.43%) were positive for at least one of the three primers for SARS-CoV-2 (N1, N2, or E) (Table 2). A total of 15 samples (30.61%) were positive for all three genes. A total of 26/49 samples (53.06%) were positive for at least two of the three primers. On comparison between the positivity rates for different primers of SARS-CoV-2, the highest positivity (61.23%) was seen for N2 gene (30/49), followed by N1 (55.10%), and the least for E gene (38.78%) (Figure 1). The positivity for N2 gene was significantly higher than for E gene (P = 0.0434). Two samples had CT value of more than 40 for N1, and therefore were considered negative.

#### Method B (PEG Precipitation)

Out of the 49 samples tested, 33 (67.35%) were positive for at least one of the three primers for SARS-CoV-2 (N1, N2 or E) (Table 2). The number of samples positive for all three genes was 15 (30.61%). A total of 25/49 samples (51.02%) were positive for at least two of the three primers. The positivity for N1 and N2 genes was 53.06% (26/49) while that for E gene was 42.86% (21/49). Four samples had a CT value of more than 40 for N1, and one sample had a CT value of more than 40 for N2.

The positivity for the month of June (96.43% for Method A, and 85.71% for Method B) was significantly higher than that for the month of July (38.10% for Method A, and 42.86% for Method B) (P value < 0.0001 for Method A and 0.0023 for Method B).

### Quantification of SARS-CoV-2

On comparison of viral quantities (gene copies per µL) for each of the primers N1, N2, and E by both Method A and Method B, it was found that the viral quantities obtained were significantly higher by Method A as compared to Method B (P = 0.0014) (Table 2).

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**Table 1.Primer-Probes used for RT-qPCR**

| Assay | Target Gene | Primer/ Probe | Sequence |
|---|---|---|---|
| N1 | Nucleocapsid (N) | Fwd | 5'-GAC CCC AAA ATC AGC GAA AT-3' |
| | | Rev | 5'-TCT GGT TAC TGC CAG TTG AAT CTG-3' |
| | | Probe | 5'-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3' |
| N2 | Nucleocapsid (N) | Fwd | 5'-TTA CAA ACA TTG GCC GCA AA-3' |
| | | Rev | 5'-GCG CGA CAT TCC GA GAA-3' |
| | | Probe | 5'-FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1-3' |
| E | Envelope (E) | Fwd | 5'-ACAGGTACGTTAATAGTTAATAGCGT-3' |
| | | Rev | 5'-ATATTGCAGCAGTACGCACACA-3' |
| | | Probe | 5'-FAM-ACA CTA GCC ATC CTT ACT GCG CTT CG-BHQ1-3' |

**Table 2.Quantification of SARS-CoV-2 RNA with N1, N2, and E Genes**

| Title | Method A | | | | | | Method B | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N1 QTY (copies /L) | N1 CT | N2 QTY (copies /L) | N2 CT | E QTY (copies /L) | E CT | N1 QTY (copies /L) | N1 CT | N2 QTY (copies /L) | N2 CT | E QTY (copies /L) | E CT |
| Mean | 8.36E+03 | 35.67 | 2.51E+04 | 36.57 | 8.39E+03 | 36.42 | 1.36E+04 | 35.63 | 1.48E+04 | 36.26 | 3.38E+03 | 36.88 |
| Standard deviation (SD) | 7.89E+03 | 1.82 | 2.97E+04 | 1.48 | 8.78E+03 | 1.70 | 3.14E+04 | 3.09 | 1.70E+04 | 1.29 | 3.50E+03 | 1.43 |
| Sample size (N) | 27 | 27 | 30 | 30 | 19 | 19 | 26 | 26 | 26 | 26 | 21 | 21 |
| Std. error of mean (SEM) | 1.52E+03 | 0.35 | 5.42E+03 | 0.27 | 2.01E+03 | 0.39 | 6.16E+03 | 0.61 | 3.33E+03 | 0.25 | 7.64E+02 | 0.31 |
| Lower 95% conf. limit | 5.24E+03 | 34.95 | 1.40E+04 | 36.02 | 4.15E+03 | 35.61 | 8.86E+02 | 34.38 | 7.98E+03 | 35.74 | 1.78E+03 | 36.23 |
| Upper 95% conf. limit | 1.15E+04 | 36.39 | 3.62E+04 | 37.12 | 1.26E+04 | 37.24 | 2.63E+04 | 36.88 | 2.17E+04 | 36.78 | 4.97E+03 | 37.53 |
| Minimum | 3.87E+02 | 33.04 | 3.10E+03 | 32.77 | 2.88E+02 | 33.35 | 1.39E+02 | 29.03 | 2.18E+02 | 33.52 | 6.56E+02 | 33.99 |
| Median (50th percentile) | 5.54E+03 | 35.12 | 1.33E+04 | 36.59 | 6.28E+03 | 36.79 | 1.68E+03 | 36.67 | 9.42E+03 | 36.30 | 1.20E+03 | 37.62 |
| Maximum | 2.65E+04 | 39.53 | 1.39E+05 | 38.82 | 3.01E+04 | 39.08 | 1.40E+05 | 40.00 | 7.57E+04 | 38.81 | 1.32E+04 | 38.83 |

