

Article

Wastewater-Based Epidemiology Monitoring for Endemics Like COVID-19 in India Through a Bi-Phase Detection Approach

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Abstract

Wastewater-based epidemiology (WBE) is increasingly recognized as a valuable tool for monitoring disease cycles, including pandemics like COVID-19. Unlike pandemics, epidemics exhibit distinct dynamics, spread patterns, multiple origin points, and varying levels of population immunity. This study evaluates the applicability of WBE for epidemic monitoring and emergency preparedness by analyzing SARS-CoV-2 presence in Jaipur's wastewater over one year post-second pandemic wave, covering a minor surge (third) and a mild resurgence (fourth) of COVID-19. A total of 1050 samples from different city localities were analyzed using a combination of two concentration methods (the direct method and the Polyethylene Glycol (PEG) method) and two detection kits (qualitative and quantitative). WBE effectively detected both minor and major epidemic outbreak cycles of SARS-CoV-2. A total of 62.91% samples out of all untreated samples tested, were found to be positive with viral genome; however, the positivity rate of any particular day did not exceed 25% even during the peaks. Notably, short-distance transportation under ambient conditions had no significant impact ($p > 0.05$) on detection, and the combination of the direct method with quantitative kits provided the highest sensitivity. Based on these findings, a cost-effective bi-phase surveillance model is proposed for year-round epidemic monitoring. This model suggests routine use of the faster, cheaper direct method, switching to the PEG concentration method during rising viral loads for enhanced resolution. Such an approach ensures sustainable, resource-efficient surveillance, particularly benefiting low- and middle-income countries facing financial constraints.

Keywords: COVID-19; epidemic monitoring; SARS-CoV-2; WBE



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1. Introduction

Wastewater-based epidemiology (WBE) is an approach of monitoring and analyzing wastewater to identify and track the presence of various biological and chemical substances, including pathogens, drugs, and other pollutants. It has become an important tool in public health surveillance, allowing for early detection and tracking of infectious disease outbreaks, drug use trends, and environmental contaminants in communities. WBE has been widely used to monitor the presence of SARS-CoV-2, the virus that causes COVID-19,

in communities [1]. WBE can provide early warning of outbreaks by detecting the virus released in sewage from infected individuals even before they show symptoms or get tested, and it can also help to track the overall prevalence of the virus in a given area. By analyzing wastewater samples from different locations within a community, public health officials can get a better understanding of the geographic distribution of the virus and use this information to guide interventions and prioritize resource allocation [2]. Due to its rapid spread and the number of cases and deaths reported in multiple countries around the world, the World Health Organization (WHO) had declared COVID-19 as a pandemic on 11 March 2020. While some regions or countries may have experienced more severe outbreaks and resultant deaths than others, the global spread of the virus continues to be a public health concern. On 5 May 2023, the WHO declared that the COVID-19 “pandemic” is no longer a Public Health Emergency of International Concern considering the current severity of the disease [3]. However, the virus continues to circulate as an endemic pathogen, with occasional local epidemic surges. As of 19 August 2025, 45,041,748 cases have been reported in India alone (WHO). These recurrent surges—often driven by new variants—highlight the ongoing need for cost-effective, population-level surveillance tools such as WBE, which can provide early warnings and guide timely interventions even in the post-pandemic phase.

WBE has already been validated as an effective approach for monitoring and surveillance of pandemics such as COVID-19. It is noteworthy that SARS-CoV-2 infectivity might persist in wastewater for several hours, with reported T_{90} values of 10.4, 10.8, and 18.3 h in unfiltered raw, filtered raw, and secondary effluent, respectively while the genomic RNA may persist even up to 18 days [4,5]. Several studies have demonstrated that the detection of SARS-CoV-2 in wastewater can serve as an early warning system for the presence of the virus in communities [6,7]. Furthermore, WBE could be a valuable approach for monitoring epidemic cycles, even in the absence of clinical samples detection awareness or when the number of reported cases is low. This is because wastewater can provide an aggregate sample of the population, including both symptomatic and asymptomatic individuals who may not seek medical attention or clinical confirmation of their infection.

WBE has already been shown to detect SARS-CoV-2 in wastewater several days before cases are reported through clinical testing [8]. By monitoring wastewater from multiple locations within a community on a routine basis, public health officials can track the overall prevalence of the virus, estimate the number of infections, and detect changes in the prevalence of the virus over time. WBE has also been used to identify the presence of new variants of SARS-CoV-2 in communities [9,10]. By analyzing wastewater samples, researchers can identify mutations in the viral genome and track the spread of new variants in a population. This guides targeted interventions and carry out resource allocation which helps manage the spread of the virus effectively.

India experienced a devastating second wave of COVID-19 cases between March and May 2021, with a peak in daily new cases exceeding 400,000 and a high death toll [11,12]. However, since then, the number of reported cases and deaths has significantly declined. The reported number of daily new cases, for example, had dropped to below 30,000, by September 2021. However, as of 20 July 2025, the WHO reported that a lower-middle-income country like India was maintaining a rate of 3.3 K COVID-19 cases and 39 deaths per 100 K population. Given the country’s vast population, this translates to a significant and concerning public health burden which must be addressed through all necessary means of preparedness.

