International Journal of Infectious Diseases 120 (2022) 210–216

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

A duplex droplet digital PCR assay for *Salmonella* and *Shigella* and its application in diarrheal and non-diarrheal samples

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ARTICLE INFO

Article history:
Received 20 August 2021
Revised 11 April 2022
Accepted 20 April 2022

Keywords:
Salmonella
Shigella
Droplet digital PCR
Real-time PCR
Children with diarrhea
Children without diarrhea

ABSTRACT

Objectives: To evaluate a duplex droplet digital polymerase chain reaction (ddPCR) assay targeting *Salmonella fimY* and *Shigella ipaH* genes.

Methods: The linear range, precision, analytical sensitivity, and analytical specificity of the ddPCR assay were analyzed. The ddPCR assay was compared with quantitative real-time PCR (qPCR) using 362 stool samples from 187 children with mild diarrhea and 175 children without diarrhea.

Results: The duplex ddPCR assay showed good linearity in the range of 5.3×10^0 to 1.24×10^5 copies/reaction for *Salmonella* and 1.9×10^0 to 1.84×10^5 copies/reaction for *Shigella*. When analyzed with spiked stool samples, the limit of detection and limit of quantification were 550 and 5500 colony-forming units per mL of stool sample for *Shigella*, respectively, whereas both were 1.0×10^4 colony-forming units per mL of stool sample for *Salmonella*. Among 362 stool samples, more samples were detected as positive by ddPCR than by qPCR. *Salmonella* load was significantly higher in diarrheal samples than in non-diarrheal samples. Determined by receiver-operating characteristic analysis, the optimal cut-off value was 1.25×10^4 copies/mL of stool sample to distinguish between symptomatic and asymptomatic *Salmonella* infections.

Conclusion: *Salmonella* and *Shigella* prevalence may have been underestimated in the past.

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Introduction

Bacteria of the genera *Salmonella* and *Shigella* are important pathogens of infectious diarrhea (Wardlaw et al., 2010). Salmonellosis was the second most commonly reported gastrointestinal infection in humans in the European Union in 2018 (ECDC, 2019). Although shigellosis is relatively uncommon in the European Union (European Centre for Disease Prevention and Control, 2018), *Shigella* spp was the second leading cause of mortality caused by diarrhea among all ages in 2016 globally, accounting for 212,438 deaths (GBD, Diarrhoeal disease collaborators 2016, 2018). *Salmonella* spp and *Shigella* spp were also frequently detected in patients with diarrhea in China (Zhang et al., 2019; Zhou et al., 2018).

Various nucleic acid detection techniques have been widely used in screening for diarrheal pathogens. Quantitative real-time polymerase chain reaction (qPCR) is one of the most commonly used techniques in this regard, and the quantification cycle (Cq) of qPCR is predictive of the quantity of input target (Heid et al., 1996). Compared with qPCR, digital PCR (dPCR) can absolutely quantify target molecules independently of both the standard curve and Cq value and is more tolerant to PCR inhibition factors and variations in PCR efficiency (Dingle et al., 2013). Moreover, dPCR displayed greater precision than qPCR in some studies (Hindson et al., 2013;

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https://doi.org/10.1016/j.ijid.2022.04.040
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Table 1
Sequences of the primers and probes used in this study.

Species	Target gene	Code	Sequence (5′–3′)	Refs.
Salmonella spp	*fimY*	SM-F	GCGGCGTTGGAGAGTGATA	(Xiao et al., 2006)
		SM-R	AGCAATGGAAAAAGCAGGATG	
		SM-P-FAM	FAM-CATTTCTTAAACGGCGGTGTCTTTCCCT-BHQ1	
Shigella spp	*ipaH* [a]	SH-F	CGCAATACCTCCGGATTCC	(Li et al. (2014))
		SH-R	TCCGCAGAGGCACTGAGTT	
		SH-P-HEX	HEX-AACAGGTCGCTGCATGGCTGGAA-BHQ1	

[a] Gene *ipaH* exists in *Shigella* and enteroinvasive *Escherichia coli* (EIEC), thus this method cannot distinguish between the two pathogens.

Pinheiro et al., 2012). Currently, the commercially available dPCR systems can be divided into two types according to the methods of partitioning: chip-based dPCR and droplet dPCR (ddPCR). dPCR has been applied to the detection of various pathogens (Kuypers and Jerome, 2017), but there has not been much data for diarrheal pathogen detection to date. In this study, we established a duplex ddPCR assay for *Salmonella* spp and *Shigella* spp and compared the ddPCR assay with qPCR to detect the two pathogens in stool samples from children with and without diarrhea.

