Surveillance of SARS-CoV-2 genome fragment in urban, peri-urban and rural water bodies: a temporal and comparative analysis

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As a result of the SARS-CoV-2 pandemic, water bodies connected to anthropogenic activities may likely reveal the presence of viral genetic material. Urban, peri-urban and rural water bodies in and around Hyderabad, Telangana, India, were monitored for the presence of SARS-CoV-2 gene fragments during the first and second wave of COVID-19 infection. The SARS-CoV-2 genes were not detected in peri-urban and rural lakes, whereas urban lakes having direct functional attributes from domestic activity showed prevalence. Distinct variability in viral load observed among five water bodies was in concordance with human activity in the catchment area. High viral load was observed during the peaks of the first and second waves, specifically in urban lakes.

Keywords: COVID-19 pandemic, domestic discharge, lake ecosystem, RNA fragments, water bodies.

THE SARS-CoV-2 posed a pandemic challenge to the existing diagnostic and clinical management. Transmission of viral genetic material through the faecal–oral route gained traction in wastewater-based epidemiology (WBE) studies[1–7]. WBE offers unbiased infection status and dynamics in a given population and provides early warning for better management of infection[8–11]. The occurrence and detection of different pathogenic viruses such as polio, SARS and MERS in wastewater have been reported previously[12,13]. Unlike the polio virus, the transmission of SARS-CoV-2 through water has not been established. So far, only a few reports have shown successful culturing of SARS-CoV-2 virus from wastewater[14]. Multiple independent studies showed the presence and persistence of SARS-CoV-2 genetic material in domestic sewage and water bodies such as lakes and rivers[14–16]. It is largely agreed that environmental factors like temperature, presence of different chemical contaminants and detergents play a detrimental role in the stability of virus/viral particles in sewage water[4,17,18]. Considering the significant human activities near water systems, it is important to conduct the long-term surveillance for possible viral contamination in water systems[14,15]. Urban water bodies function as a proxy for the anthropogenic activities surrounding them. The urban lakes are prone to higher domestic discharges due to higher population density[19]. Hence, evaluating the water bodies for viral genetic load would provide information about community infection in the catchment area. The viral genetic material in water bodies is mostly non-viable; however, the data can be used as a surveillance tool to understand infection onset and spread[20]. In this study, we monitored SARS CoV-2 genetic material in an urban lake (UL-1) for 11 months and compared it with other urban lakes (UL-2 and UL-3), a peri-urban lake (PL) and a rural lake (RL). The dynamics in viral load helped in understanding the infection rate over time and its persistence.

Materials and method

Sampling sites and collection

Water samples from selected water bodies, viz. urban, peri-urban and rural areas in and around Hyderabad, Telangana, India, were collected employing grab sampling protocol between 8:00 and 10:00 am. The samples were collected on the days when there was no rainfall for 48 h. Sterile sampling bottles were used for sampling 1 litre of lake water with 20 ml of sodium hypochlorite (0.1%)[5,9]. Three urban lakes in Hyderabad, viz. Peddacheruvu/Nacharam Lake (UL-1, 17.42°N, 78.55°E), Hussain Sagar Lake (UL-2; 17.41°N, 78.47°E) and Nizam Talab (Turkha Cheruvu) lake (UL-3, 17.52°N, 78.38°E) were chosen for study (Table 1; Figure 1). The Edulabad Lake (EL, 17.42°N, 78.69°E) and Potharaju Lake (PL, 17.40°N, 78.71°E) were sampled as referral lakes under peri-urban and rural areas respectively (Table 1). Long-term surveillance (weekly and monthly) was performed for UL-1 with two samples (lake and lake outlet) for four weeks (week-1 (7 October 2020); week-4 (28 October 2020); week-5 (4 November

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Table 1. Location, point and date of sampling of water bodies

Lake	Location	Date of sampling
Peddacheruvu (urban kake-1 (UL-1)) Sampling points – 2	17.42°N, 78.55°E	7 October 2020 28 October 2020 4 November 2020 18 November 2020 7 October 2020 4 November 2020 11 December 2020 20 January 2021 13 February 2021 2 March 2021 1 April 2021 15 April 2021 1 May 2021 17 May 2021 21 May 2021 27 May 2021 4 June 2021 18 June 2021 23 June 2021 4 July 2021 10 July 2021 14 July 2021 27 July 2021 8 August 2021
Nizam Talab Lake (UL-2)	17.52°N, 78.38°E	3 December 2020 6 January 2021 5 April 2021
Hussain Sagar (UL-3)	17.41°N, 78.47°E	14 July 2020 15 April 2021
Edulabad Lake (Peri-urban lake (PL)) Sampling points – 1	17.42°N, 78.69°E	31 March 2021
Potharaju Lake (Rural lake (RL)) Sampling points – 1	17.40°N, 78.71°E	31 March 2021

2020)); week-6 (18 November 2020)) and monthly monitoring for 11 months (October 2020 to August 2021). Week-2 (14 October 2020) and week-3 (21 October 2020) samples were not collected due to rainfall.