Our previous studies showed the importance of early detection of COVID-19 through WBE during the first and second wave in India [13,14]. As of 13 February 2023, according to Indian government figures, India has the second-highest number of confirmed cases in the world (after the United States of America) with 44,685,425 reported cases of COVID-19

infection and the third-highest number of COVID-19 deaths (after the United States and Brazil) totaling 530,753 (covid19 <http://india.org>; accessed on 1 September 2025) [14]. By 5 August 2025, however, number of reported cases had gone down remarkably to 224 nationwide with only six active cases in Rajasthan as per Ministry of Health and Family Welfare, Govt. of India [15]. This decline can be attributed to milder symptoms and home rapid tests, yet the risk of severe outcomes in patients with comorbidities persists. The mechanism and kinetics of spread of this disease have changed from being a pandemic which increases exponentially from a single point to an endemic mixed with occasional bouts of foreign origin variants. The cycles of disease waves have also become more akin to regional epidemic surges. Although decline in the number of active cases can be a positive sign, the disease still prevails, thus warranting development of suitable protocols for a continuous monitoring of sewage samples for forewarning and implementing measures to prevent further outbreaks. This is critical as COVID-19 may endemically be a long-term public health challenge that requires ongoing monitoring and response efforts [16]. The constant presence of a fractionally affected population, together with repeated epidemic outbreak cycles, points to the endemic nature of COVID-19. Although the intensity and severity of the disease have waned, community-wide patterns of spread remain important to be observed.

Since many countries are unable to afford yearlong WBE surveillance either due to lack of infrastructure or enough funds, there is a need for a suitable surveillance model. The present study sets out to accomplish two objectives. Firstly, it aims to validate the WBE based approach for endemic and epidemic surveillance. Secondly, a careful analysis has been performed to find ways of optimizing such surveillance protocols for low- and middle-income country infrastructures, which can be employed continuously in a cost-effective manner. Thus, the paradigm of a tier II city—Jaipur was taken for this purpose. This study spanned through the time points having absence and reported presence of major and minor COVID-19 waves in the city and compared the efficiency of different protocols and detection kits (qualitative or quantitative detection). Based on the findings of this comparison, it is proposed that a bi-phase detection approach can be adapted as a cost-effective method for continued utilization of WBE in monitoring and prediction of endemic or epidemic waves of pathogens even during “off-seasons”. This approach involves a combination of two phases of analysis of wastewater samples. In the proposed first phase, samples are to be directly processed for RNA extraction and qRT-PCR without any pre-processing. While the second phase involves pre-processing the samples by centrifugation, filtration, and PEG adsorption before RNA extraction (Figure 1 in the method section illustrates the details).

2. Experimental Methodology

2.1. Sample Collection and Transportation

In this study, influent samples were collected from ten municipal wastewater treatment plants located in Jaipur, covering approximately 60–70% of the city’s sewerage network, for monitoring SARS-CoV-2 (Table 1). The sampling was performed longitudinally, with samples collected weekly between 8 June 2021 and 8 July 2022. All samples were collected as one-liter grabs in sterile bottles and transported to the Environmental Biotechnology Laboratory at Dr. B. Lal Institute of Biotechnology, Jaipur, for further analysis. During the sampling process, appropriate precautions, including ambient temperature, were taken into consideration, and personnel wore standard personal protective equipment (PPE) throughout the sampling process. Additionally, the samples were stored at 4 °C for 24 h before analysis, as previously described [9].

Table 1. Wastewater treatment plant sampling sites.

S.No.	Sampling Sites	Design Capacity (MLD) ^a	Secondary Treatment ^b	Tertiary Treatment
1	Ralawata STP	30 MLD	ASP	Cl ₂ (hypochlorite)
2	Central Park Garden STP	1 MLD	SBR	Cl ₂ (bleach powder)
3	Ramniwas Garden STP	1 MLD	MBBR	UV
4	MNIT College STP	1 MLD	MBBR	Cl ₂ (hypochlorite)
5	Jawahar Circle Garden STP	1 MLD	MBBR	UV
6	Dravyavati River STP	65 MLD	SBR	Cl ₂ (hypochlorite)
7	Dhelawas STP	65 MLD	ASP	No treatment
8	Paldi Meena STP	3 MLD	SBR	No treatment
9	Jaisinghpura khor STP	50 MLD	SBR	No treatment
10	Brahmpuri STP	27 MLD	SBR	No treatment

^a. MLD = Megaliters per Day; ^b. ASP = Activated Sludge Process, SBR = Sequencing Batch Reactor, and MBBR = Moving Bed Biofilm Reactor.

2.2. Sample Processing for SARS-CoV-2 Detection via RT-qPCR

Samples were processed according to two different methods. For the direct method (namely Method B in the references cited) the protocol described by [13,17] was slightly modified for the isolation of RNA from wastewater samples. After following the initial sterilization steps, 1 mL of the sample was centrifuged at 7500 rpm for 30 min to remove debris and unwanted materials, the supernatant was processed for RNA extraction as per previous [17] protocol. The automated KingFisher™ Flex machine (Thermo Fisher Scientific, Waltham, MA, USA) was used to extract viral RNA from the processed wastewater samples via the MagMAX Viral/Pathogen Nucleic Acid Isolation Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions and internal process control (MS2 phage). The eluted RNA was then stored at −20 °C for further use.

The PEG concentration method (Method A—as dubbed in the reference cited) [13,17] involved surface sterilization of the samples as described followed by manual mixing. The samples were then heat-inactivated at 60 °C for 90 min to eliminate the virus. After heat inactivation, the samples were filtered through a 0.45 µm membrane using a vacuum filter assembly. The filtrate of each sample was then mixed with 0.9 g sodium chloride (NaCl) and 4 g polyethylene glycol (PEG) in fresh sterile 50 mL graduated conical bottom centrifuge tubes by gentle manual mixing. The samples containing PEG and NaCl were then centrifuged at 4 °C for 30 min at 7500 rpm. The resulting pellet was resuspended in 1X Phosphate Buffer Saline (PBS) and then processed for manual RNA extraction. This method of pre-processing using PEG for pelleting down and concentrating the viral particle is considered a standard practice. The total RNA extraction from the concentrated viral sample of sewage was performed manually using the ZymoBIOMICS®96 MagBead DNA/RNA Kit R2136 (Zymo Research Corporation, Irvine, CA, USA) in accordance with the manufacturer's guidelines. The easy MAG extractor stand was used for RNA extraction, and the extracted RNA was eluted in a 100 µL elution buffer [9].