Materials and methods

Bacterial strains

Salmonella enterica serovar Typhimurium strain LT2 (McClelland et al., 2001) and *Shigella flexneri* strain SH1 (isolated from a child with diarrhea in 2012) were used as positive reference strains. The following 67 strains were used to evaluate the inclusivity and exclusivity of the ddPCR assay: 9 *Shigella* spp (2 *S. flexneri* and 7 *S. sonnei*), 19 *Salmonella* spp (9 *S.* Enteritidis, 5 *S.* Typhimurium, and 5 *S.* Typhi), 16 diarrheagenic *Escherichia coli* (3 enteroaggregative *E. coli*, 3 enteropathogenic *E. coli*, 8 enterotoxigenic *E. coli*, and 2 Shiga toxin-producing *E. coli*), 11 vibrios (3 *Vibrio cholerae*, 3 *V. parahaemolyticus*, 2 *V. fluvialis*, 1 *V. alginolyticus*, 1 *V. vulnificus*, and 1 *V. mimicus*), 2 *Campylobacter jejuni*, 1 *Citrobacter freundii*, 1 *Clostridium difficile*, 2 *C. perfringens*, 1 *Edwardsiella tarda*, 1 *Listeria monocytogenes*, 2 *Plesiomonas shigelloides*, 1 *Staphylococcus aureus*, and 1 *Yersinia enterocolitica.* These strains were stored in brain-heart infusion broth containing 20% (vol/vol) glycerol at −70°C and cultured overnight on Luria-Bertani (LB) agar at 37°C. The bacterial DNA was extracted by boiling (Guan et al., 2021).

Duplex ddPCR assay

The primers and probes (Table 1) targeting either the *Salmonella fimY* gene (Xiao et al., 2006) or the *Shigella ipaH* gene (Li et al., 2014) were selected according to the Chinese food safety standard (CFDA SN/T1870-2016). The assay could not distinguish *Shigella* spp from enteroinvasive *E. coli* (EIEC), but EIEC is generally not as common as *Shigella* spp in diarrheal samples (van den Beld et al., 2019). ddPCR was performed on a QX200™ ddPCR system (Bio-Rad Laboratories, CA, USA). The 20-μL reaction mixture included 10 μL of 2 × ddPCR™ SuperMix for Probes (Bio-Rad), 1 μL of template DNA, 800 nM primers, and 250 nM probes. Droplets were created using a DG8 cartridge and the droplet generator, transferred into a 96-well plate, sealed with a PX1 PCR plate sealer (Bio-Rad), and amplified in a thermal cycler under the following conditions: 95°C for 10 min, followed by 40 cycles of 94°C for 30 s and 58°C for 60 s, and then 98°C for 10 min. The ramp speed was 2°C/s. Finally, the plate was loaded onto the droplet reader (Bio-Rad), and the data were analyzed using QuantaSoft Version 1.7.4 (Bio-Rad).

Optimization of the duplex ddPCR assay

First, the singleplex ddPCR assays were optimized by evaluating the annealing temperatures ranging from 54.5 to 62°C for *Salmonella* and 51.8–61.8°C for *Shigella*, primer concentrations (600, 800, and 1000 nM), and probe concentrations (200, 250, and 300 nM). Then, the duplex ddPCR assay was established on the basis of the optimized results of the singleplex ddPCR assays.

Comparison of the duplex and singleplex ddPCR assays

The reference strains *S.* Typhimurium LT2 and *S. flexneri* SH1 were detected in parallel in three reactions: (i) the singleplex ddPCR; (ii) the duplex ddPCR with only one template added; and (iii) the duplex ddPCR with both templates added. The templates were dilutions of genomic DNA with target genes at the concentration of $\sim 10^2$ copies/μL of reaction mixture. Each test was performed in triplicate. The difference in the three groups of measurements was compared by one-way analysis of variance.

qPCR assays

The singleplex qPCR assays used the same primers and probes as the duplex ddPCR assay. The 20-μL reaction mixture consisted of 10 μL of Premix Ex Taq (Probe qPCR) (TaKaRa, Dalian, China), 1 μL of the template DNA, and primers and probe at 250 nM each. The amplification and detection were performed with a LightCycler 96 System (Roche, Indianapolis, IN, USA) under the following conditions: 95°C for 60 s, followed by 40 cycles of 95°C for 10 s, and 60°C for 30 s.

Linearity and precision of the ddPCR assay

The DNA of strains LT2 and SH1 was extracted by boiling and serially 10-fold diluted in deionized water. Then, 1 μL of each solution (10^0–10^{-5}) was analyzed by ddPCR in triplicate. The linearity between the measured copy number and the dilution was analyzed. The intermediate precision of ddPCR was further verified with DNA dilutions at $\sim 10^2$ and $\sim 10^3$ copies/reaction (cp/r), which were stored at 4°C and tested in triplicate on three consecutive days. The repeatability and intermediate precision were measured by the coefficient of variation (CV; SD / mean × 100%) of the measured copy numbers of the replicates (World Organisation for Animal Health (OIE), 2013).

