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Protocol for detection of pathogenic enteric RNA viruses by regular monitoring of environmental samples from wastewater treatment plants using droplet digital PCR

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ABSTRACT

Background: The present comprehensive protocol is focused on the detection of pathogenic enteric RNA viruses, explicitly focusing on norovirus genogroup II (GII), astrovirus, rotavirus, Aichi virus, sapovirus, hepatitis A and E viruses in wastewater treatment plants through droplet digital PCR (ddPCR). Enteric viruses are of significant public health concern, as they are the leading cause of diseases like gastroenteritis. Regular monitoring of environmental samples, particularly from wastewater treatment plants, is crucial for early detection and control of these viruses. This research aims to improve the understanding of the prevalence and dynamics of enteric viruses in urban India and will serve as a model for similar studies in other regions. Our protocol's objective is to establish a novel ddPCR-based methodology for the detection and molecular characterization of enteric viruses present in wastewater samples sourced from Bhopal, India. Our assay is capable of accurately quantifying virus concentrations without standard curves, minimizing extensive optimization, and enhancing sensitivity and precision, especially for low-abundance targets.

Methods: The study involves fortnightly collecting and analyzing samples from nine wastewater treatment plants over two years, ensuring comprehensive coverage and consistent data. Our study innovatively applies ddPCR to simultaneously detect and quantify enteric viruses in wastewater, a more advanced technique. Additionally, we will employ next-generation sequencing for detailed viral genome identification in samples tested positive for pathogenic viruses.

Conclusion: This study will aid in understanding these viruses' genetic diversity and mutation rates, which is crucial for developing tailored intervention strategies. The findings will be instrumental in shaping public health responses and improving epidemiological surveillance, especially in localities heaving sewage networks.

1. Introduction

Diarrhea is the world's second leading cause of child malnutrition and mortality due to contaminated food and water sources. With an estimated 1.7 billion instances of childhood diarrhea, which result in the deaths of almost 525,000 children under the age of five, diarrheal illnesses remain a danger to this age group [1]. Viral diseases are the most frequent cause of diarrhea in children. Enteric viruses refer to the group of viruses that

Abbreviations: ddPCR, droplet digital PCR; GII, norovirus genogroup II; WWTPs, wastewater treatment plants; dsRNA, double-stranded RNA; qPCR, quantitative PCR; PEG, polyethylene glycol; PBS, phosphate buffer saline; NCBI, National Center for Biotechnology Information; NSP3, non-structural protein 3; RdRp, RNA-dependent RNA polymerase; HAV, hepatitis A virus; HEV, hepatitis E virus; RT-RAA, reverse transcription-recombinase aided amplification; LAMP, loop-mediated isothermal amplification.

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are competent to replicate in the gastrointestinal tract of humans or animals. When infected individuals release these viruses through their stool and sometimes urine, they can contaminate water sources. With over 200 known enteric viruses, 140 serotypes are known to infect humans [2]. Enteric viruses are responsible for causing a range of illnesses. These viruses can also induce severe acute conditions like meningitis, poliomyelitis, and non-specific febrile infections. Moreover, these viruses have been connected to persistent conditions such as diabetes mellitus and chronic fatigue syndrome [3].

Waterborne illnesses are predominantly caused by viral agents such as hepatitis A and E viruses, rotaviruses, enteric adenoviruses, caliciviruses (norovirus and sapovirus), astroviruses, and enteroviruses [4–6]. Historically, rotavirus has been the predominant cause of gastroenteritis in children under five globally [7,8]. Rotavirus infections contribute to 24 % of diarrhea cases in children under 23 months and 13 % in those aged 24–59 months [9,10]. Noroviruses cause nearly 700 million cases of gastroenteritis each year worldwide, resulting in approximately 200,000 annual deaths, primarily impacting children under five in developing countries. The introduction of rotavirus vaccines, Rotateq® and Rotarix®, in 2006 have led to a substantial decrease in the incidence of rotavirus-associated diarrhea in high-income countries [2,4,5]. However, studies from Brazil, USA, and Australia have described changes in the prevalence of serotypes as a result of post-rotavirus vaccination. Furthermore, the rotavirus vaccination has changed the etiology of viral diarrhea. In the USA and other high-income countries, norovirus is the predominant cause of diarrhea in children under five [6,11]. These observations reiterate the dynamic nature of viral gastroenteritis epidemiology and warrant monitoring this syndrome's epidemiology post-vaccination in a geographical area. Noroviruses have acquired significant attention within public health, particularly the norovirus genogroup II (GII), recognized as a predominant causative agent of acute gastroenteritis across global population. Infections with norovirus can vary in severity, but they often result in a debilitating combination of vomiting, diarrhea, and abdominal pain. With its ability to spread rapidly, primarily through fecal-oral routes, contaminated food or water, and even person-to-person, the need for efficient and early detection mechanisms has never been more paramount.