Sample fractionation

After collection, samples were packed in a disposable pack to avoid leakage during transportation. They were brought to the laboratory within 3 h of sample collection and processed within 24 h. One litre of the sample was initially filtered using 1 mm filter paper to remove larger debris, followed by 0.2 μm membrane filtration to separate suspended solids. A 60 ml aliquot of the filtrate was subjected to ultrafiltration (4000 rpm; 4°C; 10 min) using 15 ml Amicon (30 kDa Amicon® Ultra-15, Merck Millipore) to a concentration of 600 μl.

Nucleic acid extraction from concentrate fraction and RT-PCR

Next, a 150 μl of the concentrate was used for RNA extraction employing a viral RNA isolation kit (QIAamp, Qiagen) according to the manufacturer's protocol. Sterile material devoid of DNA/RNA contamination and RNase-free water was used for RNA isolation. The isolated RNA was utilized for SARS-CoV-2 detection employs an RT-PCR kit (Shanghai Fosun Long March Medical Science Co, Ltd, China) approved by the FDA (Food and Drug Administration, USA). Fosun RT-PCR contains primers, probes (chromophore-labelled) for genes encoding the envelope protein (*E*-gene, ROX), nucleocapsid (*N*-gene, JOE), and open reading frame-1ab (*ORF1ab*; FAM) of SARS-CoV-2. The RT-PCR reaction for SARS-CoV-2 detection includes reverse transcription for 15 min at 50°C and initial denaturation for 3 min at 95°C followed by 45 amplification cycles at 95°C for 5 sec and 60°C for 40 sec. Signals from the chromophore probes ROX (*E*-gene), JOE (*N*-gene), FAM (*ORF1ab*) and CY5 (internal reference) were collected by the fluorescence channels at 60°C. All the amplifications, including positive and negative controls, were provided in the Fosun RT-PCR kit. The negative controls were clean and the threshold cycle (C_T) values of positive samples matched the manufacturer's recommendation. All the reactions were performed in triplicates in a biosafety level 2 (BSL-2) laboratory.