Analytical specificity and analytical sensitivity

The analytical specificity of the primers and probes was evaluated *in silico* using the BLASTn algorithm (in non-redundant nucleotide database) and Primer-BLAST (in non-redundant database and organism limited to Enterobacterales) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and was verified with the genomic DNA of the 67 strains listed previously.

To determine the analytical sensitivity, fresh colonies of strains LT2 and SH1 were washed three times with phosphate-buffered saline (PBS; pH 7.4) buffer and then serially 10-fold diluted with PBS. The bacterial concentration of each dilution was calculated by colony count on LB agar. A healthy human stool sample was five-fold diluted with PBS and mixed with bacterial dilutions in equal volume; then, 200 µL of the mixture was used to extract nucleic acids with the EX-DNA/RNA virus extraction kit on an automatic purification system NP968 (Tianlong Science & Technology, Xi'an, China) with an elution volume of 100 µL. The nucleic acid was extracted in triplicate and analyzed with ddPCR and qPCR, respectively. Positive results were those with positive droplets present in ddPCR or Cq values available in qPCR.

Standard curves of the qPCR assays were constructed by plotting the Cq values on the y-axis and the logarithm of the expected colony-forming units (CFUs) per reaction on the x-axis. The amplification efficiency (E) was calculated: $E = 10^{-1/slope}-1$. The measured CFUs per reaction were calculated by Cq comparison with the standard curve.

The repeatability of the entire bioanalytical process (extraction and ddPCR/qPCR) was evaluated with the CV of the measured copy number or CFUs per reaction. The limit of detection (LOD) was determined as the lowest concentration at which all replicates gave positive results (World Organisation for Animal Health (OIE), 2014). The highest Cq value of the artificial samples would be used as the cut-off value for stool sample detection.

Diarrheal and non-diarrheal stool samples

In the study, 362 stool samples were analyzed by the duplex ddPCR and the singleplex qPCR assays, including 187 diarrheal and 175 non-diarrheal samples. We first conducted a pilot study with 52 characterized stool samples (36 diarrheal and 16 non-diarrheal) collected from Changning District, Shanghai, which had been tested for *Salmonella* spp and *Shigella* spp by traditional culture methods with the following media: selenite brilliant green broth (Luqiao, Beijing, China) and *Salmonella* Chromogenic Medium (Kemajia Microbe Technology, Shanghai, China) for *Salmonella* spp and MacConkey agar (Luqiao) for *Shigella* spp (Nancy A. Strockbine et al., 2015). Then, 310 surveillance samples (151 diarrheal and 159 non-diarrheal) collected from Hunan Province were tested. All samples were collected from May 2017 to March 2018 from children aged <5 years. The 187 diarrheal cases were children with mild diarrhea scored ≤10 on the Vesikari 20-point scale (Givon-Lavi et al., 2008; Ruuska and Vesikari, 1990). The 175 children without diarrhea were recruited in the community or enrolled in the same hospital as the cases who were hospitalized for reasons other than gastroenteritis.

The stool samples were stored at −80°C before nucleic acid extraction with the EX-DNA/RNA virus extraction kit. The pathogens in stool samples were then detected by the duplex ddPCR and the singleplex qPCR assays. Positive samples were those with positive droplets in ddPCR or Cq ≤36 in qPCR. For the positive samples with a result of <20 cp/r in ddPCR, the tests were repeated, and the sample was not determined as positive unless it was positive again in the repeated test.

Statistical analysis

The limit of quantification (LOQ) was determined as the lowest copy number in a reaction or the lowest concentration of pathogens in stool that could be quantified with a CV between replicates <25% (Pavsic et al., 2016; Whale et al., 2018). When comparing the distribution of *Salmonella* spp and *Shigella* spp in diarrheal and non-diarrheal samples, the difference in detection rate was analyzed by two-sided Fisher's exact test, and the difference in target concentration was analyzed by the Mann-Whitney test. Receiver-operating characteristic (ROC) curves were constructed by plotting the estimated sensitivity by 1-specificity using all diarrheal and non-diarrheal samples, which were positive by ddPCR. The measured copy number per reaction determined by ddPCR was included as the independent variable and case status (diarrheal or non-diarrheal) as the outcome and dependent variable. The Youden Index (YI = sensitivity + specificity − 1) was used to identify an optimal cut-off for the copy number per reaction. All analyses were conducted using GraphPad Prism 9 (GraphPad Software, Inc.).