The environmental persistence of the norovirus, combined with its low infectious dose, contributes to its widespread transmission [12]. One primary avenue for its dissemination, often overlooked, is wastewater [13,14]. As infected individuals shed significant quantities of the virus in feces and urine, wastewater systems inadvertently become reservoirs for these pathogens. This poses a substantial risk, especially in consideration of inadequately treated wastewater or sewage overflows, which may contaminate freshwater sources and subsequently reach large human populations [14]. Consequently, wastewater treatment plants (WWTPs) present themselves not only as potential hotspots for norovirus prevalence but also as invaluable sites for surveillance and early outbreak detection [14,15].

Detecting double-stranded RNA (dsRNA) viruses in environmental samples using quantitative PCR (qPCR) techniques is challenging due to denaturation, reverse transcription, and amplification complexities, compounded by inhibitor presence [16]. Conventional PCR methods for detecting viruses in wastewater samples are often insufficient in terms of sensitivity and accuracy, particularly when viral loads are low but still infectious [17,18]. Such limitations emphasize the need for more refined detection technologies that can provide quantifiable, reliable, and rapid results.

Droplet digital PCR (ddPCR) is a next-generation molecular detection technique that works by partitioning a sample into thousands of droplets, followed by PCR amplification within each droplet. The strength of ddPCR lies in its ability to offer absolute quantification, a feat achieved without necessitating standard curves [19]. This quality, combined with its enhanced sensitivity and precision, makes it particularly well-suited for detecting low abundance targets like the enteroviruses in environmental samples. Therefore, we would like to develop a protocol for detecting pathogenic enteric RNA viruses by regularly monitoring environmental samples from WWTPs using ddPCR with the following objectives:

(a) To establish a method of determination of pathogenic enteric viruses in effluent samples of WWTP using a novel ddPCR technology.
(b) To evaluate the circulating patterns and dynamics of pathogenic enteric RNA virus genotype distribution in Bhopal city (as a model).
(c) To establish a prototype model system for examining genetic readiness by monitoring episodic outbreaks of circulating enteric RNA virus genotypes.
(d) To explore the possibility of developing a surveillance system based on fortnightly analysis of such samples drawn from the nine different WWTPs of the city with heterogeneous effluent sources.

2. Methods

2.1. Site selection and rationalization

Our primary research setting will be Bhopal. The samples will be collected from functional WWTPs under Bhopal Municipal Corporation. Bhopal is capital of the state of Madhya Pradesh and is one of the biggest municipal corporations with a higher population density than most other central Indian cities. All the facilities and expertise needed to carry out this project are available in the institute, making the project execution more feasible.

2.2. Waste water sample collection

Fortnightly, 50 mL sewage samples from each of the nine WWTPs in the Bhopal, Madhya Pradesh Municipal Corporation area will be collected in polypropylene bottles in duplicates (Fig. 1) [13]. These WWTPs have a well-defined catchment area (Fig. 3) and are located in the following locations: Chuna Bhatti Charimli, Neelbad, Maholi Damkheda, Shirin River, Sankhedi, Maksi, Professor Colony, and Jamuniya Cheer region (Fig. 2). In order to prevent additional degradation of viral nucleic acid, samples taken from these regions will be delivered to the lab and processed for RNA isolation immediately, or refrigerated at 4 °C for processing within 24 h.

2.3. Sample processing and virus concentration by polyethylene glycol (PEG) precipitation

Virus concentration will be done using modified PEG method [20–22]. The sewage samples (50 mL) will be centrifuged at 4500×*g* for 30 min, followed by heat inactivation at (60 ± 2) °C for 90 min. The supernatant will be filtered using membrane filters (pore size: 0.22 μm), and the pH of the supernatant will be adjusted to 7–7.5. The supernatant will be then mixed with 10 % PEG 8000 and 2 % NaCl to reach a final concentration of 1 mol/L. After an overnight incubation at 4 °C, the mixture will be centrifuged for 30 min at 4500×*g*. The pellet obtained will be completely re-suspended in 500 μL of phosphate buffer saline (PBS) solution as a viral concentrate, and the supernatant will be discarded. Before extraction, the virus concentrate would either be prepared for nucleic acid extraction or kept at −80 °C for long-term storage.

