

Methods and Applications

A Quadruplex Digital PCR Assay for the Simultaneous Detection of Four Intestinal Bacterial Pathogens and Its Application in Wastewater Samples

Huihui Sun^{1,2}; Qiao Yao^{1,2}; Ximiao Zhao^{1,2}; Xia Li^{1,2}; Fuchang Deng^{1,2}; Jiayi Han^{1,2}; Lan Zhang^{1,2};
Xiaoyuan Yao^{1,2}; Biao Kan³; Jingyun Zhang^{3,#}; Song Tang^{1,2,#}

ABSTRACT

Introduction: A quadruplex digital polymerase chain reaction (dPCR) method was developed for the simultaneous detection of *Salmonella* spp., *Shigella* spp., *Vibrio cholerae*, and *V. parahaemolyticus* in wastewater to enhance pathogen identification velocity and efficiency. This study established detection limits for these bacterial pathogens and validated the method using environmental wastewater samples.

Methods: Specific primers and probes were designed targeting the *invA* gene of *Salmonella*, *ipaH* gene of *Shigella*, *tlh* gene of *V. parahaemolyticus*, and cholera toxin gene *ctxA* of *V. cholerae*. The quadruplex dPCR assay underwent rigorous evaluation for analytical sensitivity and specificity. Detection limits were determined using spiked wastewater samples, and the method's effectiveness was assessed through preliminary testing of 60 environmental wastewater samples.

Results: The quadruplex dPCR assay was optimized at an annealing temperature of 58°C. In spiked wastewater samples, the detection limits were 390 CFU/100 mL for *Salmonella*, 11 CFU/100 mL for *Shigella*, 660 CFU/100 mL for *V. cholerae*, and 640 CFU/100 mL for *V. parahaemolyticus*. Analysis of 60 municipal wastewater samples revealed pathogen concentrations ranging from 100.9–14,560 copies/100 mL for *Shigella*, 86.5–7,329 copies/100 mL for *Salmonella*, and 84.5–865.7 copies/100 mL for *V. parahaemolyticus*.

Conclusions: The developed quadruplex dPCR assay demonstrates robust capability for comprehensive surveillance of intestinal bacterial pathogens in wastewater, offering reliable detection even at low concentrations.

Wastewater-based epidemiology (WBE) has emerged as a pivotal and cost-effective public health tool, offering a swift and efficient means to evaluate the prevalence and dissemination of pathogens within a community through wastewater analysis (1). This method supports a comprehensive approach called One Sample, Multiple Analyses (OSMA) (2), allowing simultaneous monitoring of various health indicators, including antibiotic resistance genes, drug-resistant bacteria, pathogens, and chemical contaminants. This enhances traditional surveillance techniques. WBE's non-intrusive nature augments clinical surveillance by detecting pathogens in wastewater, which helps provide early warnings of disease outbreaks and track infection trends (3–4).

The utility of WBE was especially evident during the COVID-19 pandemic, where it effectively traced SARS-CoV-2 transmission, demonstrating its value in infectious disease surveillance (4–5). The monitoring of enteric pathogens including *Salmonella*, *Shigella*, and *Vibrio* species in wastewater has provided crucial epidemiological insights into foodborne and waterborne diseases. These pathogens, responsible for conditions like gastroenteritis, dysentery, and cholera, are consistently detected in wastewater systems (6–8). Surveillance of these pathogens is vital for understanding disease patterns, assessing intervention effectiveness, identifying contamination sources, quantifying disease burden, and guiding targeted surveillance efforts.

Digital polymerase chain reaction (dPCR), a third-generation PCR technology, provides exceptional accuracy and sensitivity for absolute quantification. This study establishes a quadruplex dPCR method for the simultaneous detection of *Salmonella*, *Shigella*, *V. parahaemolyticus*, and *V. cholerae* in wastewater samples. By monitoring bacterial levels in wastewater with dPCR, this approach serves as an early warning system, enabling timely identification of outbreak risks and prompt implementation of preventive measures.

METHODS

Wastewater Samples

A total of 60 wastewater samples were collected from piped wastewater networks serving large residential communities, subway stations, educational institutions, and hospitals across 4 districts of Beijing Municipality (Chaoyang, Haidian, Daxing, and Fengtai), China. The collected samples were categorized as follows: 27 from residential communities, 18 from healthcare institutions, and 15 from other locations. All samples were collected in November 2024.