In Method A, the viral quantities were significantly higher for N2 as compared to N1 (P = 0.0005), and for N2 as compared to E (P = 0.0011). In Method B also, the viral quantities were significantly higher for N2 as compared to N1 (P = 0.0062) and for N2 as compared to E (P = 0.0004).

The site-wise analysis of data for each method for all the three genes is shown in Table 3. The mean viral concentration of the seven sites in the sewage sample ranged from $3.35x10^3$ to $2.55x10^4$ gene copies per litre of sewage.

## Estimation of Sensitivity of Environmental Surveillance for COVID-19

The $LOD_{50}$ was calculated for N1 and N2. For N1, it was 0.884 gene copies per µL (CI: 0.516 to 1.514), and for N2, it was 0.545 gene copies per µL (CI: 0.342 to 0.871). Therefore it can be said that a minimum of 0.545 gene copies of N2 gene are required per µL to give a positive RT-PCR result.

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**Table 3.SARS-CoV-2 RNA Quantification in Seven Sites of Delhi**

| | Method A | | | | Method B | | | |
|---|---|---|---|---|---|---|---|---|
| | | N1 | N2 | E | | N1 | N2 | E |
| Name of the Site | No. Pos/ No. Tested | Mean Quantity (copies/L) | Mean Quantity (copies/L) | Mean Quantity (copies/L) | No. Pos/ No. Tested | Mean Quantity (copies/L) | Mean Quantity (copies/L) | Mean Quantity (copies/L) |
| Batla House | 7 of 7 | 1.13E+04 | 3.47E+04 | 1.68E+04 | 7 of 7 | 2.98E+04 | 2.36E+04 | 6.52E+03 |
| Bhalaswa Lake | 4 of 7 | 4.83E+03 | 4.60E+04 | 3.46E+03 | 4 of 7 | 3.00E+04 | 3.18E+04 | 6.69E+03 |
| Nangloi | 4 of 7 | 8.89E+03 | 9.88E+03 | 4.66E+03 | 2 of 7 | 2.79E+04 | 6.43E+03 | 9.46E+02 |
| RCH Shahdara | 3 of 7 | 1.84E+04 | 4.73E+04 | 1.68E+04 | 4 of 7 | 1.74E+03 | 8.69E+03 | 2.99E+03 |
| Sonia Vihar | 4 of 7 | 4.11E+03 | 1.18E+04 | 2.29E+03 | 4 of 7 | 9.43E+02 | 2.78E+03 | 1.78E+03 |
| Swarn Cinema | 7 of 7 | 3.48E+03 | 1.37E+04 | 5.53E+03 | 7 of 7 | 4.80E+03 | 8.95E+03 | 9.18E+02 |
| Wazirpur JJ Colony | 6 of 7 | 9.77E+03 | 1.50E+04 | 2.45E+03 | 5 of 7 | 4.45E+03 | 1.64E+04 | 3.59E+03 |