2.3.1. RNA extraction and quantification

RNA will be isolated from the virus concentrate using the All Prep Power Viral DNA/RNA Kit (Qiagen, Germany) based on the guanidine isothiocyanate method. The All Prep Power Viral DNA/RNA Kit from Qiagen is designed to extract viral DNA and RNA efficiently and rapidly from various types of samples. Viral RNA will be specifically bound to a

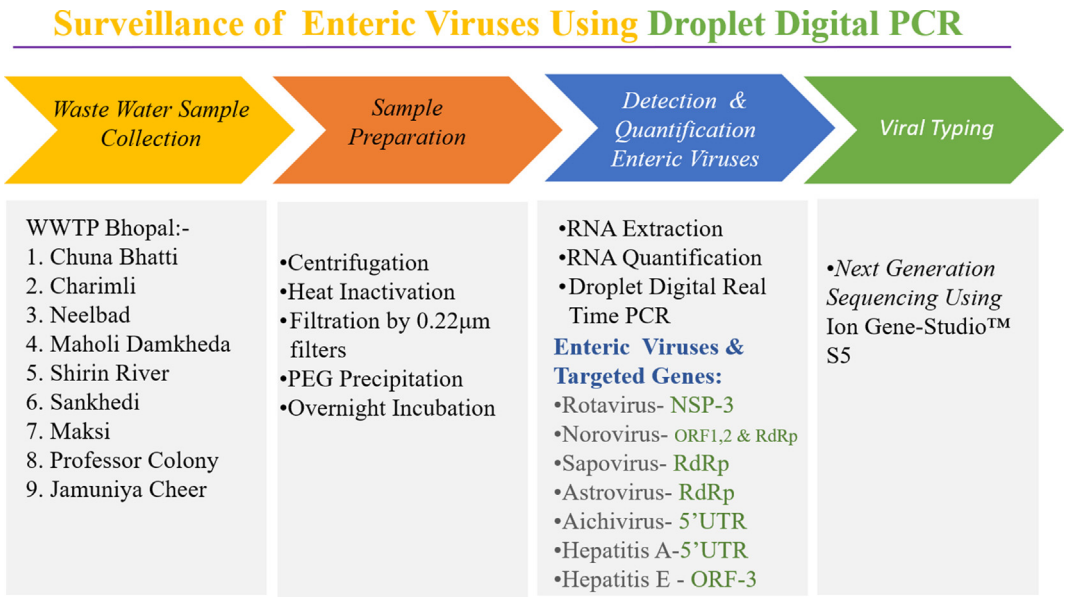

Fig. 1. Proposed workflow for the surveillance of enteric viruses using droplet digital PCR. Abbreviations: WWTP, wastewater treatment plant; PEG, polyethylene glycol; NSP, non-structural protein; ORF: open reading frame; RdRp, RNA-dependent RNA polymerase; UTR, untranslated regions.

silica-gel membrane, and PCR inhibitors like divalent cations and proteins will be effectively removed during the washing stages [23,24]. This kit typically includes a range of components such as lysis buffers to break open cells (PM1) and viruses, binding buffers (PM3 and PM4) that allow nucleic acids to adhere to a solid phase for purification, wash buffers (PM5) to remove impurities, and elution buffers to release the purified DNA/RNA. Additionally, the kit contains collection tubes, spin columns with magnetic beads (MB spin column) for the extraction process, and enzymes (possibly proteinase K) to aid in the breakdown of proteins and facilitate nucleic acid release. Viral RNA will bind specifically to the silica-gel membrane of the MB spin column while contaminants pass through. All PCR inhibitors, including divalent cations and proteins, will be removed during washing processes, leaving only pure viral RNA that may be eluted in water or a buffer included within the kit by following the manufacturer's recommendations. To extract RNA, lysate pellet dissolved in PBS will be added to a solution of PM1 containing β-mercaptoethanol in a 1.5 mL microcentrifuge tube and incubated for 10 min. After centrifugation, the RNA-containing supernatant will be collected and mixed with the IRS solution. Next, the sample will be transferred to an MB spin column and washed with PM4 and PM5 solutions. After elution, the RNA present in the flow-through will be stored at −80 °C for subsequent use later. The quality and concentration of the extracted RNA will be evaluated by determining the absorbance at 260 nm using the Multiskan Sky High Spectrophotometer (Thermo Scientific, USA). The purity of the RNA will be ascertained by observing the 260/280 absorbance ratio.