Results

Establishment of the duplex ddPCR assay

We first optimized the primer/probe concentrations and annealing temperatures in the singleplex ddPCR assays (Figures S1 and S2). According to the fluorescence values of positive and negative droplets, we set the primer/probe concentration to 800/250 nM and the annealing temperature to 58°C. The duplex ddPCR assay under the same conditions could clearly distinguish positive from negative droplets. The results obtained by the duplex reaction were comparable to those obtained by the singleplex reactions ($P > 0.05$) (Figure S3). Therefore, it is feasible to quantify the two target genes in a duplex reaction.

Linearity and precision of the ddPCR assay

Evaluated with extracted DNA of strains LT2 and SH1, the duplex ddPCR showed good linearity ($R^2 > 0.99$) in the range of 5.3×10^0–1.24×10^5 cp/r for *Salmonella* and 1.9×10^0 to 1.84×10^5 cp/r for *Shigella*. Furthermore, when LT2 and SH1 were analyzed simultaneously, the duplex ddPCR maintained good linearity within the above range ($R^2 > 0.99$).

The repeatability of the duplex ddPCR assay (measured by CV) decreased with the decrease of target concentrations (Table 2). In the evaluation of the intermediate precision, the measured concentrations (mean ± SD) were 5,377.8 ± 330.1 cp/r (CV 6.1%) and 532.2 ± 52.0 cp/r (CV 9.8%) for *Salmonella* and 5,735.6 ± 150.3 cp/r (CV 2.6%) and 320.9 ± 68.2 cp/r (CV 21.3%) for *Shigella*. The LOQs (with a CV <25%) of the ddPCR for *Salmonella* and *Shigella* were conservatively estimated at the level of 10^2 cp/r.

Analytical specificity and analytical sensitivity

According to the *in silico* specificity analysis results, the target sequences from most *Salmonella* and *Shigella*/EIEC strains were identical to the primer and probe oligonucleotides. However, some strains harbor 1–3 mismatches in the target sequences of the primers and probes. Organisms other than target bacteria cannot be matched by the primers and probes. The *in vitro* testing of the 67 strains confirmed the analytical specificity: all but 9 *Shigella* spp and 19 *Salmonella* spp were negative when tested by the duplex ddPCR assay.

Next, the analytical sensitivity of the duplex ddPCR assay was evaluated with artificially spiked stool samples (Table 3). The LOD was 550 and 1.0×10^4 CFU/mL of stool sample for *Shigella* and *Salmonella*, respectively. The LOQ (with a CV <25%) of the entire bioanalytical process (extraction and ddPCR) was 5500 and 1.0×10^4 CFU/mL of stool sample for *Shigella* and *Salmonella*, respectively.

The LODs of qPCR were the same as those of ddPCR for both pathogens. The standard curves of qPCR were established (Figure S4) and the bacterial load per reaction was calculated (Table 3). The LOQ of the entire bioanalytical process (extraction and qPCR) was 5500 CFU/mL of stool sample for *Shigella* (same as that of

Table 2
The repeatability of the duplex ddPCR assay in detecting strain nucleic acid measured by the variation in replicate results.

	Dilutions	One target in the reaction			Both targets in the reaction		
		Mean (cp/r)	SD (cp/r)	CV (%)	Mean (cp/r)	SD (cp/r)	CV (%)
Genomic DNA of *Salmonella enterica* serovar Typhimurium LT2	10^0	1.24×10^5	1.09×10^3	0.9	1.34×10^5	4.99×10^2	0.4
	10^{-1}	1.17×10^4	8.99×10^1	0.8	1.23×10^4	1.61×10^2	1.3
	10^{-2}	1.10×10^3	2.47×10^1	2.3	1.06×10^3	4.88×10^1	4.6
	10^{-3}	1.00×10^2	1.18×10^1	11.8	1.26×10^2	1.52×10^1	12.0
	10^{-4}	1.77×10^1	4.50×10^0	25.4	3.23×10^1	8.40×10^0	26.0
	10^{-5}	5.30×10^0	3.00×10^0	56.6	2.90×10^0	2.50×10^0	86.2
Genomic DNA of *Shigella flexneri* SH1	10^0	1.84×10^5	1.63×10^3	0.9	1.82×10^5	3.34×10^3	1.8
	10^{-1}	1.46×10^4	4.99×10^1	0.3	1.39×10^4	8.22×10^1	0.6
	10^{-2}	1.54×10^3	4.51×10^1	2.9	1.45×10^3	5.07×10^1	3.5
	10^{-3}	1.81×10^2	2.29×10^1	12.6	1.37×10^2	2.17×10^1	15.8
	10^{-4}	2.93×10^1	5.70×10^0	19.5	1.84×10^1	5.40×10^0	29.3
	10^{-5}	1.90×10^0	8.00×10^{-1}	42.1	9.00×10^{-1}	1.30×10^0	144.4

Mean, average copy values ($n = 3$) in a final reaction volume of 20 µL. CV between replicates ($n = 3$); CV = SD/Mean × 100%.
CV, coefficient of variation; cp/r, copies/reaction; ddPCR, droplet digital polymerase chain reaction.