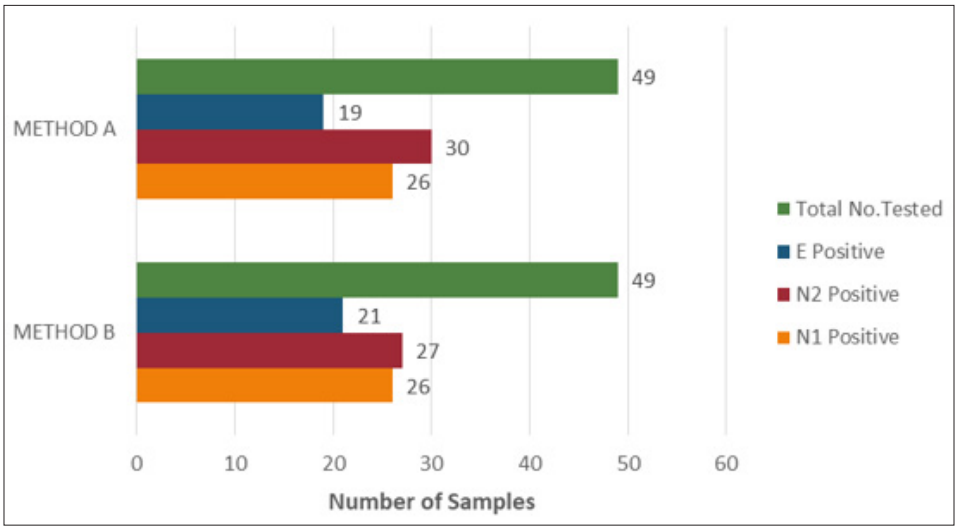


**Figure 1.Positivity for different Gene Targets**

This study shows that sewage samples can test positive for SARS-CoV-2 even when the prevalence of the disease is as low as approximately 7 active cases per 1000 population. This is calculated on the basis of the average amount of viral RNA shed per gram of stool, the average amount of stool produced per day, the per capita wastewater generated per day, the recovery rate of viral RNA from sewage samples, and the sensitivity of RT-qPCR test for SARS-CoV-2 (N2 gene) as described before.

## Discussion

The first case of COVID-19 was detected in India on 27 January 2020 from Thrisur, Kerala in a student who had returned from Wuhan.[5] Delhi reported the first case on 02 March 2020 in a person with a travel history from Italy. The cases gradually increased in number and till 31 March 2021, there were 1,21,49,335 confirmed cases and 1,62,468 deaths in India. In the National Capital Territory of Delhi, there were 6,60,611 cases and 11,016 deaths.[34] The Government of India was very proactive and scaled up measures to halt the progress of the epidemic well before the declaration of a pandemic by the WHO. As the number of cases increased, the capacity of testing of suspected samples was found wanting, as was the situation all over the world. The existing laboratories were upgraded and an increasing number of laboratories were recruited to meet the increasing demand for testing by RT-PCR. The protocols for the treatment of COVID-19 patients with various grades of severity and different underlying risk factors were prepared and disseminated. Health care facilities were increased in number and a high percentage were designated exclusively for COVID-19 patients. A high percentage of non-essential medical care was put on hold and resources were diverted into COVID-19 care. The provision of ventilators and other specialised ICU equipment

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was enhanced to full potential, along with the identification and modification of alternate methods to substitute for such equipment. Various degrees of lockdown and containment measures were implemented as per the situation in different regions of the country, and this was continuously monitored and modified resulting in evolving policy. In addition to testing of cases by RT-PCR, sero-surveys were carried out to determine the extent of population exposure to the virus.[31]

Testing clinical samples from all suspects and timely delivery of reports are challenging, even for the economically developed countries with relatively good health infrastructure. This challenge increases greatly in a large country like India which is highly populated, very diverse, and has significant resource constraints. Efforts are being made all over the world to find alternative methods of surveillance that are cost-effective, fast, require minimal resources, and are applicable throughout the country. Waste-water Based Epidemiology (WBE) has been well established for diseases like polio.[44] Researchers globally are trying to evaluate the role of sewage based environmental surveillance for COVID-19.[49]

The present study is an effort to evaluate sewage based environmental surveillance for COVID-19 in Delhi, India. It was done with the dual aim of detection as well as quantification of SARS-CoV-2 in wastewater samples specifically for Delhi. Though there are reports of detection of SARS-CoV-2 in sewage, this is the first study in India, which goes a step further to quantify the detected virus.