2.3.2. *Droplet digital real-time PCR*

RNA samples will be reverse-transcribed to identify and quantify the norovirus GII by targeting virus-specific genes and internal process control by droplet digital real-time PCR machine (QX200 BioRad, USA) using One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad, USA), as per the manufacturer's protocol (Fig. 4). The sequences of the primers and probes are derived from previously published articles (Table 1). The primers and probes were aligned with nucleotide sequences available at National Center for Biotechnology Information (NCBI) using the BLAST program for validation. The primers and probes sharing maximum homology with Asian enteric RNA virus isolates, targeting the highly

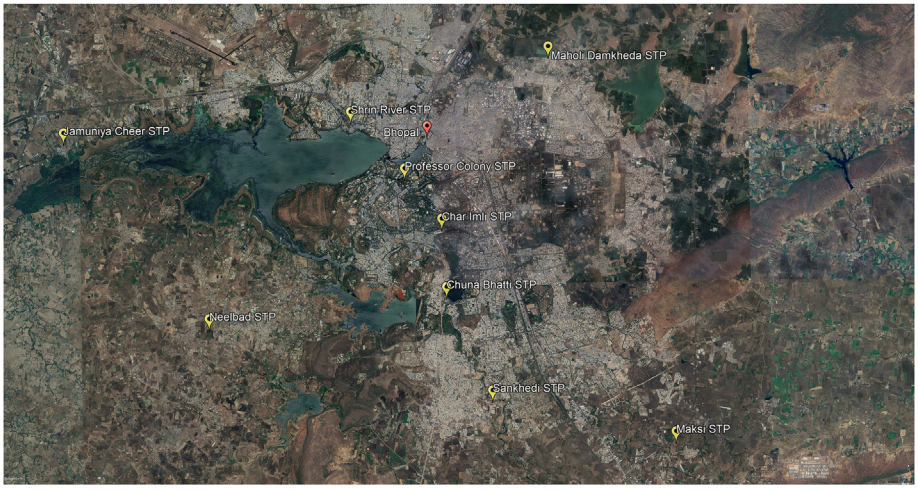

Fig. 2. Google map showing the location of WWTPs in the Bhopal district. Map source: Google Earth Map. Abbreviations: WWTPs, waste water treatment plants; STP, sewage treatment plant.