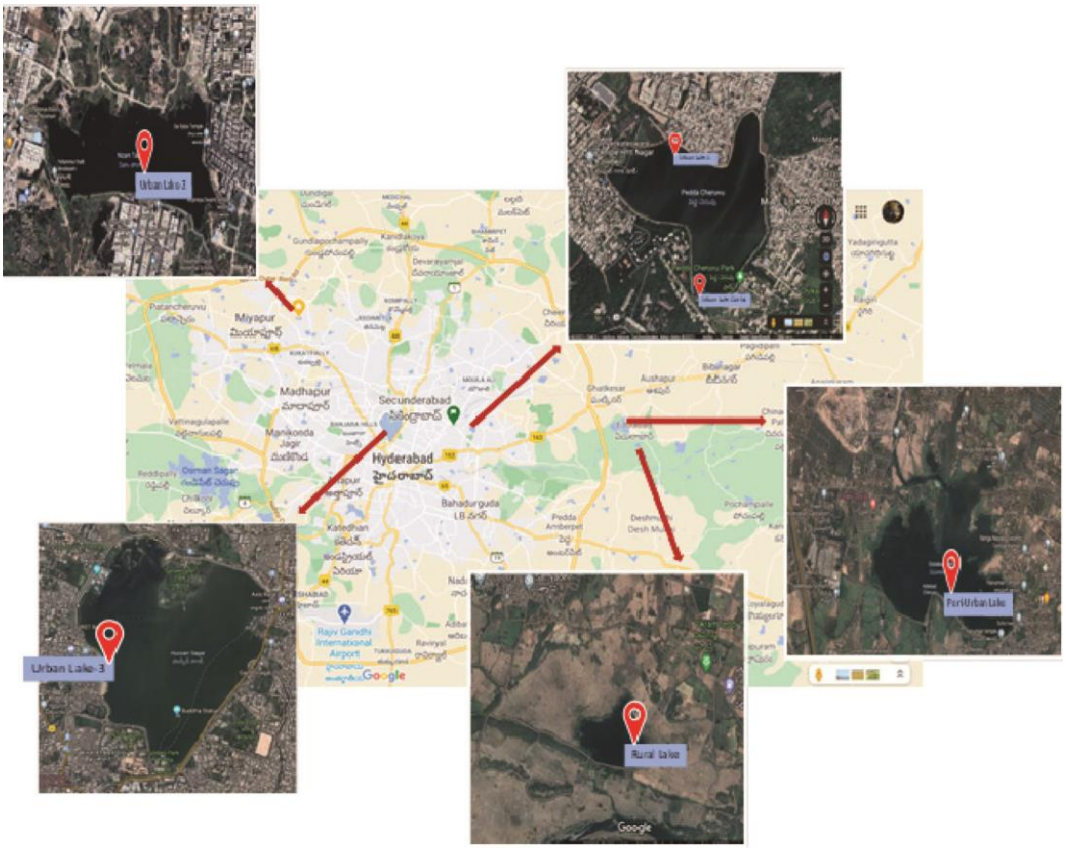

Figure 1. Map illustrating the points of sample collection with reference to lakes in and around Hyderabad, Telangana, India (courtesy: Google Maps).

Statistical analysis

From the obtained C_T values, RNA copies/l of water were calculated using the linear fit equation of *E*-gene (eq. (1)).

$$\log \text{RNA copies for volume of RNA used for RT-PCR} = \frac{C_T \text{ of } E\text{-gene} - 33.696}{-3.2839}. \quad (1)$$

Relative standard deviation (RSD) was calculated for the C_T value of individual genes based on the eq. (2)[8,9].

$$\text{RSD} = 100 * S/\bar{X}, \quad (2)$$

where $\bar{X}$ is the mean of the C_T values and S is the standard deviation.

Lake-water characteristics

Selected physico-chemical characteristics, viz. total dissolved solids (TDS), total suspended solids (TSS) and chemical oxygen demand (COD; closed reflux titrimetric method) of the lake-water samples were evaluated according to standard protocols[21].

Results and discussion

Surveillance of targeted gene fragments – UL-1

UL-1 selected for long-term viral RNA surveillance is an artificial lake spreading over 90 acres and surrounded by a population of ~1,000,000. The catchment area of UL-1 is subjected to anthropogenic activities and receives a treated discharge of 10 MLD capacity from the sewage treatment plant (STP). The collected UL-1 sample showed a yellowish tinge while the lake appeared dark grey in colour, which may be because of eutrophication. Water from UL-1 showed COD of 152 mg/l, TDS of 800 mg/l and TSS of 110 mg/l (Table 2).

Weekly monitoring: A total of eight samples over a period of six weeks were collected (7 October 2020 (week 1); 28 October 2020 (week 4); 4 November 2020 (week 5); 18 November 2020 (week 6)) accounting for four samples each from UL-1 and its outlet point. Samples during week 2 (14 October 2020) and week 3 (21 October 2020) were not collected due to multiple rainfall events. All the lake samples showed positive signals for the targeted genes (*E*-gene, *N*-gene and *ORF1ab*). The C_T value of *E*-gene in lake samples varied between 28.16 ± 0.46 and 23.04 ± 12.02

Table 2. Lake-water characteristics

Parameters	Nacharam Lake	Nizam Talab Lake	Hussain Sagar	Edulabad Lake	Pothuraj Lake
pH	8.6	8.05	7.6	8.0	7.0
Total dissolved solids (TDS) (mg/l)	800	524	720	443	337
Total suspended solids (TSS) (mg/l)	110	–	125	61	46
Chemical oxygen demand (COD) (mg/l)	152	134	225	176	127
Visible colour (after filtration)	Yellowish tinge	Colourless	Green colour	Light yellowish tinge	Colourless