**Inactivation of Sewage and Safety Considerations**

In the current study, wastewater was inactivated at 56ºC for 30 minutes to minimise the risk of exposure to laboratory workers. Inactivation of the virus before processing increases the safety for the laboratory workers handling the sewage sample. Wang et al. have reported that heating at 56°C for 30 minutes, effectively inactivated the virus while preserving the stability of viral RNA in both human sera and sputum samples.[41] Liu Y et al. also reported that heat inactivation at 56ºC for 30 min, 56ºC for 60 min, 60ºC for 30 min, 60ºC for 75 min, and 100ºC for 10 min does not affect the detection results of Real-Time Reverse Transcription PCR of SARS-COV2.[27]

**Concentration of Wastewater Sample**

A large amount of raw sewage needs to be effectively concentrated so that the virus particle of interest is collected in a relatively smaller volume (in the range of 2 to 10 ml) before extraction. Concentration of the sewage is an extremely important step enabling identification of the virus even in a low prevalence area. An ideal concentration method for environmental surveillance of COVID-19 should be sensitive, technically simple, economical, reproducible, less time consuming, should use equipment that is readily available in a laboratory, and should have a high viral recovery.

Many different concentration methods have been reported in the literature, for the concentration of SARS-CoV-2 in sewage. Some of the popular methods are ultrafiltration, ultracentrifugation, polyethylene glycol (PEG) precipitation, use of an electronegative membrane, glass wool filtration, and the two-phase separation method used for the concentration of polioviruses.[49]

The recovery rate for a concentration method is the proportion of viral RNA obtained after concentration to the total quantity of virus present in the raw sewage sample, usually expressed as a percentage. There is a significant variation (2-73%) in the reported recovery rates for different concentration methods for SARS-CoV-2 by different researchers.[22,30,20] Threshold recovery yield is the minimum recovery rate that is acceptable for a concentration method. Currently, there is no consensus on the threshold recovery yield for concentration methods.[30]

The recovery rate for ultrafiltration was considered to be 28%[2] for the purpose of calculating the sensitivity of environmental surveillance of COVID-19. La Rosa et al. reported the recovery rate for the two-phase separation method (with certain modifications) to be 2.04% which is lower as compared to other methods[24] and this might be the possible reason for extremely low positivity in the samples processed by Method C in our study.

**Gene Targets for SARS-CoV-2 RT-PCR**

As per the CDC guidelines on testing methods for wastewater surveillance, primers and probes targeting regions of the SARS-COV-2 N (N1 and N2, by CDC) and E genes (E_Sarbeco, Charite protocol, Berlin) have been taken to be sensitive and specific for quantifying SARS-CoV-2 RNA in wastewater.[7] As per the WHO, the detection of SARS-CoV-2 by RT-PCR can be done with the targets on the E, RdRP, N and S genes.[45] The gene targets used in this study are N1 and N2 genes (CDC assay) and E gene (Charite protocol, Berlin) for SARS-CoV-2.[6,10]

In the present study, the positivity for N1, N2, and E targets for Method A was found to be 55.10%, 61.23% and 38.78% respectively. In Method B, the positivity for N1 and N2 was 53.06%, and for E gene, it was 42.86%. The $LOD_{50}$ for N1 and N2 genes was 0.884 and 0.545 g.c./µL respectively indicating a higher sensitivity for N2 assay as compared to N1 assay. In both the methods, the virus quantities were significantly higher for N2 as compared to N1 and E gene assays.

Several studies involving gene targets for SARS-CoV-2 have observed differences in the assays. Medema et al. noted discrepancies between CDC N1, CDC N2, CDC N3, and E_Sarbeco assays for several wastewater samples.

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As per their study, the N1 primer/ probe set started to produce a signal in sewage samples when the observed COVID-19 prevalence was around or even below 1.0 case in 100,000 people, and the N3 and E set started to yield positive signals when the observed prevalence was 3.5 case per 100,000 people or more.[28] Ahmed W et al. used five different RT-qPCR assays (targeting different regions of RNA from SARS-CoV-2 genome) amongst which CDC N1 assay was found to be most sensitive.[3] Randazzo et al. have observed discrepancies among N1, N2, and N3 assays for several water samples.[38] Sherchan et al., who also noted a discrepancy between N1 and N2 assays, have discussed the possible factors responsible for this inconsistency, namely, the sequences of the primers and probes, assay sensitivity, low levels of SARS-CoV-2 RNA in wastewater, and sub-sampling error.[39]

**SARS-CoV-2 Titres in Wastewater**

In the current study, quantification for SARS-CoV-2 was done for each of the gene targets N1, N2, and E for both, Method A and Method B (Table 2). It was found that the mean concentration for N1 was 8.68 x $10^3$ and 1.42 x $10^4$ for Method A and Method B respectively. The mean concentration for N2 was 2.55 x $10^4$ and 1.41 x $10^4$ for Method A and Method B respectively. The mean concentration for E gene was 7.43 x $10^3$ and 3.35 x $10^3$ for Method A and Method B respectively.