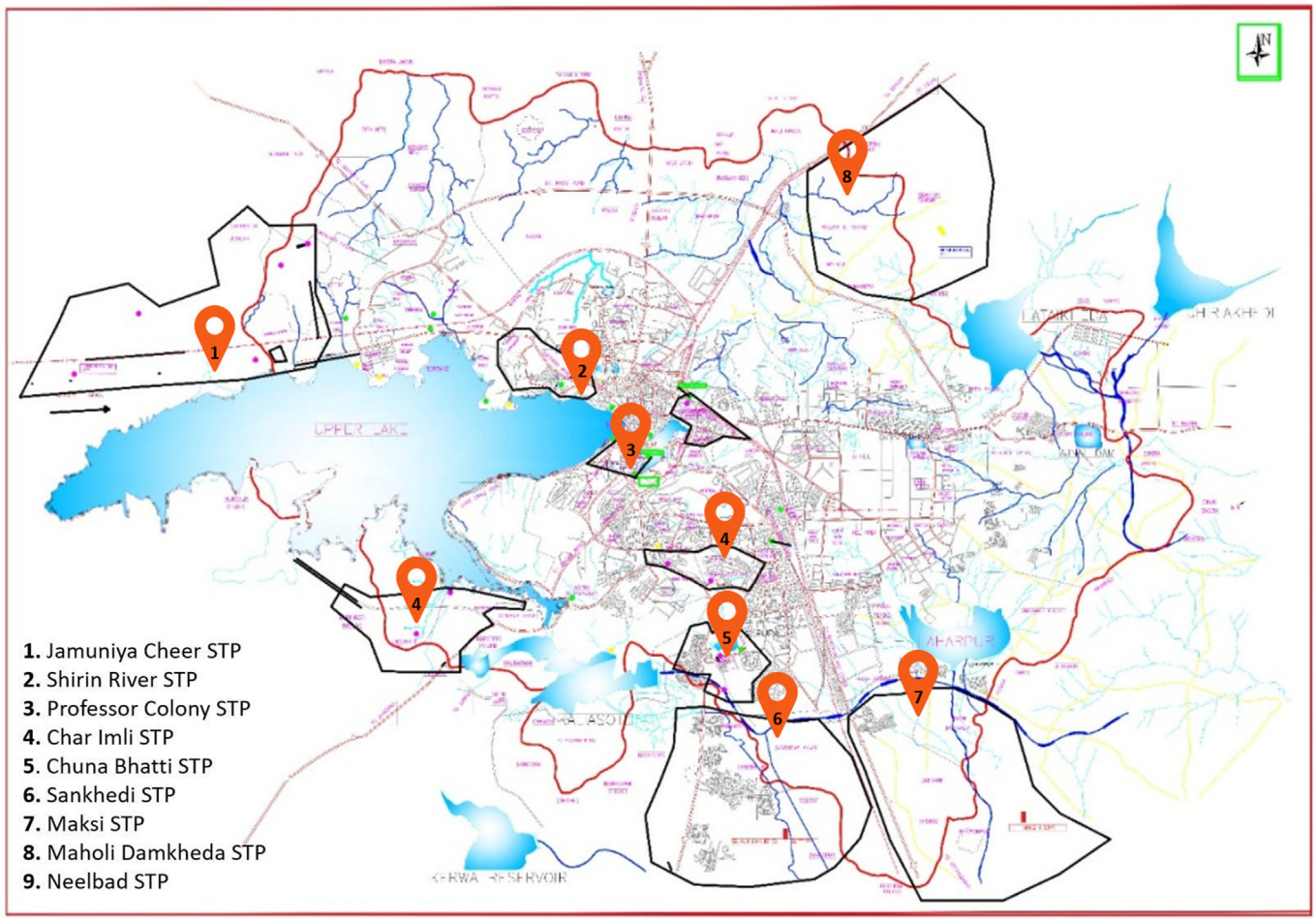

Fig. 3. Map showing the catchment area of WWTPs in Bhopal district. Map source: Office of Bhopal Municipal Corporation. Bhopal. Abbreviations: WWTPs, waste water treatment plants; STP, sewage treatment plant.

conserved regions, were selected. The TaqMan probe was labeled with 5′-FAM (a fluorescent reporter dye) and 3′-BHQ-1 (a quencher). RNAs will be analyzed for the presence and quantification of seven pathogenic enteric viruses (hepatitis A & E viruses, rotavirus, astrovirus, norovirus, sapovirus, and Aichi virus) through targeting virus-specific genes and internal process control by ddPCR machine (QX200 BioRad, USA) using One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad, USA), as per the manufacturer's protocol (Fig. 4). For rotavirus, non-structural protein 3 (NSP3) gene region is targeted [25]. Sapovirus and astrovirus will be detected by targeting the RNA-dependent RNA polymerase (RdRp) in ddPCR assay [26,27]. Detection of hepatitis A virus (HAV) and Aichi virus will be done by targeting the 5′ untranslated regions (UTR) [28,29] and hepatitis E virus (HEV) via open reading frame 3 (ORF3) region [30], respectively. After the PCR run, the Quanta Soft software (BioRad, USA) will be employed for data analysis.

2.3.3. Quality control

In every PCR assay, positive and negative controls will be incorporated. The positive control will be a PCR product from a previously successful PCR assay, ensuring that the assay can detect the target virus. The negative controls will include: (a) an extraction control, which will undergo the entire extraction process without containing any enterovirus, ensuring that no contamination occurs during extraction; (b) a reaction tube with distilled water instead of the test template, which will serve as a no-template control to check for any contamination in the reagents or environment; and (c) a sample that has previously shown a negative result in a PCR test, verifying that the assay does not produce false-positive results with known negative samples. These controls will be processed alongside the test samples, and the validity of each run will be confirmed only if all controls provide the expected outcomes, thus ensuring the accuracy of the ddPCR results and minimizing the risk of non-specific signals leading to false positives.

2.3.4. Next-generation sequencing

All samples testing positive for the pathogenic viruses will undergo next-generation sequencing for detailed viral genome identification. Leveraging the Ion GeneStudio™ S5 Prime System sequencing platform (Thermo Scientific, USA), we aim for >99 % genome coverage at a 20 × depth. The RNA samples will be reverse transcribed, followed by a sequence of library preparation steps. The final amplicons will undergo sequencing, with the resultant raw data processed through a Torrent Suite Server. With its specialized plug-ins for the Ion Ampliseq viral panel, this server will facilitate the trimming of low-quality reads. Eventually, sequences will be mapped to enteric reference sequences, followed by the derivation of a consensus sequence. Annotations and genotyping for the enteric viruses will be performed on these sequences.