Artificially Spiked Wastewater

Fresh bacterial colonies of the four positive control strains were washed three times with phosphate-buffered saline (PBS, pH 7.4) and subjected to serial twofold dilutions in PBS. For each dilution, 1 mL was spiked into 100 mL of pre-screened wastewater that had tested negative for all four target pathogens.

Bacterial Strains

The positive control strains utilized for method development were *V. cholerae* strain N16961, *Salmonella enterica* serovar Typhimurium strain LT2 (9), *S. flexneri* strain SH1, and *V. parahaemolyticus* strain ICDC-VP1329. The specificity of the dPCR assay was validated using a comprehensive panel of 90 bacterial strains, comprising 51 target strains and 39 non-target strains. The panel included 16 *Salmonella* strains (5 *S. Enteritidis*, 5 *S. typhi*, and 6 *S. Typhimurium*); 9 *Shigella* strains (2 *S. flexneri* and 7 *S. sonnei*); 11 *V. parahaemolyticus*; 13 *V. cholerae* (7 O1 serogroup and 6 O139 serogroup, with 12 *ctxA*-positive strains); 2 Enteroinvasive *Escherichia coli* (EIEC); 20 other pathogenic *E. coli* (excluding EIEC); and various other species including 1 *V. alginolyticus*, 3 *V. fluvialis*, 2 *V. mimicus*, 2 *V. vulnificus*, 1 *Aeromonas hydrophila*, 3 *Plesiomonas shigelloides*, 1 *Yersinia enterocolitica*, 2 *Clostridium perfringens*, 1 *Edwardsiella tarda*, 1 *Listeria monocytogenes*, 1 *Citrobacter sp.*, and 1 *Clostridium difficile*. All strains were maintained in laboratory collection.

Primers and Probes

Target gene sequences were selected based on comprehensive sequence alignments from the NCBI NR database (<https://www.ncbi.nlm.nih.gov/nucleotide/>). All primers and probes were designed using Beacon Designer V8.20 and synthesized by

Sangon Biotech (Shanghai, China).

The primer and probe sequences (5' to 3') for pathogen detection are as follows: for the *invA* gene of *Salmonella*, the forward primer is CCGCCAAACCT AAAACCAG, the reverse primer is GGCTCTTCG GCACAAGTA, and the probe is FAM-CGCCAA TCAGTCCYAACGACGACCCTT-BHQ1; for the *ipaH* gene of *Shigella*, the forward primer is GCAGAG AACTTCAGCTCTC, the reverse primer is CAGT GCGGAGGTCATTTG, and the probe is HEX-TCACTCCCGACACGCCATAGAAACGC-BHQ1; for the *tlh* gene of *V. parahaemolyticus*, the forward primer is CGAACGAGAACGCAGACATTA, the reverse primer is GCAACCACTTTGTTGATTTG ATCT, and the probe is ROX-TTCTTCGCCGCTG ACAATCGCTTCTCA-BHQ2; and for the *ctxA* gene of *Vibrio cholerae*, the forward primer is AGGGGCTACAGAGATAGATATTACA, the reverse primer is GCGGTGCATGATGAATCCA, and the probe is Cy5-ACCTGCCAATCCATAACCATCTG CTGC-BHQ3.

Quadruplex dPCR Assay

The quadruplex dPCR analysis was performed using the multiplex QIAcuity Digital PCR system (QIAGEN, Hilden, Germany). Reactions were conducted using the QIAcuity Probe PCR Kit (QIAGEN) according to manufacturer specifications. Each 40 µL reaction mixture contained 10 µL of 4× dPCR™ Supermix for Probes (QIAGEN), 10 µL of template DNA, 800 nM of each primer, 400 nM of each probe, and 6.25 units/mL EcoRI-HF® (New England Biolabs, United Kingdom). The reaction mixture was distributed into a 24-well 26k QIAcuity Nanoplate (QIAGEN), sealed, and analyzed using the QIAcuity dPCR instrument (QIAGEN). Each sample was analyzed in triplicate, with a no-template control (NTC) serving as the negative control. The thermal cycling protocol consisted of initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 58 °C for 30 s. Positive results were determined by the presence of positive droplets in the dPCR assay.

Analytical Specificity

The specificity of primers and probes underwent comprehensive evaluation using both the BLASTn algorithm (within the non-redundant nucleotide database) and Primer-BLAST (within the non-redundant database restricted to Enterobacterales)

(<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Subsequent validation was performed against genomic DNA extracted from 90 bacterial strains, comprising 51 target strains and 39 non-target strains as detailed above.