Table 3
Results of ddPCR and qPCR for the detection of artificially spiked stool samples.

	Expected CFU/mL of stool sample	Expected CFU/reaction	ddPCR			qPCR				
			Mean (cp/r)	SD (cp/r)	CV (%)	Mean of Cq	SD of Cq	Measured CFU/reaction		
								Mean	SD	CV (%)
Shigella flexneri SH1	5.50×10^5	1.10×10^3	1.23×10^4	2.44×10^3	19.9	22.4	0.2	1.28×10^3	1.85×10^2	14.4
	5.50×10^4	1.10×10^2	9.50×10^2	1.00×10^2	10.5	26.4	0.1	9.76×10^1	7.00×10^0	7.2
	5.50×10^3	1.10×10^1	8.40×10^1	1.59×10^1	18.9	30.0	0.4	9.50×10^0	2.20×10^0	23.2
	5.50×10^2	1.10×10^0	1.23×10^1	4.90×10^0	39.8	33.0	0.7	1.40×10^0	6.00×10^{-1}	42.9
	5.50×10^1	1.10×10^{-1}	3.00×10^0 [a]	3.50×10^0	116.7	36.0 [a]	1.3	NA	NA	NA
Salmonella enterica serovar Typhimurium LT2	1.00×10^7	2.00×10^4	1.78×10^4	1.78×10^3	10.0	26.5	0.4	2.49×10^4	7.52×10^3	30.3
	1.00×10^6	2.00×10^3	1.84×10^3	4.28×10^2	23.3	30.0	0.3	1.89×10^3	3.71×10^2	19.7
	1.00×10^5	2.00×10^2	2.34×10^2	1.72×10^1	7.3	33.5	0.7	1.44×10^2	7.82×10^1	54.4
	1.00×10^4	2.00×10^1	2.37×10^1	4.00×10^0	16.9	35.7	0.4	2.72×10^1	7.00×10^0	25.7
	1.00×10^3	2.00×10^0	1.50×10^0 [a]	1.60×10^0	106.7	-	-			

CV between replicates ($n = 3$). Data were NA because Cq was outside the range of the standard curve. Expected CFU/mL of stool sample and expected CFU/reaction: the bacterial concentration calculated by colony counting results. Measured CFU/reaction: the bacterial concentration calculated by Cq comparison with the standard curve. The standard curve equation was $y = -3.104x + 40.124$ ($R^2 = 0.9898$, amplification efficiency = 110.0%) for *S.* Typhimurium LT2 and $y = -3.54x + 33.407$ ($R^2 = 0.9960$, amplification efficiency = 91.6%) for *S. flexneri* SH1.
CFU, colony forming unit; cp/r, copies/reaction; Cq, quantification cycle; CV, coefficient of variation; ddPCR, droplet digital polymerase chain reaction; NA, not available; qPCR, quantitative real-time polymerase chain reaction.
[a] Of the three replicates, two were positive and one was negative (the measured copy number was 0 in ddPCR, or Cq was not available in qPCR).

ddPCR), but it was difficult to determine the LOQ for *Salmonella*. In addition, a Cq of 36 was used as the cut-off value for both targets in detecting stool samples.

Detection of Salmonella *spp and* Shigella *spp in the diarrheal and non-diarrheal stool samples*

The 52 characteristic samples in the pilot study were selected on the basis of culture results, of which 16/36 (44.4%) diarrheal and 4/16 (25.0%) non-diarrheal samples were positive for *Salmonella*, whereas none were positive for *Shigella*. When detected by ddPCR and qPCR, 29/52 (55.8%) and 14/52 (26.9%) *Salmonella*-positive samples, and 11/52 (21.2%) and 0 *Shigella*-positive samples were reported, respectively (Table 4). Among the 20 *Salmonella* culture-positive samples, 17 were ddPCR-positive and 12 were qPCR-positive; among the 32 culture-negative samples, 12 were ddPCR-positive and two were qPCR-positive.

Among the 310 stool samples, ddPCR revealed more positives than qPCR (Table 5). There was no significant difference in the detection rate of *Salmonella* spp between the diarrheal and non-diarrheal samples, regardless of whether it was detected by ddPCR (5.3% and 2.5%, $P = 0.2471$) or qPCR (2.0% and 0.6%, $P = 0.3601$). The detection rate of *Shigella* spp was not significantly different between the two groups (1.3% and 0.6%, $P = 0.6143$).