2.3.5. Data analysis

All quantitative data will be analyzed using the Microsoft Excel software.

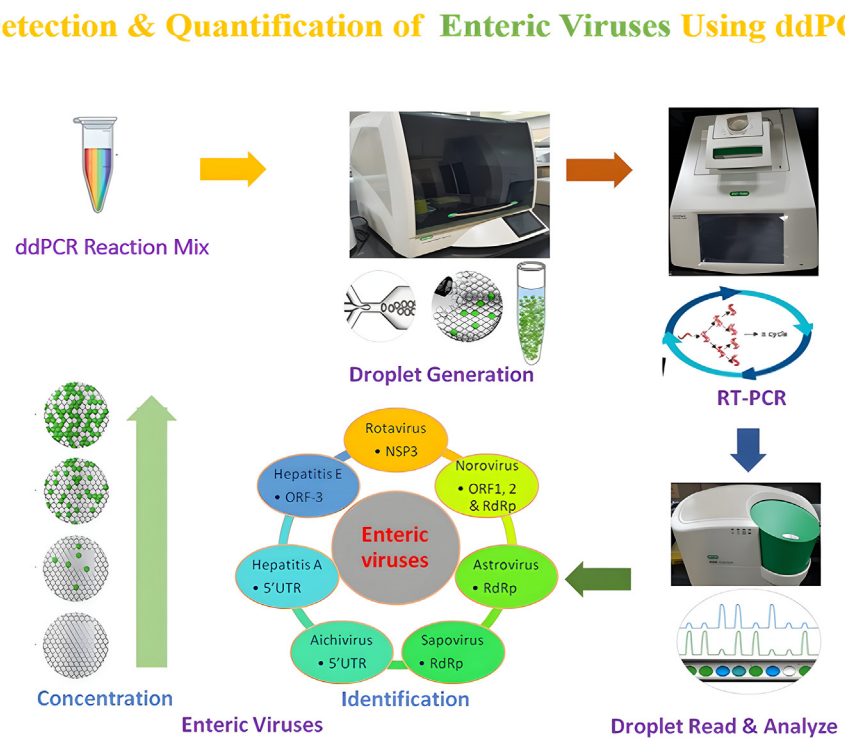

Fig. 4. Proposed workflow of droplet digital PCR. Abbreviations: ddPCR, droplet digital PCR; RT-PCR, reverse-transcript PCR; NSP, non-structural protein; ORF: open reading frame; RdRp, RNA-dependent RNA polymerase; UTR, untranslated regions.

3. Discussion

To our knowledge, this study is unique in the sense that it plans to comprehensively study the enteric viruses (viz. norovirus, rotavirus, sapovirus, astrovirus, Achivirus, hepatitis A & E enteric viruses) in urban settings, particularly in cities like Bhopal where a sewage treatment system is well established. The biweekly sampling approach offers a cost-effective and manageable method for trend analysis and early outbreak detection by correlating wastewater viral concentrations with epidemiological data [31,32]. Selecting Bhopal as the study site is strategic, considering its demographic characteristics, urban infrastructure, public health significance, and logistical advantages. This setting provides a robust platform for understanding the dynamics of enteric viruses in urban wastewater, which is crucial for developing effective public health policies and interventions. As urbanization accelerates, the need for advanced monitoring techniques becomes increasingly pressing. Noroviruses, particularly the GII genogroup, have emerged as one of the significant pathogens responsible for acute gastroenteritis globally [33]. Swiftly detecting and responding to these outbreaks can drastically reduce the healthcare burden while improving the community's well-being.

Table 1
Primer and probe sequences used for detection of enteric viruses in ddPCR.