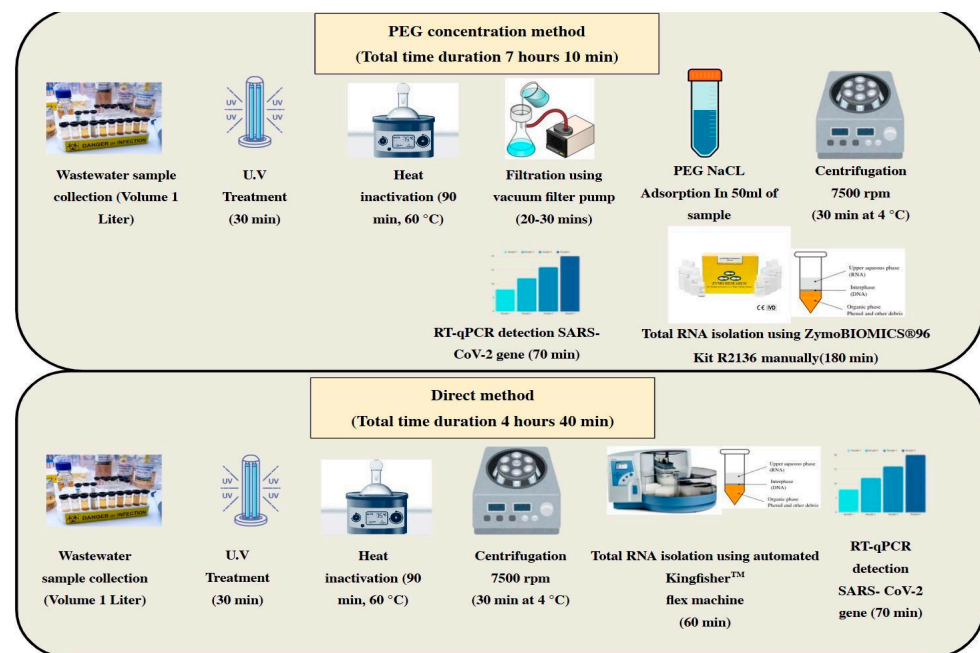


Figure 1. Description of the PEG concentration method and the direct method used for SARS-CoV-2 monitoring.

2.3. RT-PCR Based Qualitative and Quantitative Detection of Viral Genome

The presence and amount of SARS-CoV-2 RNA in the extracted wastewater RNA samples were analyzed through detection of multiple genetic targets either qualitatively or quantitatively using two different commercial RT-PCR kits on a CFX 96 Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). For qualitative detection, Kit 1, Allplex™ 2019-nCoV Assay RT-PCR (Seegene Inc. Seoul, South Korea) was used, targeting the E gene, N gene, and RdRp gene with internal controls on two different fluorophore channels. The RT-PCR reaction mixture was prepared by mixing 11 µL RNA with 14 µL master mix, followed by thermal cycling and detection using Bio-Rad CFX96 Real Time system. The analysis of readings was performed using Bio-Rad CFX Manager Dx software (version 3.1, Bio-Rad Laboratories, Inc., Hercules, CA, USA). The presence of at least two genes (out of three tested namely RdRp, N and E) with Ct values was considered positive.

For quantitative detection, Kit 2, InnoDetect One Step COVID-19 (Innodx Solutions Private Limited, Gurgaon, India), was used. Two different plasmid DNAs were used to prepare a standard curve ranging from 10 pg/µL to 0.01 fg/µL for N gene and ORF1ab gene. The kit used three different fluorophore channels for individual gene identification. The RT-PCR reaction mixture was prepared using the master mix, primer probe, and isolated RNA, followed by thermal cycling and detection. The samples with the quantitative presence of either of the two genes (N or ORF1ab) or both the genes were considered positive. Positive samples were selected for further processing [2,7]. MS2 phage was taken as internal process control, template positive control and template negative controls were set along with every run in qualitative and quantitative detection.

2.4. Statistical Analysis

A paired *t*-Test analysis was performed to evaluate the efficiency of the two methods as well as the number of positive samples detected in two different transportation methods. A two-tailed, unequal variance *t*-Test was performed on the samples collected at the cold chain and the ambient normal temperatures using MS excel for statistical analysis. The data analysis package of the Excel in MS office 2007 was used to calculate *p* values.

3. Results and Discussion

3.1. WBE Approach Could Catch Epidemic Cycles Even in Absence of Clinical Detection

While WBE has reliably provided early warnings during the COVID-19 pandemic, its effectiveness under post-pandemic conditions—where localized epidemic cycles dominate—requires further examination. Since the pattern of epidemic is different from that of a pandemic in terms of origin of the infection, kinetics and the intensity of spread; the approaches to detect these two, might be different. Epidemics usually have multiple focal points of origin, and the disease spreads radially through the community from those points. In contrast, pandemics typically have a single point of origin and spread globally. This means that monitoring for epidemics may require a more localized approach, while monitoring for pandemics may require a more global approach. The agents that cause epidemics are often not new to the population, and there may be some level of acquired immunity in the community. This can result in lower peak infections and less severe outbreaks compared to pandemics. The goals of monitoring epidemics may differ from those of monitoring pandemics. For example, in an epidemic, the priority may be to quickly identify and contain the outbreak preventing it from spreading. In a pandemic, however, the goal may be to track the spread and kinetics of the disease globally and accordingly develop interventions to mitigate the impact of the disease on public health and society. Overall, the approaches to detecting and monitoring epidemics and pandemics may differ based on the unique characteristics of each type of outbreak.