In the first round of ddPCR screening of the 362 samples, 61 were positive for *Salmonella* and 43 were positive for *Shigella.* The samples with a result of <20 cp/r were tested by ddPCR repeatedly (Table 6). Finally, 41 samples were positive for *Salmonella* spp and 14 samples were positive for *Shigella* spp. The load of *Salmonella* spp was significantly higher in the diarrheal samples than in the non-diarrheal samples ($P = 0.0358$), whereas no significant difference in the load of *Shigella* ($P = 0.8142$) was observed between the two groups (Fig. 1).

We performed ROC analysis to define the optimal cut-off value for distinguishing between symptomatic and asymptomatic infections (Fig. 2). For *Salmonella*, the area under the ROC curve (AUC), which quantifies the overall ability of the test to discriminate between cases and non-cases, was 0.71 (95% confidence interval, 0.54–0.87). The optimal cut-off value with the highest YI was 25.0 cp/r (that is, 1.25×10^4 copies/mL of stool sample) when the sensitivity and specificity were 37.9% and 100.0%, respectively. The AUC value was 0.55 (95% confidence interval, 0.24–0.86) for *Shigella*, and this assay could not distinguish case status.

Discussion

qPCR has been widely used in detecting diarrheal pathogens, including *Salmonella* spp and *Shigella* spp (Mackay, 2004). Quantitative analysis by qPCR depends on the standard curve and Cq value (Bustin et al., 2009). In contrast, dPCR directly gives the number of copies in the reaction. *Salmonella* spp and *Shigella* spp are among the most common diarrhea-associated pathogens.

Table 4
Comparison of qPCR and ddPCR for detecting 52 characterized stool samples from Changning.

Pathogens	Results	Diarrheal samples ($n = 36$)			Non-diarrheal samples ($n = 16$)		
		Culture + n, (%)	Culture − n, (%)	Subtotal n, (%)	Culture + n, (%)	Culture −n, (%)	Subtotal n, (%)
Salmonella spp	ddPCR +, qPCR +	9 (25.0)	1 (2.8)	10 (27.8)	3 (18.8)	1 (6.3)	4 (25.0)
	ddPCR +, qPCR -	4 (11.1)	7 (19.4)	11 (30.6)	1 (6.3)	3 (18.8)	4 (25.0)
	ddPCR -, qPCR +	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	ddPCR -, qPCR -	3 (8.3)	12 (33.3)	15 (41.7)	0 (0.0)	8 (50.0)	8 (50.0)
	Subtotal						
	ddPCR +	13 (36.1)	8 (22.2)	21 (58.3)	4 (25.0)	4 (25.0)	8 (50.0)
	qPCR +	9 (25.0)	1 (2.8)	10 (27.8)	3 (18.8)	1 (6.3)	4 (25.0)
	Total	16 (44.4)	20 (55.6)	36 (100.0)	4 (25.0)	12 (75.0)	16 (100.0)
Shigella spp	ddPCR +, qPCR +	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	ddPCR +, qPCR -	0 (0.0)	8 (22.2)	8 (22.2)	0 (0.0)	3 (18.8)	3 (18.8)
	ddPCR -, qPCR +	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	ddPCR -, qPCR -	0 (0.0)	28 (77.8)	28 (77.8)	0 (0.0)	13 (81.3)	13 (81.3)
	Subtotal						
	ddPCR +	0 (0.0)	8 (22.2)	8 (22.2)	0 (0.0)	3 (18.8)	3 (18.8)
	qPCR +	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	0 (0.0)	36 (100.0)	36 (100.0)	0 (0.0)	16 (100.0)	16 (100.0)

ddPCR, droplet digital polymerase chain reaction; qPCR, quantitative real-time polymerase chain reaction.

Table 5
Comparison of qPCR and ddPCR for detecting 310 stool samples collected from Hunan.