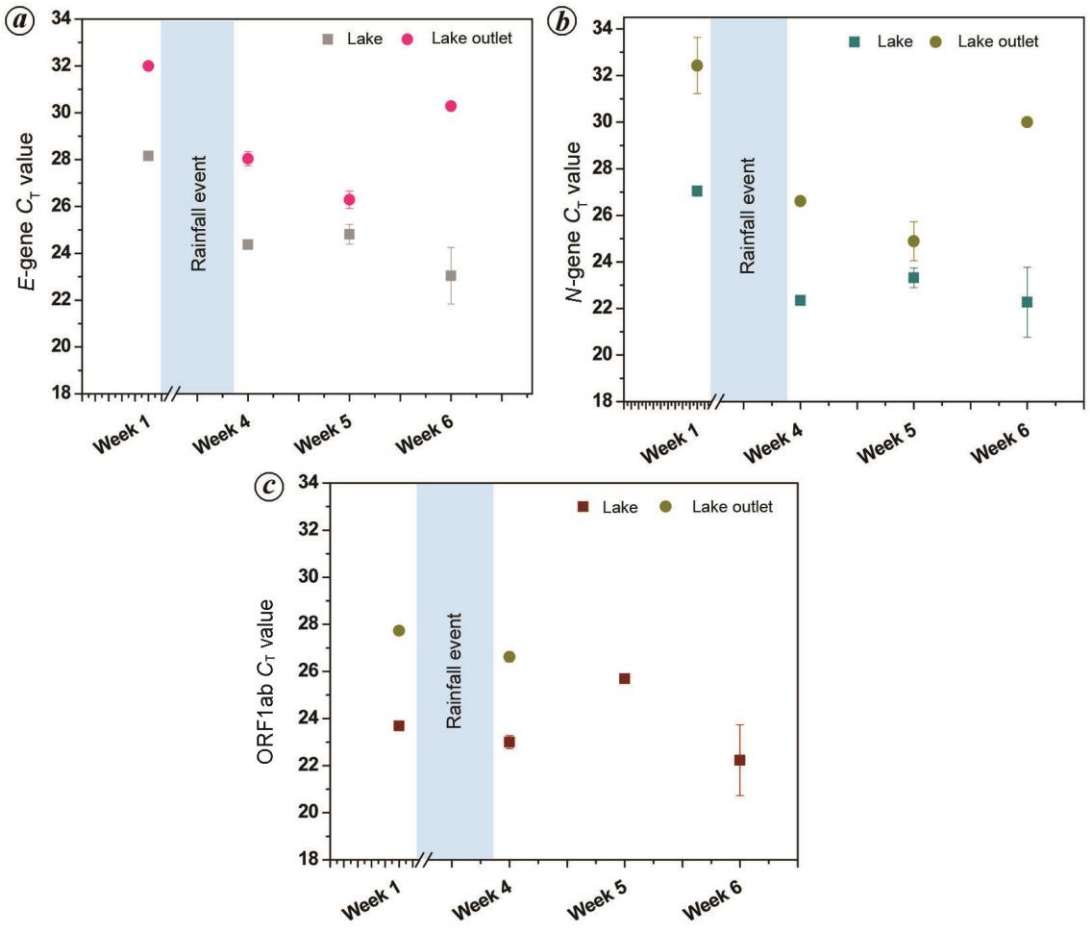

Figure 2. C_T values of (***a***) *E*-gene, (***b***) *N*-gene and (***c***) *ORF1ab* of samples collected weekly from UL-1 and its outlet point (all values represent $\bar{X}$ + SD).

with continuous reduction from week-1 (28.16 ± 0.46), week-4 (24.37 ± 1.23), week-5 (24.81 ± 1.70) and week-6 (23.04 ± 12.02). A similar trend was observed in both *N*-gene (week-1: 27.04 ± 0.81; week-4: 22.35 ± 0.62; week-5: 23.32 ± 1.83; week-6: 22.27 ± 11.54) and *ORF1ab* (week-1: 27.73 ± 0.75; week-4: 26.62 ± 0.80; week-5: 25.69 ± 1.86; week-6: 22.23 ± 10.21) (Figure 2; Supplementary Table 1). The continuous reduction in C_T of all the targeted genes represents an increase in viral load in the community surrounding UL-1 with time. The increase in viral RNA copies/l with respect to time was observed for lake samples, which ranged between 26,927 and 975,668. The highest RNA copies/l was observed during week-6 (975,668), followed by week-5 (282,039), week-4 (383,969) and week-1 (26,927) (Figure 3; Supplementary Table 2). The range of RNA copies correlated well with the infection spread in the community near UL-1 (ref. 8). Interestingly, 75% of the lake outlet samples showed positive signals for the viral genetic material. The *E*-gene was detected in all the outlet samples, while the *N*-gene was detected in weeks 1, 4 and 5, and *ORF1ab* gene was detected in weeks 1 and 2 samples only. The trend of C_T values showed a relatively lower viral load in outlet samples when compared to lake samples. Comparatively lower RNA copies/l (1823) were observed during week-1, while week-5 showed higher RNA copies/l (99,914) (Figure 3 and Supplementary Table 2).