The present study aimed to investigate whether WBE is capable of detecting the dynamics of a typical epidemic. To achieve this, wastewater samples were collected weekly from different locations in the city from August 2021 to June 2022. This period covered the monitoring of wastewater samples after the second wave of COVID-19 had declined in the city. By analyzing the wastewater samples during this period, this study aimed to identify any potential epidemic cycles or trends that could be useful for monitoring and controlling the spread of COVID-19 in the city.

The majority of the population in the city where this study was conducted had either been vaccinated or had developed immunity due to prior infection with COVID-19. As a result, there was a decrease in the severity of symptoms and hospitalizations, leading to a relaxation of government regulations and a decrease in the necessity of testing. The use of home testing kits was also allowed, which further led to a decrease in reported cases. However, in order to investigate the sensitivity of WBE for epidemic monitoring, the presence of viral genome was checked in wastewater samples. As shown in Figure 2, this study found that there were two minor waves of COVID-19 spread, with a low third wave observed between December 2021 to February 2022, and a minor spike observed in August to September 2021. These waves were not publicly reported by the city health care system. Although the caseload did increase during these waves, the proportion of positive untreated wastewater samples collected citywide on any given day, did not even exceed 25%. Furthermore, the overall intensity was much lower than that of the second wave, and there was a decrease in mortality and genome load (Supplementary Table S1). This decrease in intensity could be in part attributed to a wide-scale vaccination drive which had ensured that millions of residents had received at least one dose of the vaccine before December 2021 and the use of rapid home testing kits, which could have led to decreased testing and reporting.

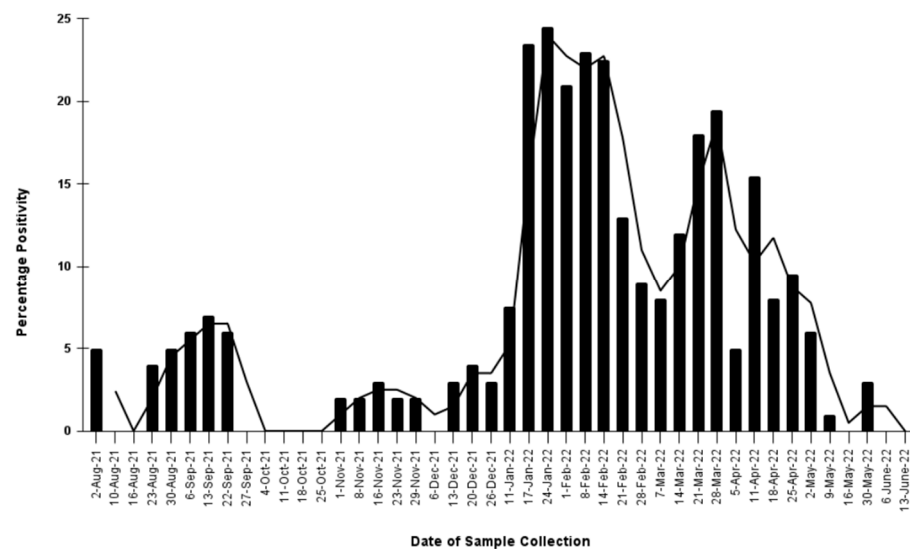


Figure 2. Percentage positivity showing minor to low rate of infection increase across the city. Each bar depicts the percentage of sites tested positive on the given date in the city. The solid line represents the moving average.

3.2. Different Phases of the Epidemic Cycle Showed Differences in Target Detection

It is possible that detection of target sequences by RT-qPCR in the WBE approach could vary during different phases of epidemic cycles. This could be owing to factors such as changes in the prevalence and distribution of the virus in the population, differences in the viral load shed in feces during different stages of the disease, and differences in the performance of the RT-qPCR assay over time [18]. However, more research is required to confirm if this is the case.

As mentioned above, this study collected wastewater samples from various sites (Table 1) in the city over a period of time when the second wave of COVID-19 had subsided and post-vaccination drive. Out of 755 untreated wastewater samples tested, 475 were found to be positive for COVID-19 viral genome. The highest log values observed during the windows of third and fourth waves were in order of 10^8 copies/L while the lowest were in order of 10^4 copies/L. Different local infection wave patterns were observed in different communities, and the WBE approach was capable of distinguishing local, sub-city, or community-level disease spread cycles. The details of positive samples detected are given in Table 1.

Like a typical epidemic, several local infection wave patterns were observed in different communities as shown in Figure 3. It was observed that although the third wave of COVID-19 showed its presence all across the city, the minor spike observed during August and September did not spread evenly to all localities of the city. For example, Sites 1, 2 and 6 remained unaffected by this spike while sites 3 and 10 were only minimally affected. This pattern was different from that of sites 4, 5, 7, 8 and 9 where the spike was very consistently detected. This result underscores the local exposures and viral spreads in the city since each of these sites caters to a different catchment area. Interestingly, although the sites showed similar epidemic patterns they are not geographically adjacent (Figure 3). This pattern clearly shows that WBE is capable of distinguishing local, or community level disease spread cycles and is not limited to city hotspot detection.

In the RT-PCR testing, it was observed that during a minor third and a mild fourth wave in the city all the tested targets did not show the same detection pattern during the span of wastewater monitoring. Some genetic targets like ORF1ab and N were detected during more phases of epidemic cycle than others like E, which showed less detection during the period between two different waves or RdRP, which was not detected in any

phase except for the peak duration of the fourth wave in the city (Figure 4). This observation may be explained easily by the sensitivity of different targets used; alternatively, this pattern may also be an indicator of some key variables affecting the kinetics of pathogen spread as all the targets belong to genes, which have different functions during the viral life cycle and thus may have different degradation kinetics [19]. Regardless of the aforementioned reason, the data highlight the importance of checking for multiple target regions to get a comprehensive picture from the wastewater monitoring.