Pathogens	Results	Diarrheal samples ($n = 151$) n (%)	Non-diarrheal samples ($n = 159$) n (%)	Total ($n = 310$) n (%)
Salmonella spp	ddPCR +, qPCR +	3 (2.0)	1 (0.6)	4 (1.3)
	ddPCR +, qPCR -	5 (3.3)	3 (1.9)	8 (2.6)
	ddPCR -, qPCR +	0 (0.0)	0 (0.0)	0 (0.0)
	ddPCR -, qPCR -	143 (94.7)	155 (97.5)	298 (96.1)
	Subtotal			
	ddPCR +	8 (5.3)	4 (2.5)	12 (3.9)
	qPCR +	3 (2.0)	1 (0.6)	4 (1.3)
Shigella spp	ddPCR +, qPCR +	2 (1.3)	1 (0.6)	3 (1.0)
	ddPCR +, qPCR -	0 (0.0)	0 (0.0)	0 (0.0)
	ddPCR -, qPCR +	0 (0.0)	0 (0.0)	0 (0.0)
	ddPCR -, qPCR -	149 (98.7)	158 (99.4)	307 (99.0)
	Subtotal			
	ddPCR +	2 (1.3)	1 (0.6)	3 (1.0)
	qPCR +	2 (1.3)	1 (0.6)	3 (1.0)

ddPCR, droplet digital polymerase chain reaction; qPCR, quantitative real-time polymerase chain reaction.

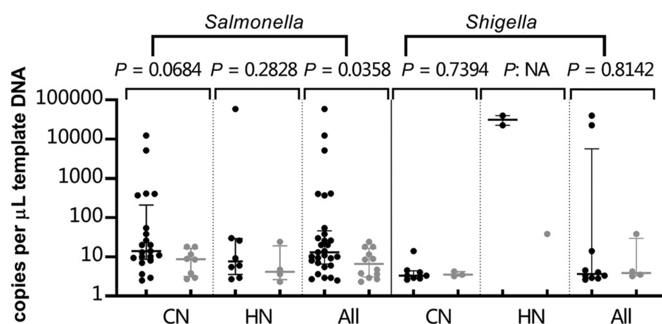

Fig. 1. The concentration of the target genes in the nucleic acid extracted from diarrheal and non-diarrheal stool samples measured by ddPCR.
Solid black circle (●), positive detections in the diarrheal samples; solid gray circle (●), positive detections in the non-diarrheal samples; short bar and line (┼), median with interquartile range. *P* values were calculated by the Mann-Whitney test.
CN, samples from Changning in the pilot study; ddPCR, droplet digital polymerase chain reaction; HN, samples from Hunan; NA, not available.

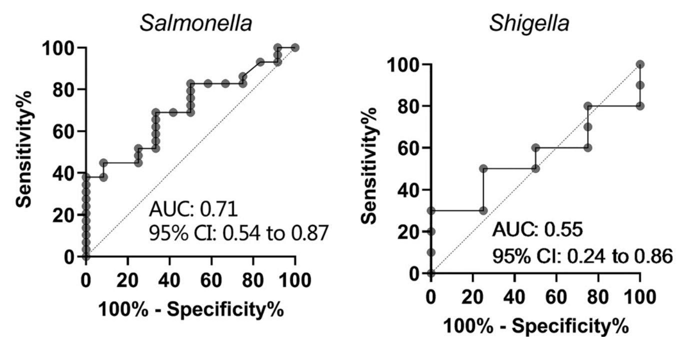

Fig. 2. ROC curve for case status versus gene quantities per reaction measured by the duplex ddPCR.
Curves were plotted by calculating the sensitivity and 1-specificity of ddPCR compared with case status. Only samples positive in ddPCR were included. The area under the ROC curve (AUC) quantifies the overall ability of the test to distinguish between cases and controls.
ddPCR, droplet digital polymerase chain reaction; ROC, receiver-operating characteristic.

Here, we established a duplex ddPCR assay for *Salmonella* spp and *Shigella* spp and used it to analyze a group of diarrheal and non-diarrheal samples. Compared with culture and qPCR, ddPCR revealed more positive samples.

The primers and probes used in the duplex ddPCR assay have previously been used in singleplex dPCR assays (Wang et al., 2018; Yang et al., 2020). The ddPCR assay for *Shigella* spp exhibited higher analytical sensitivity than qPCR in detecting *Shigella* spp from artificial mouse feces (Yang et al., 2020). Similarly, compared with qPCR, the ddPCR assay for *Salmonella* spp was more sensitive in detecting genomic DNA, pure strain culture, and spiked milk samples (Wang et al., 2018). However, in our study, ddPCR and qPCR showed the same analytical sensitivity in detecting artificially spiked stool samples. The LODs and LOQs might be refined with two-fold serial dilutions and more replicates.

Table 6
The measured concentration of target genes by the duplex ddPCR assay in the diarrheal and non-diarrheal samples.