Monthly monitoring: Totally 15 samples were collected from October 2020 to June 2021. As there was a rise in COVID-19 positive cases from March 2021, the sampling was performed twice in April 2021 (1 and 15, mid-April); four times in May 2021 (1, 17, 21 and 27) and three times in June 2021 (4, 18 and 23). All the lake samples were positive for all the three targeted genes. The *E*-gene C_T value varied significantly between 31.94 ± 2.15 and 24.81 ± 1.70, and correlated well with the first and second wave scenarios. A C_T value of 28.16 ± 0.46 was detected for the October 2020 sample, which further decreased to 26.49 ± 0.94 and 24.81 ± 1.70 in November 2020 and December 2020 respectively. A sharp rise in C_T values was observed for the January 2021 and February 2021 samples, which were 31.94 ± 2.15 and 31.34 ± 1.97 respectively, with a slight increase of ~0.6. From February 2021, a marked decrease in C_T was noticed followed by March 2021 (30.66 ± 0.48), April 2021 (1 April 2021 – 28.75 ± 0.54; 15 April 2021 – 27.38 ± 2.00) and May 2021 samples (1 May 2021 – 28.25 ± 0.36; 17 May 2021 – 28.38 ± 0.68) representing the onset of the second wave (Supplementary Table 3). The samples from 21 May to 23 June 2021 showed an increasing trend of *E*-gene C_T values (21 May 2021 – 29.02 ± 0.92 to 27 May 2021 – 30.28 ± 2.41; 4 June 2021 – 31.10 ± 2.07; 18 June 2021 – 31.75 ± 1.05; 23 June 2021 – 31.95 ± 0.18) (Figure 4 and Supplementary Table 3). A similar trend was observed for the other two targeted genes. The samples from October to December 2020 showed a decrease in C_T values for the *N*-gene, viz. 27.04 ± 0.81 (October 2020), 25.38 ± 1.05 (November 2020) and 23.32 ± 1.83 (December 2020), followed by an increment in January 2021 (30.10 ± 1.62). A further decrease was observed from February to mid-May 2021 (February 2021 (29.04 ± 1.58); March 2021 (28.47 ± 0.09); 1 April 2021 (27.11 ± 0.62); 15 April 2021 (26.03 ± 2.09); 1 May 2021 (26.89 ± 2.34); 17 May 17 May 2021 (25.19 ± 1.48)). The samples from 21 May 2021 to 23 June 2021 showed an increasing trend from 25.35 ± 0.59 to 31.10 ± 0.23, with no detection on the 18 June 2021 sample (Figure 4; Supplementary Table 3). For *ORF1ab*, October 2020 C_T value was observed to be 27.73 ± 0.75, while a decrease in C_T value was observed in November 2020 (25.72 ± 0.55) and December 2020 (25.69 ± 0.68) (Figure 4; Supplementary Table 3). No detection of *ORF1ab* was observed in January 2021 samples. Whereas, the detection was observed in February 2021 samples which showed a persistent decrement till May 2021 (Figure 4; Supplementary Table 3). The samples from 21 May 2021 showed a marginal decrease from 25.96 ± 1.29 to 32.76 ± 1.66, with no detection in the 18 June 2021 sample. An increase of ~2 was observed in C_T values from 1 April 2021 to 15 April 2021 which showed C_T value of 27.13 ± 2.17 in the lake samples.

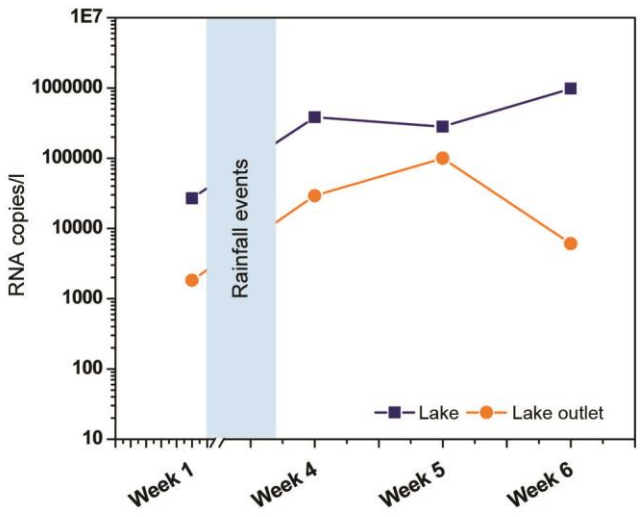

Figure 3. RNA copies calculated based on linear fit equation of *E*-gene of samples collected weekly from UL-1 and its outlet point.