Limit of Detection (LOD)

The analytical sensitivity of the assay was determined using artificially spiked wastewater samples. Bacterial concentrations in each dilution were quantified by colony-forming unit (CFU) counting on LB agar plates. The spiked wastewater samples underwent centrifugation at 10,000 g for 30 minutes, and DNA was extracted from the resulting pellet using a DNeasy® PowerSoil Kit (QIAGEN, USA) according to the manufacturer's protocol. The extracted DNA was resuspended in 100 µL of nuclease-free water, with 10 µL used as template for dPCR detection. The entire process, from spiked sample preparation through dPCR detection, was performed in triplicate. The LOD was defined as the lowest CFU concentration in wastewater that yielded positive results across all replicates (10). Process repeatability was assessed using the coefficient of variation (CV) of the measured copy number per reaction.

Statistical Analysis

The dPCR data were analyzed using the QIAcuity Software Suite (version 2.5.0.1; Qiagen, Germany). A database was established using Microsoft Excel 2010. Statistical analysis and visualization were conducted using R software (version 4.4.2, R Core Team, Vienna, Austria). Differences in target gene concentrations in nucleic acid extracted from 100 mL wastewater samples across different sampling sites were assessed using the Wilcoxon test, implemented with the “wilcox_test” function from the “rstatix” package. Whisker plots were created using the “ggplot2” package.

RESULTS

Establishment and Optimization of the Quadruplex dPCR Assay for Four Bacterial Pathogens

Following primer and probe design, the amplification efficiency of each target gene using serial dilutions of positive control strain was initially evaluated DNA via real-time PCR. The analysis

demonstrated optimal amplification efficiency (90%–110%) for each target gene in both singleplex (single target gene with corresponding primers and probe) and quadruplex (all four target genes with corresponding primers and probes) systems. After confirming amplification efficiency, the quadruplex dPCR reaction conditions were optimized. Through systematic evaluation of annealing/extension temperatures between 58°C and 62°C, 58°C provided optimal discrimination between positive and negative partitions. Based on these results, 58°C was the optimal annealing and extension temperature for the quadruplex dPCR assay (Figure 1).

Analytical Specificity and Sensitivity

In silico specificity analysis confirmed high target specificity for all primers and probes across the four target genes. However, two important considerations emerged: the *ipaH* gene is present in both *Shigella* and EIEC, and while the *ctxA* gene is predominantly found in *V. cholerae*, it can occasionally occur in other species such as *V. mimicus*. Subsequent experimental validation using 90 bacterial strains (51 target and 39 non-target) demonstrated robust analytical specificity of the quadruplex dPCR assay. The assay showed complete concordance with expected results for target organisms, with no observed cross-reactivity or false-positive results across the tested strain panel.

Using artificially spiked wastewater samples, the following LOD values were established for the quadruplex dPCR assay: 390 CFU/100 mL for *Salmonella*, 11 CFU/100 mL for *Shigella*, 660 CFU/100 mL for *V. cholerae*, and 640 CFU/100 mL for *V. parahaemolyticus* (Table 1).

Pathogen Detection and Quantification in Municipal Wastewater Samples Using Quadruplex dPCR Assay

The quadruplex dPCR assay was employed to analyze four bacterial pathogens across 60 municipal wastewater samples collected from the pipe network. Analysis revealed differential detection rates among the target pathogens: *Shigella* exhibited the highest prevalence at 78.3% (47/60), followed by *Salmonella* at 46.7% (28/60), and *V. parahaemolyticus* at 33.3% (20/60) (Table 2). No *V. cholerae* was detected in any of the samples. Quantitative analysis demonstrated pathogen-specific concentration ranges: *Shigella* showed the highest concentrations at 100.9–14,560 copies/100 mL, followed by *Salmonella* at 86.5–7,329