The average concentration of the viral RNA copies per litre of sewage for the seven different sites is as follows: Sewage Pumping Station Batla House - 2.04 x $10^4$, Bhalaswa Lake Drain - 2.05 x $10^4$, Nangloi - 9.79 x $10^3$, Red Cross Hospital Shahdara - 1.60 x $10^4$, Sonia Vihar - 3.96 x $10^3$, Swarn Cinema - 6.23 x $10^3$, and Wazirpur JJ Colony - 8.61 x $10^3$. The average concentration calculated for Delhi, using data of the seven sites, was 1.25 x $10^4$. Thus there was not much variation in the concentration of the virus observed from different sites. This is consistent with the fact that the entire city of Delhi was affected by the pandemic. When the situation is under control, this methodology would be useful for identifying pockets affected by the disease.

The titres of the virus per litre of sewage sample vary from as low as 19 RNA copies/L to as high as $10^5$ RNA copies/L.[4,51,37] The titres in different countries are not comparable due to the lack of uniformity in variables such as geographical location, environmental temperature, the wastewater system, the nature of sampling, and prevalence of the disease in an area.

The first case of COVID-19 was reported in Delhi on 2 March 2020. The cases gradually increased with intermittent waxing and waning of the epidemic. The total number of confirmed COVID-19 cases in Delhi by the end of May was 18,549 which rapidly increased to 85,161 by the end of June and then to 1,34,403 by the end of July. The number of new cases reported per day in the month of June was significantly higher than in July. The number of active cases as on 30 June 2020 was 26,246 cases whereas that on 31 July was 10,743.[34] This probably explains the fact that the positivity rate and the quantity of virus in the samples collected in the month of June were significantly higher than in July. This also indirectly suggests that continuous monitoring of the sewage virus quantities will give a signal of the increasing or decreasing trend of the epidemic in the community.

Different investigators have used Grab Method[16,38,21] or Composite Method[23,29,51] for sewage sample collection. The average concentration of viral RNA in wastewater by composite samples was lower (approximately 1500 viral genomes/ L) than those in grab samples (approximately 2 x $10^4$ viral genomes/ L).[30] Gerba et al. reported that peak concentration in the sewage should be considered rather than average concentrations obtained by 24-hour composite samples.[14] In the current study, Grab sampling was used. The average concentration for different sites in Delhi in our study was in the range of $10^3$ to $10^4$ gene copies per litre, which is similar to other studies that have used Grab samples for testing.[38,39]

Nemudryi A et al. reported that viral titres varied considerably between repeated samples from the same site when the samples were collected manually. This variation is less when the samples were collected by an autosampler; and the noted variability was due to the sampling method, rather than inconsistencies associated with RNA extraction or the RT-qPCR assay. Though the autosampler decreased the variability, the viral titres were higher in samples collected manually during peak flow.[33]

The quantity of viral RNA detected in wastewater samples depends on a number of variables[30,20] such as prevalence of the disease, type of sampling (grab or composite), per capita volume of wastewater generated, amount of virus shed per gram of faeces, temperature conditions, storage conditions, time interval between collection and testing, the concentration method used, the extraction method used, presence of PCR inhibitors and their neutralisation, the primer-probes used in RT-PCR, and their limit of detection. Due to the large number of variables on which the value depends, there can be a variation in the viral quantity obtained from the same site at the same time.