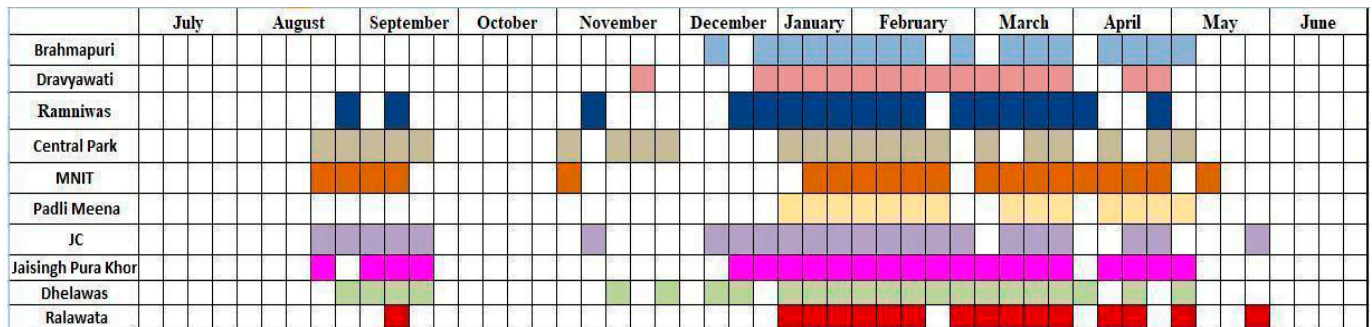


Figure 3. Heat map showing site-wise viral detection during the study window. Different colors depict site-wise positivity over the year.

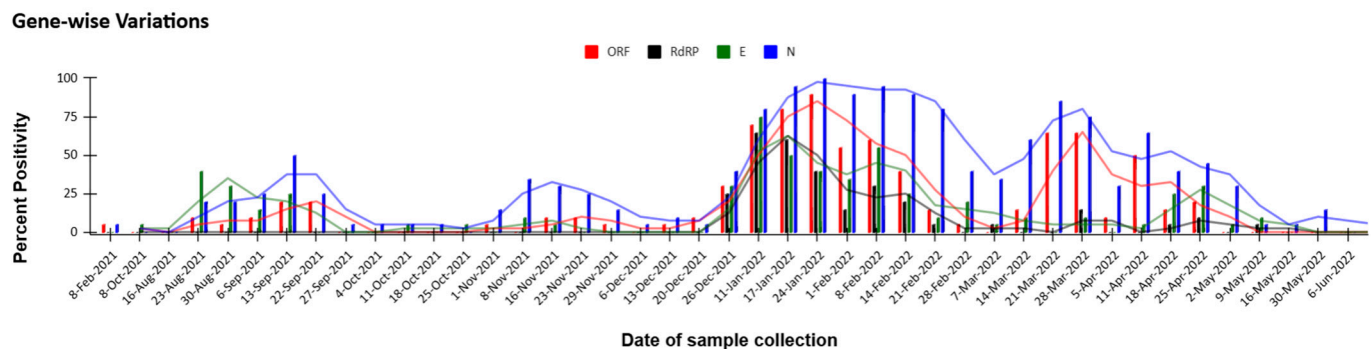


Figure 4. Variation in detection of genes during various phases of epidemic in community. The Y axis shows the number of positive sites for the given gene on a given date. Solid lines of colors depicting respective genes represent the moving averages.

Although, in contrast to the patients' testing, where the presence of multiple positive targets or Ct values lower than 35 is considered to be a good cut-off for considering a sample as positive, the detection of even one target gene in wastewater should be considered a "low infection number" possibility in the community. It has been observed that during the rise and peak of infection waves, more than one target may be detected by RT-PCR, while samples collected from time points right after these phases and until the next rise usually tested positive for only one target. This indicates that there could be a possibility that any wastewater sample from a community with a low number of infected people might only be retaining degraded or very diluted representation of pathogen genomes in the community wastewater, thus making it easy to miss intact genomes during collection [19].

Although monitoring is only useful on untreated wastewater from a community, this study looked at the presence of genome load in treated wastewater as an indicator of health risk. It was observed that as compared to previously reported data [2,13], there were more numbers of effluent samples detected positive with the genome load of SARS-CoV-2 than observed during initial phases of COVID-19 spread. It was observed that as compared to influent samples where N and ORF genes could be quantified in 29.89% and 18.59%, respectively; percentages of positive effluents were less (25.48% and 15.71%, respectively).

One possible reason for such observation could be the tendency of viral particles or genome copies to show retention at various steps of the treatment plants leading to their slow release at later time [20]. Given the absence of evidence for COVID-19 transmission via treated wastewater containing viral genomes, the potential health risk posed by SARS-CoV-2 in such contexts appears minimal. However, this is an observation worth exploring in case of other pathogenic causal agents of epidemics which might be present in treated wastewater.

3.3. Impact of Kit Sensitivity and Pre-Processing Methods on Target Detection

As discussed in Section 3.2, a difference in target detection was observed during different phases. These observations raise the question about the influence of other variables in this detection. In order to investigate this, we considered variables like target sensitivity and the impact of PCR inhibition (owing to a difference in the pre-processing treatments). Two different RT-PCR kits were employed targeting four different genes overall (Kit 1 = E gene, RdRp gene and N gene; Kit 2 = N gene, ORF gene with N gene as a common target). Both kits were applied to samples processed using the two pre-processing approaches described in the Methods section. It is expected that a sample where viral particles are concentrated will give better yields and will be overall a more sensitive approach for detection of viral presence. It was, however, observed that the percentage positivity of the samples detected by either approach was not significantly different.