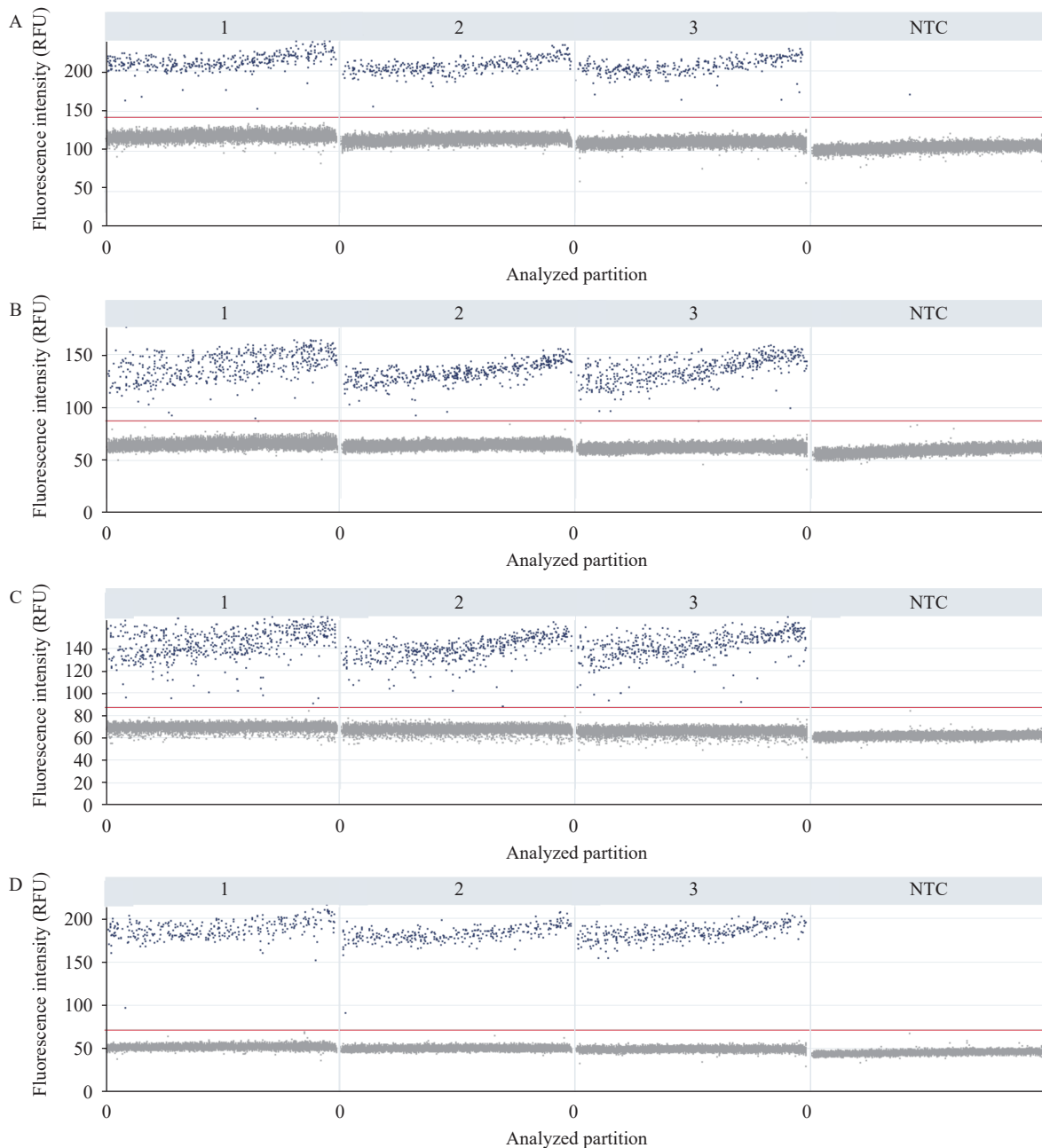


FIGURE 1. The fluorescence intensity of the target gene by digital PCR. (A) the *ipaH* gene of *Shigella* SH1; (B) the *invA* gene of *Salmonella* LT2; (C) the cholera toxin gene *ctxA* of *Vibrio cholerae* N16961; (D) the *tlh* gene of *Vibrio parahaemolyticus* ICDC-VP1329. 1, 2, and 3: Three replicates.

Note: The red horizontal lines denote the fluorescence threshold. Partitions represented by blue dots above this threshold line are interpreted as positive, while those represented by grey dots positioned below the threshold line are classified as negative.

Abbreviation: NTC=no-template control; PCR=polymerase chain reaction; RFU=relative fluorescence unit.

copies/100 mL, and *V. parahaemolyticus* at 84.5–865.7 copies/100 mL (Figure 2).

DISCUSSION

Current approaches for pathogen detection in

wastewater encompass traditional culture-based methods, molecular techniques such as PCR, and next-generation sequencing (11). However, these methodologies exhibit significant limitations in sensitivity, specificity, and their capacity to detect low pathogen concentrations in complex wastewater matrices. dPCR has emerged as a transformative

TABLE 1. Results of dPCR for the detection of artificially spiked wastewater samples.