The outlet of UL-1 was positive for two (*E*-gene and *N*-gene) among the three targeted genes. The detection was from October 2020 to February 2021, whereas the March and April 2021 samples showed no detection of the targeted genes. The decrease in C_T value of the *E*-gene C_T was from 32 ± 0.89 (October 2020) to 30.28 ± 0.03 (November 2020), which later showed an increasing trend till February 2021 (32.32 ± 0.09) (Figure 4 and Supplementary Table 3). Similarly, C_T of the *N*-gene was observed to decrease from 32.43 ± 10.82 (October 2020) to 28.01 ± 3.47 (December 2020), which later showed an increasing trend till February 2021 (32.26 ± 0.06; Figure 4 and Supplementary Table 3) with no detection of *ORF1ab* in the outlet samples. The viral load might be affected by the ecological conditions of the lake as well as the amount of domestic/wastewater discharge and the prevailing climatic conditions[22,23].

Higher RNA copies of 282,029/l were detected for December 2020, representing peak infection during the end of the first phase. A lower copy number was observed in January 2021 (3128 RNA copies/l) and February 2021 (2881 RNA copies/l), followed by a slight increase in March 2021 (4665 RNA copies/l) (Figure 5 and Supplementary Table 4). The April 2021 samples showed an increase in trend from 17,804 RNA copies/l (1 April 2021) to 46,217 RNA copies/l (15 April 2021), while there was a decreasing trend in the May and June 2021 samples (1 May 2021 (25,307) of RNA copies/l; 17 May 2021 (23,028); 21 May 2021 (14,733); 27 May 2021 (6103); 4 June 2021 (3426); 18 June 2021 (2165) and 23 June 2021 (1882) RNA copies/l) (Figure 5; Supplementary Table 4). RNA copies of lake outlet samples followed the same trend, as the highest copies were observed in November 2020 (99.914 RNA copies/l) followed by December 2020 (6090 RNA copies/l) (Figure 5; Supplementary Table 4). The eleven months' surveillance data of the water body covered both the first wave (second half) and second wave. The