Although the PEG method (which included concentration of the sample) detected a mere 2% more positive samples per total tested sample (which was statistically insignificant) as compared to the direct method; two interesting observations were made in the data. Firstly, there is a log reduction of an order of 10^1 to 10^2 in viral copy numbers detected per liter in the samples processed through the PEG concentration method. Secondly, it was observed that whenever there was a period of low case reports in the city the direct method (which did not include concentration) seemed to be detecting better than the PEG concentration method which failed to detect viral presence despite concentration of the sample (Figure 5). This observation may be explained by either of two factors: (a) additional filtration steps used to remove gravel and grit may also have removed viral particles, or (b) concentration of the sample by adsorption may have co-concentrated inhibitors, which interfered with RT-PCR detection when the inhibitor-to-genome ratio was high. Since the direct method showed better detection at low caseload time points, it was important to understand whether this behavior was somehow affecting the detection pattern of target genes observed (Figure 4). Upon looking at the specific method wise gene detection it was found that the observation differences were not limited to just the gene target tested but the pattern of detection was also unique for each Method for every gene (Figure 6). These findings highlight a critical insight in wastewater-based epidemiology. The results indicate that assessing the effectiveness of a detection method should not be solely based on percent positivity (i.e., number of positive samples / 100 samples tested). Here, overall PEG concentration showed slightly higher positivity from that of the samples pre-processed by the direct method. However, as can be observed by Figures 5 and 6, sensitivity of the direct method was more in the absence of peak sessions of the disease waves.

Moreover, although the quantitative kit (Kit 2) was overall more sensitive, samples pre-processed using the direct method during periods of lower community caseload were most consistently detected with this kit. (Figure 5). This indicates that the choice of kit as well as pre-processing approach can have an impact on the detection of viral presence in wastewater.

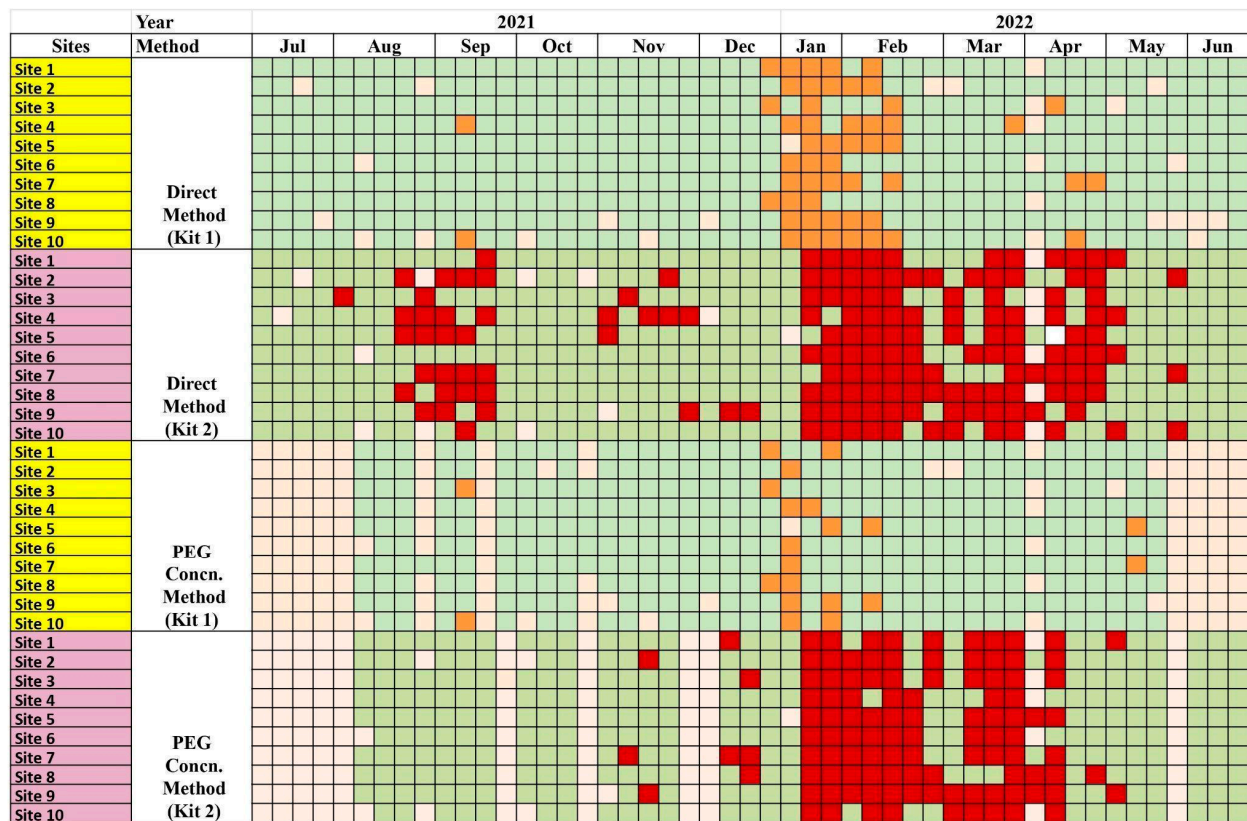


Figure 5. Heat map depicting the variations in target detections in various combinations of pre-processing and kits used. Top 20 rows show samples pre-processed by the direct method and last 20 rows show detection in PEG concentration method-processed samples. Green cells represent samples which tested negative. Orange and maroon depict the samples testing positive for Kit 1 and Kit 2, respectively. Peach cells are points that were not tested.