Bacterial strains	Expected CFU/mL of wastewater sample	Mean±SD (cp/μL DNA)	CV (%)	LOD (CFU/100 mL wastewater sample)
<i>Shigella flexneri</i> SH1	0.22	3.13±0.31	9.78	11
	0.11	1.31±0.36	27.49	
	0.05	1.16±0.33	28.65	
<i>Salmonella enterica</i> serovar Typhimurium strain LT2	7.80	9.65±1.01	30.5	390
	3.90	3.33±1.02	30.5	
	1.95	0.95±0.33	35.22	
<i>Vibrio cholerae</i> N16961	33.00	37.48±15.81	42.17	660
	6.60	2.23±1.39	62.30	
	3.30	0.15±0.13	87.12	
<i>Vibrio parahaemolyticus</i> ICDC-VP1329	12.80	11.45±1.11	9.7	640
	6.40	4.56±0.40	8.83	
	3.20	1.38±0.54	38.79	

Notes: Mean, average copy values ($n=3$) in a final reaction volume of 40 μL. CV between replicates ($n=3$), $CV=SD/Mean \times 100\%$. Expected CFU/mL of wastewater sample: the bacterial concentration calculated by colony counting results.

Abbreviation: CV=coefficient of variation; SD=standard deviation; LOD=limit of detection; CFU=colony-forming unit.

* In three replicates, a negative well appeared.

TABLE 2. Comparison of the quadruplex dPCR for detecting 60 wastewater samples of different types (community $n=27$, hospital $n=15$, other $n=18$) collected from Beijing Municipality, China.

Type of Place	<i>Shigella</i> spp.		<i>Salmonella</i> spp.		<i>Vibrio parahaemolyticus</i>		<i>Vibrio cholerae</i>	
	POS (%)	POS/SUM	POS (%)	POS/SUM	POS (%)	POS/SUM	POS (%)	POS/SUM
Community	88.9	24/27	63.0	17/27	40.7	11/27	0.0	0/27
Hospital	53.3	8/15	13.3	2/15	13.3	2/15	0.0	0/15
Other	83.3	15/18	50.0	9/18	38.9	7/18	0.0	0/18
Total	78.3	47/60	46.7	28/60	33.3	20/60	0.0	0/60

Notes: Positive well: a well is considered positive when the number of positive droplets is greater than or equal to 3. Positive sample: a sample is determined to be positive when two or more of its three replicate wells are positive.

Abbreviation: POS=number of positive samples. SUM=the sum of positive and negative samples.

technology for wastewater surveillance, offering superior sensitivity and quantitative precision for pathogen detection (12). Its capacity for absolute quantification without calibration curves renders dPCR particularly advantageous for wastewater analysis, especially given its robust performance in the presence of PCR inhibitors commonly encountered in complex environmental matrices (12–13).

Previous studies have demonstrated the efficacy of multiplex dPCR for bacterial detection. A duplex dPCR assay developed for *Shigella* and *Salmonella* detection in fecal samples achieved detection limits of 12.3 copies/r and 23.7 copies/r, with corresponding CFU/mL values of 550 and 10,000 (14), respectively. In this study, the detection limits were 13.1 copies/r and 33.3 copies/r. Comparatively, this method demonstrates enhanced sensitivity, with detection limits of 11 and 390 CFU/100 mL for these bacteria in

wastewater matrices. These performance differences may be attributed to variations in sample type and processing procedures. While another triplex dPCR assay reported a detection limit of 0.23 copies/μL for *Salmonella* nucleic acid in food samples, our method achieved 3.33 copies/μL in wastewater matrices. This sensitivity variation likely reflects differences in both methodological approaches and matrix-specific bacterial recovery rates. The disparity underscores the critical importance of optimizing wastewater sample pretreatment protocols for real-world applications. In this analysis of environmental samples, the elevated detection rates for *Shigella* and *Salmonella*, with concentrations predominantly ranging from 394.55 to 1300 and 131.9 to 527.3 CFU/100 mL, respectively, indicate potential public health concerns that warrant enhanced surveillance measures. This sensitivity variation is likely attributable to differences in