## Sensitivity of Environmental Surveillance for COVID-19

In the current study, it was determined that sewage samples can test positive for SARS-CoV-2 even when the prevalence of disease is as low as approximately 7 active cases per 1000 population. This was calculated on the basis of the

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average amount of viral RNA shed per gram of stool, the average amount of stool produced per day, the per capita wastewater generated per day, the recovery rate of viral RNA from sewage samples, and the sensitivity of RT-PCR test for SARS-CoV-2 genes. The factors on which calculation of the number of infected individuals depends are subject to immense variation and it is based on a number of assumptions. Faecal shedding in individuals can range from $10^5$ to $10^8$ RNA copies/ gram of stool.[25,50] Prolonged faecal shedding for up to four weeks after the first symptom onset has been reported in some studies.[52,19,54] Ling et al. have reported that viral RNA could be detected in the faeces of 81.8% of the cases even with a negative swab report.[26] The per capita wastewater generated is also bound to vary as it depends on various factors such as geographical location of a place, socio-economic status of people in a particular area, supply of water, season of the year etc.

The practical limit of detection of the virus in wastewater is low enough to make this a highly sensitive method for catching the presence of SARS-CoV-2 in a specified area. However, our estimate is based on a number of assumptions that are subject to significant uncertainty as discussed above and therefore need not be accurate. Considering the wide variability of these factors, it is difficult to estimate the exact number of people infected. However, if environmental surveillance is carried out over a continuous period, it is possible to detect the trend in the quantity of viral RNA and can give an early warning sign of an upcoming upsurge in cases.

**Role of Sewage in Transmission**

The presence of viral RNA in sewage samples raises the possibility that it may also contribute to the transmission of infection. However, the presence of a live virus is essential for the transmission of infection and mere detection of viral RNA may not be sufficient. Wölfel et al. have reported that culturable virus was not isolated from the faeces of patients despite high viral RNA concentrations.[50] Two studies have however demonstrated the presence of culturable SARS-CoV-2 in faecal samples from COVID-19 patients.[41,56] Though the involvement of gastrointestinal system is very high in SARS-CoV-2 patients, looking for the virus in faecal samples of patients is not a very definite or cost-effective method for clinical testing, but testing for this virus in sewage is an important method for surveillance.

Coronaviruses die off very rapidly in wastewater, with a 99.9% reduction in 2-3 days.[42] Conventional wastewater treatment processes should inactivate SARS-CoV-2, and multiple barriers used in drinking water treatment plants should suffice to remove SARS-CoV-2 to non-detectable levels.[20] As per WHO, no infectious SARS-CoV-2 virus has been recovered from untreated or treated sewage. Given the myriad pathogens routinely expected to be found in untreated sewage and the commensurate precautions normally taken, sewage sampling in the context of COVID-19 is not expected to engender any additional infection risk to workers. Laboratory processing of wastewater samples should follow existing biosafety standards for handling SARS-CoV-2, i.e., BSL-2.[49]

**Advantages of Wastewater Surveillance for COVID-19**

Wastewater systems offer a practical approach to identify viruses excreted in the faeces of a population, and is a promising tool for COVID-19 surveillance that can effectively complement the existing surveillance mechanisms.[20,49]

The detection of the virus in sewage, even when the COVID-19 prevalence is low, indicates that sewage surveillance could be a sensitive tool to monitor the circulation of the virus in the population.[29]

A BSL-2 facility is sufficient for wastewater testing for disease surveillance.[49] Highly sophisticated infrastructure and equipment are not essential. This can ensure countrywide use of this approach for COVID-19 surveillance.

Environmental surveillance can provide a snapshot of the situation in the catchment area by testing a single sample of wastewater. This results in the optimisation of limited testing resources to catch the presence of disease in that area. On the other hand, sero-surveillance requires testing of a large number of samples which translates into more time, higher cost, essential co-operation from the community, and greater dependence on inter-sectoral coordination and logistics.

If the occurrence of the disease is plotted as a linear graph, it is well known that the following three points would be important:

- Point X: Entry of virus into the body
- Point Y: Appearance of first clinical symptom
- Point Z: Appearance of detectable antibodies

The snap-shot of sero-surveillance catches the infection only at point Z, missing out the entire period from point X to point Z.[15,11] On the other hand, analysis of wastewater enables us to catch the presence of disease a little after point X. Therefore, even if it was possible to get a snap-shot of the infection by sero-surveillance, it would be at point Z, and this is much later than the snap-shot obtained by wastewater surveillance, which is at a point little after X. So environmental surveillance can provide a headway of 4 to 7 days over confirmed case data to the policymakers, health care managers, and executers to take immediate and proactive action for prevention and control of disease.[49] The impending upsurge in cases can thus be better managed by continuous environmental surveillance by quantification of the virus.[53,38,36]

Sewage surveillance could also serve as an early warning

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of (re-)emergence of COVID-19 in cities, much like the sewage surveillance for poliovirus that has been used for this purpose.[29] It may give a chance to catch the districts not exhibiting too many cases but are at risk.[21]

SARS-CoV-2 RNA detection in sewage is independent of the access to clinical testing and health-seeking behaviour. This can be very helpful in areas where clinical testing, for whatever reason, is low.