Copies/reaction (copies/μL of DNA)	Copies/mL of stool	Number of positive samples			
		Round 1	Round 2	Diarrheal samples	Non-diarrheal samples
Salmonella fimY					
5.1×10^3–5.8×10^4	2.6×10^6–2.9×10^7	3	/	3	0
368–412	1.8×10^5–2.1×10^5	3	/	3	0
20–54	1.0×10^4–2.7×10^4	8	/	7	1
10–18	5.0×10^3–9.0×10^3	8	8	5	3
<10	$<5.0 \times 10^3$	39	19	11	8
	Subtotal	61		29	12
Shigella ipaH					
2.2×10^4–4.0×10^4	1.1×10^7–2.0×10^7	2	/	2	0
14	7×10^3	1	1	0	1
<10	$< 5 \times 10^3$	40	11	8	3
	Subtotal	43		10	4

ddPCR, droplet digital polymerase chain reaction.

In the analysis of stool samples, more positives were reported by ddPCR than by culture. ddPCR (and qPCR) could not distinguish between dead and live bacteria, thus the positive results did not prove the presence of a viable strain. Three samples were positive for *Salmonella* by culture but negative by ddPCR. The inconsistency might be related to the nucleic acid degradation during sample preservation and be reduced by some measures, such as timely detection of fresh samples and reduction of the number of freeze-thaw cycles and cryopreservation time for frozen samples or nucleic acids. The ddPCR assay revealed more positive samples than qPCR, especially in detecting samples carrying low-load bacteria. The prevalence of the two pathogens may be much higher than that obtained by culture indicated in previous surveillance data (Long et al., 2019; Zhou et al., 2018).

In our study, a small amount (<10) of positive droplets sometimes appeared in supposedly negative channels. Similar phenomena once occurred in other studies (Pinheiro et al., 2012; Racki et al., 2014). These "false positives" could be attributed to low-level contamination (cross-contamination between samples or carry-over contamination from the previous amplified product) during the preparation of the reaction mixture. Therefore, nucleic acid contamination should be controlled by wiping down workbenches, physically separating the different parts of the process, using personal protective equipment properly, etc. Nevertheless, the samples with weak positive results (e.g., <10 cp/r) needed further validation. In this study, more than half of the samples with a concentration of <10 copies/μL of DNA were negative in the second test. These inconsistencies might reflect the uncertainty of ddPCR in detecting low-concentration target genes, but it might also be related to nucleic acid contamination. Only samples positive in both rounds of testing were considered positive; thus, the reliability of the results was improved, but it is possible that some positive samples were ignored.

Salmonella spp and *Shigella* spp are diarrhea-associated pathogens. The greater the pathogen load, the higher the strength of association (Liu et al., 2014; Liu et al., 2016). A qPCR-based study (Liu et al., 2016) found that the pathogens were associated with diarrhea when there were $>2.1 \times 10^6$ copies of *Shigella ipaH* gene or $>2.2 \times 10^5$ copies of *Salmonella ttr* gene (one copy per genome, the same as *fimY*) per gram of stool. In our study, the optimal cut-off value to distinguish between symptomatic and asymptomatic infections was 1.25×10^4 copies/mL of stool for the *Salmonella fimY* gene. For *Shigella*, the number of positive samples was too small, and the target concentration range was too narrow to propose a cut-off value.

Low-load samples lack clinical significance and sometimes may be false positive, thus antimicrobial therapy for patients with low-load pathogens may not be appropriate. Meanwhile, the low load may be due to the patient being at a specific stage of infection or errors in sample collection and preservation. The possibility of this pathogen as the cause of diarrhea cannot be easily ruled out. Re-sampling and testing will help eliminate false positives, and comparing the changes in bacterial load will help understand the clinical significance of pathogens. The interpretation of low-load positive samples will be an important issue in real sample detection.

Limitations of ddPCR include the narrower linear dynamic range compared with qPCR (Baker, 2012; Wang et al., 2018) and its inability to distinguish between DNA from viable and dead cells (as qPCR). The main limitation of this study was the small number of samples, especially the lack of *Shigella*-positive samples. In addition, different annealing temperatures, primer and probe concentrations, and reaction reagents were used in ddPCR and qPCR. These differences might affect the comparison of the two methods.

Declaration of Competing Interest

None.

Ethical approval

The protocol was approved by the Ethics Committee of the National Institute for Communicable Disease Control and Prevention of the Chinese Center for Disease Control and Prevention.

Acknowledgments

This work was supported by the Ministry of Science and Technology of the People's Republic of China (2018ZX10712001-014 to JZ) and the Health Commission of Hunan Province (20200249 to ZZ).

Author contributions

Jingyun Zhang: study design, data analysis, and paper writing. **Yuanming Huang:** data analysis and paper writing. **Panpan Xue:** nucleic acid extraction and droplet digital polymerase chain reaction detection. **Zhifei Zhan** and **Zheng Huang:** sample collection.

Jie Li and **Baowei Diao:** bacterial culture and sample storage. **Biao Kan:** study design and paper review.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2022.04.040.

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