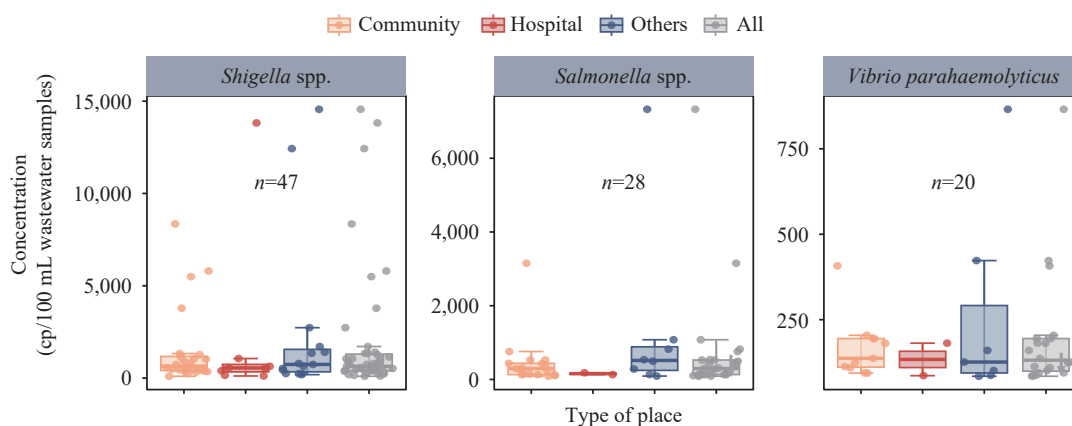


FIGURE 2. Whisker plots of the concentration of the target genes in the nucleic acid extracted from 100 mL wastewater samples from different sampling sites measured by quadruplex dPCR. Upper and lower fences display the 5th and 95th percentiles. The boxes display the interquartile range and median. Abbreviation: dPCR=digital polymerase chain reaction.

methodological approaches, as well as potential variations in DNA extraction and PCR amplification efficiency, which may be influenced by matrix differences. These discrepancies highlight the critical need for standardized wastewater sample pretreatment methods in surveillance systems, particularly when comparing data across different sentinel sites. Since the *ipaH* gene is present in both *Shigella* and EIEC, and the *ctxA* gene can occasionally be found in other species, for positive detection of *Shigella* and *Vibrio cholerae*, it is recommended to isolate and culture the strains before performing biochemical identification. Alternatively, detection can focus on specific genes of EIEC and vibroid-like bacteria.

This study presents the first application of multiplex dPCR for simultaneous monitoring of these four pathogens in wastewater environments. The developed quadruplex dPCR method enhances detection throughput and facilitates large-scale wastewater monitoring programs. The method's capacity to detect pathogens at low concentrations enables earlier identification of potential disease outbreaks.

Despite these advantages, several challenges and limitations remain in implementing multiplex dPCR for wastewater monitoring. First, the technology has not yet achieved full process automation and still relies on operator technical proficiency, potentially affecting result accuracy. Second, samples with extremely low concentrations often exhibit high CV values across repeated measurements, with discrepancies between detected values and actual copy numbers — a phenomenon which was also encountered in this experiment.

Therefore, to enhance result reliability and accuracy

in practical applications, it is recommended to perform at least three replicate measurements and calculate the average. Furthermore, while dPCR assays remain relatively costly and the complexity of analysis may pose barriers for laboratories with limited resources, potentially hindering widespread application in public health monitoring, exploring collaborations with external laboratories to share equipment or providing simplified data analysis software could help lower entry barriers. Due to the nascent stage of wastewater monitoring in our country and the absence of standardized protocols for multiplex dPCR in wastewater surveillance, result variability across different laboratories has hindered cross-study comparability. Therefore, establishing national standards promptly is essential to unify workflows and practices among laboratories.

Conflicts of interest: No conflicts of interest.

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Ethical statements: This study did not involve human or animal subjects, thus, no ethical statement was required.

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* Corresponding authors: Jingyun Zhang, zhangjingyun@icdc.cn; Song Tang, tangsong@nieh.chinacdc.cn.

¹ National Key Laboratory of Intelligent Tracking and Forecasting for Infectious Diseases, National Institute of Environmental Health, Chinese Center for Disease Control and Prevention, Beijing, China; ² China CDC Key Laboratory of Environment and Population Health, National Institute of Environmental Health, Chinese Center for Disease Control and Prevention, Beijing, China; ³ National Key Laboratory of Intelligent Tracking and Forecasting for Infectious Diseases, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, China.

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