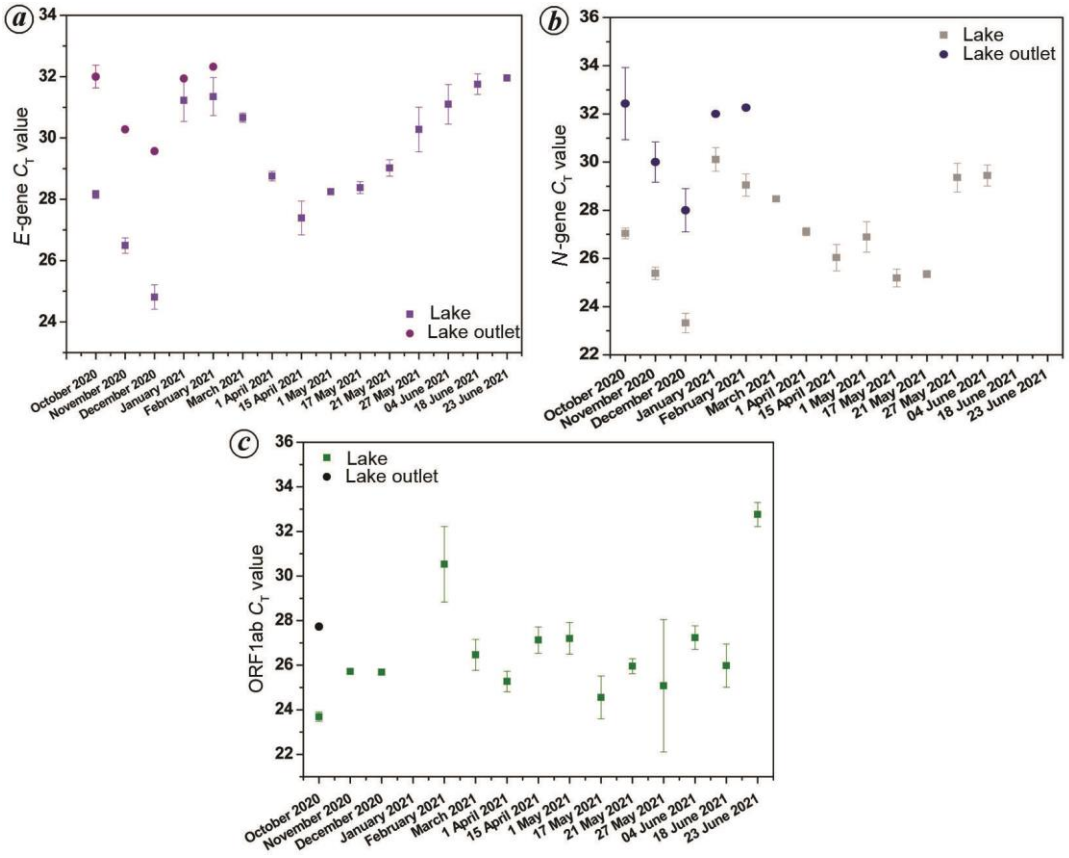

Figure 4. C_T values of (***a***) *E*-gene, (***b***) *N*-gene and (***c***) *ORF1ab* of samples collected monthly from UL-1 and its outlet point (all values represent, $\bar{X}$ + SD).

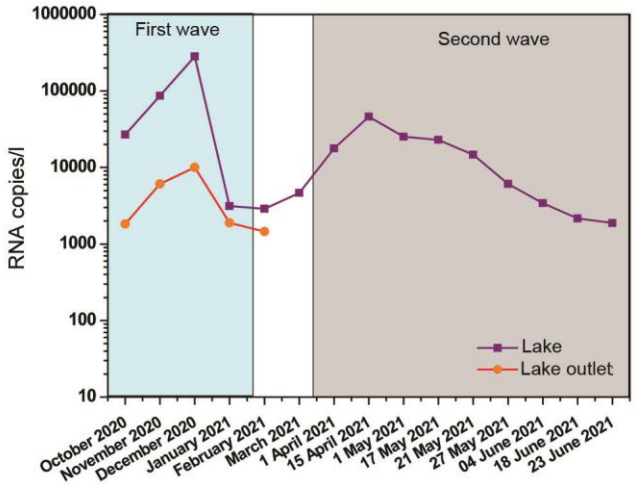

Figure 5. RNA copies calculated based on linear-fit equation of *E*-gene of samples collected monthly from UL-1 and its outlet point.

February 2021 samples showed the onset of the second wave, which was supported by the March–June 2021 data. This long-term study highlights the importance of water bodies in surveillance, which was eventually observed during the onset of the second wave.

Surveillance of targeted gene fragments–urban lake-2

UL-2 located in Pragathi Nagar area of Kukatpally, Hyderabad, covers nearly 35 acres with a population of ~150,000 in the catchment area. The lake has three-point sources (domestic discharges) and one outlet. Visually it is transparent without any signs of eutrophication. The samples were collected for three months, from the end of the COVID-19 first wave till the onset and persistent phase of the second wave (December 2020, January 2021 and February 2021). The lake samples showed the presence of *N*-gene and *ORF1ab*. A decrease in the trend of C_T for the *N*-gene and *ORF1ab* was observed. For the *N*-genes, C_T decreased from December 2020 (33.62 ± 1.33) to January 2021 (30.02 ± 1.69) and February 2021 (29.08 ± 1.65) (Table 3). Whereas C_T values of *ORF1ab* showed a marginal decrease from December 2020 to January 2021. RNA copies were not calculated as all the samples showed no detection of the *E*-gene. The lake outlet samples did not contain the three targeted genes. Being a relatively young lake with no signs of eutrophication and self-regeneration capacity might be the probable reason for not detecting all the genes in the lake water samples, even though untreated domestic sewage discharge was released. Also, the

Table 3. SARS-CoV-2 RNA C_T values of UL-2 and UL-3

Lake	Sampling time	*E*-gene	*N*-gene	*ORF1ab*	RNA copies per litre wastewater
UL-2	December 2020	ND	33.62 ± 1.33	32.38 ± 1.95	–
	January 2021	ND	30.02 ± 1.69	31.16 ± 1.79	–
	February 2021	ND	29.08 ± 1.65	ND	–
UL-3	July 2020	30.82 ± 0.06	31.18 ± 0.11	ND	4160
	April 2021	33.44 ± 0.12	31.96 ± 0.16	ND	664

ND, Not detected.

composition of domestic discharges, specifically with surfactants will also influence the presence of viral genetic material.