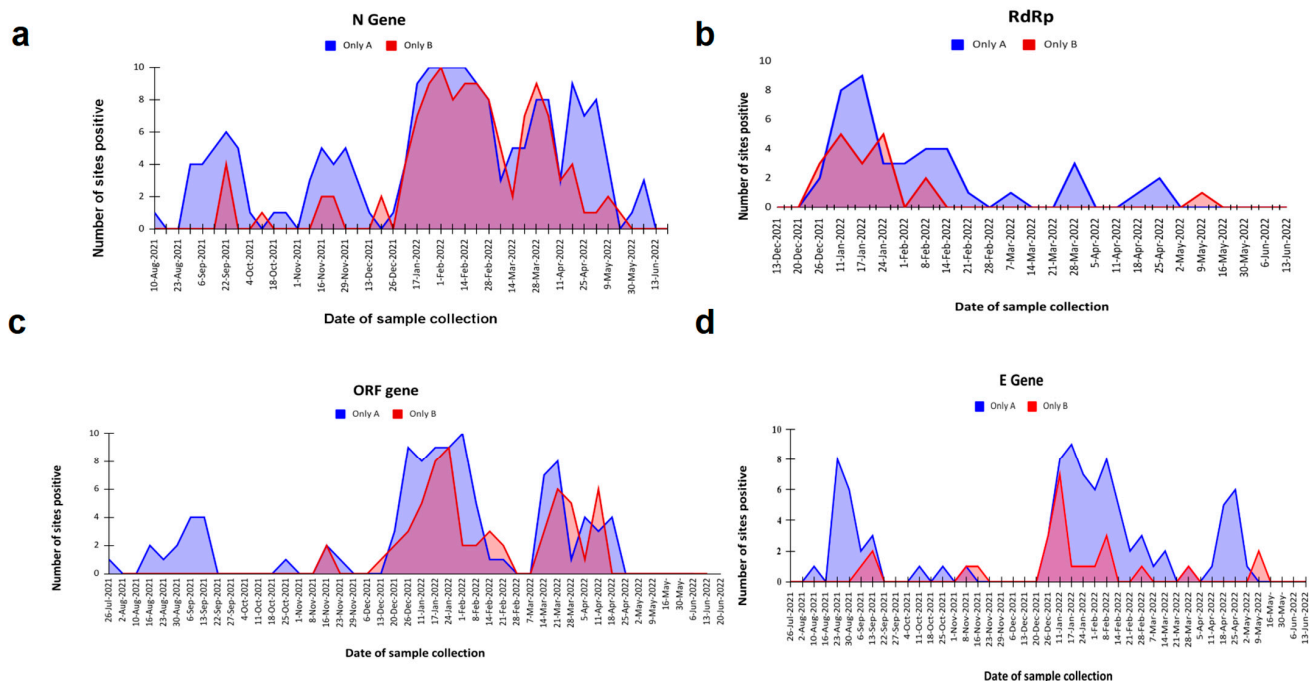


Figure 6. (a–d): Graphs showing the number of sites in which the target gene tested positive by only the direct method or only through the PEG concentration method (depicted in blue and pink, respectively).

3.4. Local Monitoring Could Potentially Eliminate the Need for Cold Chain Transport

Cold chain transportation is another critical variable influencing the detection [21] in WBE surveillance, and is therefore a standard practice for collecting and transporting the samples related to wastewater-based epidemiology. However, to maintain a cold chain transport is an investment in itself and may add up to a large amount in day-to-day operations, so it should be acknowledged that this practice can incur substantial costs. Since this study focused on variables affecting the regular monitoring of the endemic, cold chain transport, which is currently recognized as a crucial factor in ensuring precise COVID-19 detection, was also investigated. Opportunistically, this study is based in Rajasthan, where environmental conditions exhibit extreme high ambient temperatures. Our investigation thus examined whether transporting samples at ambient temperature, instead of under cold chain conditions, compromises the sensitivity of COVID-19 detection.

To check this, we designed a sampling protocol encompassing all the sites described above, (Table 1). Samples were collected together; however, one set of sample containers was transferred into refrigerated boxes immediately after noting the sample temperature while the other set was kept at the ambient temperatures for the transportation. Upon collection, the samples' temperature was immediately recorded on-site (29 °C to 34 °C). Subsequently, temperature measurements were again acquired upon the samples' arrival at the Environmental Biotechnology Laboratory, located at Dr. B. Lal Institute of Biotechnology, Jaipur, followed by transportation through both cold-chain (which was recorded between 23 °C to 32.5 °C) and ambient temperature methods (which was 29 °C to 34 °C). The ensuing procedures remained consistent with the aforementioned methodology.

Both sets of samples were processed similarly and tested for positivity. Interestingly, experimental results indicated that there was no statistically significant difference between the number of positive samples between two methods of transportation when the samples were transported locally (Table 2 and Figure 7a,b). Both methods showed similar performance in terms of positivity rate and viral load (log values). A *t*-test comparing the number of positive samples from cold chain versus ambient temperature transport yielded a *p* value of 0.70, exceeding the significance threshold of 0.05 (Supplementary Table S2).

Table 2. Results of comparison of cold chain vs. normal (ambient) temperature transport. The numbers represent the samples tested positive out of the total samples tested.

Out of 204 Samples Tested (Each)	Cold Chain Transportation	Normal-Temperature Transportation
Total positive samples (Direct and PEG)	41	37
Positive samples detected in both the methods	16	19
Samples testing positive only in PEG method	7	6
Samples testing positive only in direct method	18	12

Based on the study findings, it is proposed that since there is no significant difference in the positivity of sample transportation between the cold chain and normal-temperature methods protocols in distances within a city, using the ambient temperature transport method should be adapted as a cost-efficient alternative to the cold chain approach. This would increase the affordability of WBE for the cities which currently do not have elaborate sampling and processing setups for such monitoring.