No.	Name	Specification	Reference
1.	Rotavirus forward primer	5′-GACGGVGCRACTACATGGT-3′	[52]
2.	Rotavirus reverse primer	5′-GTCCAATTCATNCCTGGTGG-3′	[52]
3.	Rotavirus probe	FAM-5′-CCACCRAAYATGACRCCAGCNGTA-3′-BHQ	[52]
4.	Norovirus GII forward primer	5′-CARGARBCNATGTTYAGRTGGATGAG-3′	[53]
5.	Norovirus GII reverse primer	5′-TCGACGCCATCTTCATTCACA-3′	[53]
6.	Norovirus GII probe	FAM-5′-TGGGAGGGCGATCGCAATCT-3′-BHQ	[53]
7.	Norovirus GI forward primer	5′-CGYTGGATGCGNTTYCATGA-3′	[53]
8.	Norovirus GI reverse primer	5′-CTTAGACGCCATCATCATTYAC-3′	[53]
9.	Norovirus GI probe	FAM-5′-AGATYGCGATCYCCTGTCCA-3′-BHQ	[53]
10.	Hepatitis E virus forward primer	5′-GGTGGTTTCTGGGGTGAC-3′	[54]
11.	Hepatitis E virus reverse primer	5′-AGGGGTTGGTTGGATGAA-3′	[54]
12.	Hepatitis E virus probe	FAM-5′-TGATTCTCAGCCCTTCGC-3′-BHQ	[54]
13.	Hepatitis A virus forward primer	5′-CTCTTTGATCTTCCACAAGRGGT-3′	[29]
14.	Hepatitis A virus reverse primer	5′-GCCGCTGTTACCCTATCCAA-3′	[29]
15.	Hepatitis A virus probe	HEX-5′-AGGCTACGGGTGAAAC-3′-BHQ	[29]
16.	Aichi virus forward primer	5′-CCCAGTGTGCGTAACCTTCT-3′	[28]
17.	Aichi virus reverse primer	5′-GTACCTGCCTGGCATYCCTA-3′	[28]
18.	Aichi virus probe	HEX-5′-ACGCCCTGTGCGGGATGAAA-3′-BHQ	[28]
19.	Astrovirus forward primer	5′-GCTTCTGATTAAATCAATTTTAA-3′	[26]
20.	Astrovirus reverse primer	5′-CCGAGTAGGATCGAGGGT-3′	[26]
21.	Astrovirus probe	FAM-5′-CTTTTCTGTCTCTGTTTAGATTATTTTAATCACC-3′-BHQ	[26]
22.	Sapovirus forward primer	5′-GACCAGGCTCTCGCYACCTAC-3′	[27]
23.	Sapovirus reverse primer	5′-CCCTCCATYTCAAACACTAWTTTG-3′	[27]
24.	Sapovirus probe	FAM-5′-TGGTTYATAGGYGGTAC-3′-BHQ	[27]

Abbreviations: ddPCR, droplet digital PCR; GII, genogroup II; FAM, carboxyfluorescein; BHQ, Black Hole Quencher®.

Recent wastewater-molecular-based surveys have indicated that people start excreting these viruses in their feces and urine even before showing disease symptoms. Thus, sewage surveillance can be a vital early indicator for the spread of the virus in the human population [34].

Wastewater-based epidemiology is an effective tool for monitoring viral outbreaks and complementing clinical testing [35]. It is proven to be successful in detecting outbreaks of HAV, HEV, norovirus, rotavirus, and SARS-CoV-2 in communities before clinical testing. To monitor COVID-19 in communities, the Center for Disease Control has launched a Nationwide Wastewater Surveillance System [36,37]. Hepatitis A and E viruses are the leading causes of infectious hepatitis, which are primarily transmitted through contaminated food and water. Developed countries such as Italy, Norway, and the UK have conducted sewage surveillance of HEV to track its spread and prevent outbreaks [38–42]. A study conducted in South Africa reported sapovirus concentrations in wastewater suggested that levels exceeding 10^4 copies/mL may require public health intervention [43]. However, the threshold limit for other enteric viruses in sewage water must be established to initiate rapid public health interventions.

Recent advancements in molecular biology techniques have enabled the detection and quantification of a diverse range of pathogenic viruses in environmental samples [44]. However, despite the application of tools like qPCR, several challenges persist in accurately quantifying RNA viruses. These challenges arise from the multiple steps involved in the process, including reverse transcription, and the influence of external factors like inhibitors present in the environmental samples. Consequently, these factors pose limitations to achieving accurate viral quantification [16]. The recent emergence of ddPCR has revolutionized the field of viral detection and quantification. This innovative technique eliminates the requirement for standard curves [45], offering a significant advantage over traditional methods like qPCR. Unlike qPCR, ddPCR is independent of PCR efficiency, potentially leading to more precise measurements. Due to its versatility, ddPCR has found widespread application in clinical research [46]. The use of ddPCR in the diagnosis of viral diseases has been increasing in recent years [47]. However, its use in environmental studies for detecting and quantifying enteric viruses remains limited, with only a few studies published to date [16,48]. ddPCR is a reliable and scalable method for detecting and quantifying low levels of viral nucleic acids, making it a valuable tool for early diagnosis and monitoring of viral infections [47]. The ddPCR assay is also more sensitive than other molecular detection methods, such as reverse transcription-recombinase aided amplification (RT-RAA) and loop-mediated isothermal amplification (LAMP). Testing of clinical samples revealed a higher positivity rate detected by ddPCR (5.6 %) compared to qPCR (4.4 %) [49]. This study on enteric viruses in Bhopal's wastewater will provide valuable insights into viral epidemiology. It will help identify high-risk areas and populations, prioritize vaccination and preventative measures, and improve vaccine effectiveness. Early detection of viral outbreaks will enable rapid public health interventions and to establish novel benchmarks in surveillance precision. The sophisticated methodologies utilized in this study are expected to impact public health policies and resource distribution, while findings are anticipated to shape global response towards viral infections, and this model can be implemented in similar urban settings across the globe [50].