Surveillance of targeted gene fragments – urban lake-3

UL-3 is an artificial lake with a spread of 1409 acres and a depth of 32 ft. This lake has point sources (treated sewage) of 50 MLD from two STPs. The presence of aquatic microflora was evident with signs of eutrophication. The samples were collected from this lake during the two infection peak (waves) phases of COVID-19 (July 2020 and April 2021). Both the samples showed amplification of two genes (*E*-gene and *N*-gene). The detected samples showed higher C_T values of the *E*-gene of 30.82 ± 0.06 and 33.44 ± 0.12 for July 2020 and April 2021 respectively. Whereas C_T values of the *N*-gene were 31.18 ± 0.11 (July 2020) and 31.96 ± 0.16 (April 2021) (Table 3). RNA copies of 4160 and 664 RNA copies/l were observed in July 2020 and April 2021 respectively (Table 3). The lake receives majority of treated domestic water which upon reaching the water body gets more diluted due to which the sample might have resulted in low RNA copies.

Surveillance of targeted gene fragments – peri-urban/ rural water bodies

The peri-urban lake is located near Ghatkesar and covers about 1236 acres. The lake catchment area includes villages and agricultural fields. It has non-point and point sources accounting for domestic discharges and agricultural run-off. A rural lake, i.e. Pothuraju Lake (RL) which is located to the southeast of PL is surrounded by agricultural fields and majorly receives non-point run-off. Samples from both PL and RL were collected during the second COVID-19 wave (1 April 2021). Both the lakes were used as referral water bodies for comparison. Samples from PL and RL were negative for the targeted genes, which might be because of the nature of these lakes, since PL is known to receive less domestic sewage and more agricultural run-off and RL is devoid of domestic sewage.

Proxy analysis

Comprehensive long-term monitoring of different types of lentic water bodies located in urban, semi-urban and rural areas showed the presence of RNA genetic material of the virus contributing to the associated functional activities of the lake catchment area. Urban lakes encompassing domestic activities showed the prevalence of viral load and may be considered as a proxy for WBE studies to assess the community infection rate. Domestic discharge from the population residing around a catchment area forms a major basis for this. The UL-1 samples showed positive signals for all three genes indicating high viral load, whereas the UL-1 outlet samples showed positive signals for *E*-gene and *N*-gene and did not detect *ORF1ab*. The UL-2 and UL-3 samples showed positive signals for two out of three targeted genes (UL-2 *N*-gene and *ORF1ab*; UL-3 *E*-gene and *N*-gene). The referral lakes PL and RL did not detect any targeted genes. From the above results, it is clear that urban lakes are impacted probably due to non-treated sewage discharge resulting in high viral load, whereas the rural lakes PL and RL devoid of domestic discharge are negative to SARS-CoV-2 RNA fragments.

The trend of the RNA copies curve (with reference to UL-1) correlated with the dynamics of COVID-19 cases corresponding to the first and second waves in India[24]. This observation indicates a clear-cut function of the water bodies to act as a proxy for surveillance studies to predict an epidemic/pandemic as an early warning signal (as part of WBE studies), to assess infection rates during the ongoing pandemic and to understand the dynamics of viral load pertaining to the community in a catchment area. The genetic materials/fragments in sewage or water bodies will be in non-viable form and therefore do not infect the community. However, it can be used as a surveillance tool to understand the infection's onset and spread. If the monitoring of water bodies/wastewater can be implemented in the urban and semi-urban areas, the infection and rate of spread can be known prior to the outbreak. This study indicates the need for regular monitoring of water bodies/wastewater to understand the outbreak and spread of pathogens in the community and functions as a proxy for the surrounding domestic activities. The surge in viral gene load from the February 2021 samples suggests the

onset of the second wave, which correlated well with the prevailing pandemic situation.

Conclusion

This study reveals that the urban water bodies linked with domestic activity function as a proxy for epidemiological studies. The SARS-CoV-2 gene fragments were detected in urban lakes surrounded by anthropogenic activities. The surge in the February 2021 samples showed the onset of the second wave of COVID-19 infection, which correlated well with the prevailing pandemic situation. The reference water bodies (peri-urban and rural lakes) were devoid of SARS-CoV-2 genome fragments. The dynamics of the viral load helps to understand the infection rates and serves as an early warning signal.

Conflict of interest: The authors declare that they have no conflict of interest.

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