It can facilitate pooled testing for mass gatherings and closed residential settings (e.g. nursing homes, prisons, etc.). In such situations, it would not be feasible to test every individual, and it would be much easier to detect the presence of a virus by wastewater testing. Thus public health response can be quickly implemented in an unobtrusive manner.

Environmental surveillance can be used to determine the circulating strains of the virus in a particular area. To get an overview of the circulating strains in a country, a large number of clinical samples will have to be tested. This is an extremely costly affair, burdening the scarce resources which can be effectively used elsewhere. Sewage sample, being representative of an entire catchment area, will enable the identification of different circulating strains in an area.

Virus genome sequencing can give an early signal of entry of a new mutant strain into an area. It can detect variations in the viral strains via phylogenetic analysis, providing a substantial advantage for recognising virus trees that have evolved.[30]

It can contribute to understanding the dynamics of a viral epidemic. This is useful for the evaluation of control measures.[48]

Historical wastewater samples can be analysed for evidence of SARS-CoV-2 circulation in the past. SARS-CoV-2 virus was detected in wastewater in mid-December 2019 in Italy[24] and in November 2019 in Brazil.[13]

**Limitations of Wastewater Surveillance**

Wastewater testing is a technically complicated procedure, requiring specialised equipment, and intensive training. A high volume of sewage needs to be processed for better sensitivity and handling. Frequent sampling (on a weekly basis) is required to provide actionable data. It is not useful for part of the population that is not linked to the sewerage system. It will not represent certain facilities such as prisons, hospitals etc. that treat their waste before discharge into the common sewerage system. The amount of rise in titre, required to raise an alarm for the public health system is not yet established. The proportion of individuals that are infectious or symptomatic cannot be determined. Low levels of infection in a community may not be detected. Also, a negative wastewater test result does not mean that nobody in the area is infected with SARS-CoV-2. Quality assurance and proficiency testing mechanisms are not in place and need to be established.

**Policy**

Waste-water Surveillance as a Public Health Tool to influence Policy.

The COVID-19 pandemic has demonstrated how novel pathogens can rapidly emerge and spread through the human population and eventually cause severe public health crises. There is always a possibility of similar threats in the future. Wastewater based screening is a cost-effective tool for monitoring the circulating pathogens in an area. An effort needs to be made globally to establish environmental surveillance for different pathogens on a regular basis. The establishment of a sewage bank can help in retrospective analysis which can help us in understanding the origin and progress of a disease in a particular area and the evolution of epidemics.

As per a WHO report, the Netherlands plans to incorporate daily sewage surveillance into its national COVID-19 monitoring. A similar approach to using environmental surveillance as part of the routine COVID-19 surveillance package is being studied in Germany and has been initiated in Australia and New Zealand.[49]

Quality of results of wastewater surveillance would be an important issue if this is used at a big scale for monitoring and control of diseases. The multiple processes required for this (sample collection, concentration, nucleic acid extraction, and RT-qPCR) are tedious, technically demanding, and require intense training and supervision.

Wastewater surveillance would be useful if there is an action plan in place to utilise the information generated. A protocol should be evolved to describe the immediate steps to be taken in the event of a significant rise in titres. Also, various personnel involved in undertaking these processes and in the supervision of the same should be clearly identified along with the chain of command.

## Conclusion

SARS-CoV-2 RNA could be successfully isolated from sewage samples in Delhi. The average concentration calculated for Delhi, using data of the seven sites, was $1.25 \times 10^4$ gene copies per µL of sewage. Ultrafiltration and PEG precipitation can be used for concentration of the virus from sewage samples. The practical limit of detection of the virus in wastewater is low enough to make this a highly sensitive method for detecting the presence of SARS-CoV-2 in a specified area. Environmental surveillance by testing wastewater has great potential as a tool for monitoring and control of the disease.

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