This study sets out to ascertain the prevalence of common enteric viruses in the Bhopal region, utilizing the novel ddPCR-based methodology offering a detailed and precise quantification approach that traditional methods might overlook. This precision is crucial, given the potentially high genetic diversity and mutation rates of RNA viruses like the norovirus GII. Understanding this genetic makeup can provide insights into the virus's transmission dynamics, virulence, and resistance patterns, allowing for more tailored intervention strategies. Studies have shown that increased population density can lead to a higher prevalence of enteric viruses in wastewater, particularly in areas with insufficient sanitation infrastructure or improper sewage treatment systems [51]. While our study primarily focuses on the Bhopal region, its implications resonate more broadly. The integration of advanced molecular techniques with traditional epidemiological approaches represents a paradigm shift in infectious disease surveillance. The lessons gleaned from our research, centered on noroviruses GII, can be extrapolated to other pathogens, ushering in an era of more proactive, informed, and responsive public health strategies.

The study on enteric viruses in Bhopal's wastewater faces several limitations, notably the lack of direct clinical correlation. This absence makes it difficult to precisely link the detection of viruses in wastewater with actual health outcomes or disease prevalence in the community. Additionally, the representativeness of wastewater samples may not encompass the entire population, particularly those in areas not connected to the main sewage system. The findings are also constrained by time and location, limiting their generalizability to other periods or areas. Environmental factors affecting virus survival and detection in wastewater could further impact the accuracy of results.

4. Conclusions

In this upcoming study within the Bhopal Municipal Corporation, we plan to employ a pioneering methodology using ddPCR to detect and characterize pathogenic RNA enteric viruses in WWTPs. We anticipate that our findings will highlight the significant presence of viruses in the region, including norovirus GII, rotavirus, HAV, and HEV, emphasizing continuous monitoring. Our study is the first to screen for Aichi virus, astrovirus, and sapovirus in this area. Beyond mere detection, the integration of next-generation sequencing into our research is expected to provide invaluable insights into the genetic makeup of these viruses, deepening our understanding of their epidemiology. When augmented with innovative techniques like ddPCR, the potential of wastewater-based epidemiology is poised to be a game-changer, enabling communities to address viral outbreaks proactively.

Therefore, this research will likely underscore the importance of non-clinical data in public health decision-making, offering a broader perspective on disease spread by capturing both symptomatic and asymptomatic cases. In the context of Bhopal and similar settings, this study aims to suggest a path forward: the development of a responsive, data-driven public health framework that capitalizes on wastewater surveillance. Such a strategy is expected to pave the way for early outbreak detection and provide communities with actionable insights to enhance vaccination campaigns and refine containment measures.

CRediT authorship contribution statement

Ram Kumar Nema: Writing – review & editing, Writing – original draft, Project administration, Methodology, Funding acquisition, Conceptualization. **Surya Singh:** Writing – review & editing, Visualization, Methodology. **Ashutosh Kumar Singh:** Writing – review & editing, Visualization. **Devojit Kumar Sarma:** Writing – review & editing. **Vishal Diwan:** Writing – review & editing. **Rajnarayan R. Tiwari:** Writing – review & editing, Resources. **Rajesh Kumar Mondal:** Writing – review & editing. **Pradyumna Kumar Mishra:** Supervision, Conceptualization.

Ethical approval and ethical considerations

Ethical approval for carrying out this study has been obtained from the Institutional Ethics Committee, ICMR- National Institute for Research in Environmental Health, Bhopal, India (No. ICMR-NIREH/BPL/IEC/2023-24 Dated 10.07.2023).

Patient and public involvement

Patients or the public Will not be involved in the design, or conduct, or reporting, or dissemination plans of our research.

Funding

No external funding was received for this study.

Availability of data and materials

This manuscript does not report data generation or analysis.

Declaration of competing interest

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

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