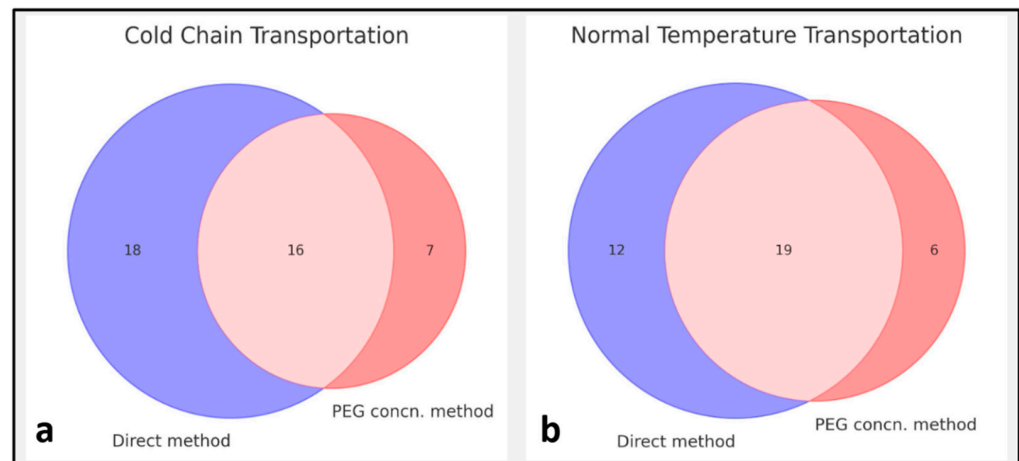


Figure 7. Venn diagrams showing the number of positive samples in (a). Cold chain transportation or (b). transportation at ambient temperature. Blue shows positive in only the direct method, red depicts positive only in the PEG concentration method and peach shows the number of samples testing positive in both the direct and PEG concentration methods.

3.5. Epidemic Monitoring Can Be Pragmatically and Cost-Effectively Applied as a Bi-Phase Model

This investigation aimed at developing a pragmatic protocol that is sustainable for low- and middle-income countries and the countries with underdeveloped infrastructure and resources towards yearlong continuous pathogen monitoring via WBE. Several combinations of steps and variables were assessed to understand and propose the optimal step by step procedure. Since the previous report [13] and results discussed above indicate that there was no significant difference in the detection based on percentage positivity between the two methods of sample pre-processing and that they perform nearly the same, cost calculation was performed for both the approaches. Considering the only difference in these approaches was in pre-processing steps and semi-automation for RNA extraction the cost of these two were compared. It was found that pre-processing of samples using the PEG concentration method alone could take the cost of this approach to more than 330 times that of the direct method.

Given that the PEG concentration method is only ever so slightly better (39.54% vs. 37.35% by the direct method) in target detection it does not seem to be a pragmatic yearlong epidemic monitoring approach for any country where resources are limited and can be better utilized. Further the cost incurred (additional 2–3% to reagents cost) in maintaining a cold chain transport within local distances may be optional and invested only when more sensitivity in detection needs to be ensured. Since epidemics are cyclic and rise from within the community, yearlong monitoring is also equally vital for disease management. Therefore, it is proposed that WBE is used as a monitoring system in parallel to the sentinel system, using the pre-processing steps of the direct method during the “off sessions”, i.e., when detection is either absent or is sparse. PEG concentration procedure can thus be employed as soon as first signs of epidemic detection as an “urgent response protocol” against the epidemic on rise. This will allow the resource limited regions to afford continuous low cost monitoring while reserving the resources only to be employed during the surge waves of infection. Since this strategy will allow yearlong monitoring through WBE, this strategy will not be dependent on patient cohorts to show symptoms or to be reported to hospitals or sentinel clinics. Additionally, looking out for more than one target in a pathogen from community wastewater will ensure an early detection which is the major advantage of using the WBE (Figure 4). This bi-phase monitoring approach will give a comprehensive epidemic monitoring in a cost-efficient manner.

Therefore, this study suggests using the direct method, which is sensitive for low pathogen presence, for year-long monitoring of wastewater as a parallel system to the sentinel system, and switching to the PEG concentration method, which gives good positivity rate during the rising waves; as an “urgent response protocol” during epidemics. This bi-phase monitoring approach will provide a cost-efficient and comprehensive epidemic monitoring system.

4. Conclusions

This study demonstrates that wastewater-based epidemiology (WBE) is a cost-effective, practical strategy for epidemic monitoring, with particular applicability in low- and middle-income countries (LMICs). By comparing two pre-processing methods and detection kits, we found that combining the direct method with a quantitative kit provides the highest sensitivity for SARS-CoV-2 detection. Importantly, short-distance ambient transport did not significantly reduce detection rates compared to cold chain transport, offering a feasible option to reduce infrastructure costs in resource-limited settings.

Based on these findings, we propose a bi-phase surveillance framework: routine monitoring with the faster, lower-cost direct method, and a switch to the more sensitive PEG concentration method when rising sample positivity or genome loads indicate a potential outbreak. This approach captures both minor fluctuations and major epidemic waves, and allows timely, targeted allocation of resources. Distinguishing between epidemic and pandemic dynamics such as differing spread patterns, multiple origin points, and varied immunity levels has proved to be essential in interpreting results and tailoring interventions.

The proposed framework in this study offers a scalable, adaptable model for sustainable year-round WBE surveillance, enabling public health systems to respond rapidly to emerging threats while optimizing limited resources. This study thus shows that a simple, cost-effective bi-phase WBE approach can reliably track epidemic trends, even in resource-limited settings.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/covid5090147/s1>, Table S1: COVID-19 intensity comparison to second wave; Table S2: Raw data of cold chain vs. normal temperature transportation.

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Conflicts of Interest: Authors Tamanna Pamnani, Komal Sharma and Sandeep K. Shrivastava are affiliated with Dr. B. Lal Clinical Laboratory Pvt. Ltd. The authors declare that there are no conflicts of interest.

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