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Application of droplet digital PCR in detection of seed-transmitted pathogen *Acidovorax citrulli*

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Abstract

Bacterial fruit blotch caused by *Acidovorax citrulli* is a serious threat to cucurbit industry worldwide. The pathogen is seed-transmitted, so seed detection to prevent distribution of contaminated seed is crucial in disease management. In this study, we adapted a quantitative real-time PCR (qPCR) assay to droplet digital PCR (ddPCR) format for *A. citrulli* detection by optimizing reaction conditions. The performance of ddPCR in detecting *A. citrulli* pure culture, DNA, infested watermelon/melon seed and commercial seed samples were compared with multiplex PCR, qPCR, and dilution plating method. The lowest concentrations detected (LCD) by ddPCR reached up to 2 fg DNA, and 10^2 CFU mL^{-1} bacterial cells, which were ten times more sensitive than those of the qPCR. When testing artificially infested watermelon and melon seed, 0.1% infestation level was detectable using ddPCR and dilution plating method. The 26 positive samples were identified in 201 commercial seed samples through ddPCR, which was the highest positive number among all the methods. High detection sensitivity achieved by ddPCR demonstrated a promising technique for improving seed-transmitted pathogen detection threshold in the future.

Keywords: bacterial fruit blotch, *Acidovorax citrulli*, droplet digital PCR, seed detection, quantitative real-time PCR

1. Introduction

Bacterial fruit blotch (BFB), caused by *Acidovorax citrulli*, is a major yield-limiting disease of cucurbitaceous crop worldwide (Bahar *et al*. 2010; Burdman and Walcott 2012; Giovanardi *et al*. 2018). The bacterial pathogen can lead to devastating losses of the marketable fruit. Since the first BFB outbreak occurred in watermelon in 1987, it has become a serious threat to the cucurbitaceous crop production (Wall and Santos 1988). The originate sources of *A. citrulli*

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dispersal include contaminated seed, infected transplants and alternative hosts (Choi *et al*. 2016). *Acidovorax citrulli* can be easily spread by irrigation, machinery, workers and other media in the hot, wet conditions and overwinters in seed. Seed-transmitted *A*. *citrulli* represents the most important source of inoculum for BFB outbreak (Rane and Latin 1992). To alleviate the effects of seedborne *A*. *citrulli*, the seeds were produced in cool and dry conditions. Since the high threat of BFB outbreak, it is important and necessary to use the sensitive detection techniques to exclude the contaminated seed lots.

Droplet digital polymerase chain reaction (ddPCR) is an emerging and powerful molecular biotechnology to quantify the absolute number of target DNA presents in a sample. The advantage of ddPCR compared with quantitative real-time PCR (qPCR) is that it is needless for a calibration curve in quantifying target nucleic acids. Unlike qPCR, ddPCR measures fluorescence signals at the end-point and partitions PCR reaction into thousands of nanoliter-sized discrete reactions which is water-in-oil droplets. Since every discrete reaction contains zero or at least one copy of the target DNA, post-PCR droplets are analyzed individually in a binary (positive or negative) outcome (Hindson *et al*. 2011; Pinheiro *et al*. 2012). Fluorescent and non-fluorescent droplets are defined as positive (presence of target sequence) or negative (absence of target sequence), respectively. The number of target DNA molecules in the original sample can be determined by counting the number of positive droplets and Poisson statistics (Hindson *et al*. 2011). Recently, more and more efforts have focused on the role of ddPCR in diagnosis of cancer, routine analysis of the presence of genetically modified organism (GMO) in food, and quantification of viruses and bacterial pathogens (Dobnik *et al*. 2016). High accuracy and precision were achieved by ddPCR assay in detecting human protozoan parasites *Plasmodium falciparum* and *Plasmodium vivax* (Koepfli *et al*. 2016), and viral pathogen BK virus (Bateman *et al*. 2017). Additionally, several studies have demonstrated the application of ddPCR in detecting several plant pathogens, including *Erwinia amylovora*, *Ralstonia solanacearum*, *Xanthomonas citri* subsp. *citri*, and *Candidatus* Liberibacter asiaticus (Dreo *et al*. 2014; Zhao *et al*. 2016; Zhong *et al*. 2018). Compared with qPCR assay, the ddPCR assay showed higher resilience to PCR inhibitors in detection of *X*. *citri* subsp. *citri*, the pathogen causing citrus bacterial canker (Zhao *et al*. 2016).

The aim of this study was to develop ddPCR assay to detect *A*. *citrulli* in melon and watermelon seeds. We adapted and optimized qPCR assay for *A*. *citrulli* to ddPCR format. The sensitivity and stability of ddPCR assay in the detecting the target bacteria cells, bacterial DNA and infested watermelon/melon seed were assessed and compared with other molecular-based assays, including multiplex PCR, qPCR and dilution plating.

2. Materials and methods

2.1. Bacterial strains and cell suspension preparation

Acidovorax citrulli strains were divided into two groups based on BOXPCR fingerprint (Walcott 2004). Representative *A*. *citrulli* strains NM-4 (Group I) and strain Xu 3-14 (Group II) were used in this study for melon and watermelon seed inoculation, respectively. Strain Xu 3-14 was originally isolated from watermelon seedlings in a greenhouse in Beijing in 2014 and strain NM-4 was isolated from melon in Inner Mongolia of China in 2012. Both strains were maintained in 15% glycerol at –80°C and re-streaked on PF (Schaad 1999) medium plate to obtain fresh cultures.

To prepare bacterial suspension at different concentrations for molecular assays, a single colony of *A*. *citrulli* strain Xu 3-14 was inoculated in TBY broth and cultured at 28°C, 120 r min^{-1} for 24 h. Bacterial cells were collected by centrifugation and resuspension in sterilized distilled water, the concentration was adjusted to OD_{600}=0.5. Ten-fold serial dilutions were prepared in 1-mL sterilized water and 100 μL was plated on PF medium plate for confirmation of concentration, the rest were stored at –20°C for molecular assays. The original concentration of bacterial suspension was 5.2×10^8 CFU mL^{-1} (OD_{600}=0.5), calculated based on serial dilution plating on PF medium.

2.2. Seed inoculation and commercial seed sample collection

Artificially infested seeds were prepared by inoculating seed with strain Xu 3-14 (watermelon) and NM-4 (melon). Briefly, the bacterial strain was grown overnight in 100-mL TBY broth at 30°C under 180 r min^{-1}. Pre-tested healthy watermelon seeds and melon seeds (n=100) were inoculated by soaking seeds in bacterial suspension (10^6 CFU mL^{-1}) at 28°C under 120 r min^{-1} for 30 min. The inoculated seeds were air dried at room temperature. Seed samples at different infection levels were prepared by mixing a single inoculated seed with 100, 500 and 1 000 healthy seeds for both melon and watermelon, respectively. Five replications of seed samples with infestation level of 1, 1/100, 1/500, and 1/1 000 were prepared.

For testing of commercial seed samples, total of 201 seed samples from 62 seed lots were collected from Beijing seed market, each sample contained 500 seeds. None of the collected seed sample was coated with fungicide.

2.3. Seed sample detection by dilution plating

The watermelon/melon seed samples were soaked in phosphate buffer (pH=7.4) at 28°C for 4 h, the seed soaking buffer was collected and centrifuged at 8 000 r min^{-1} for 10 min. The supernatant was discarded and pellet was resuspended in 1-mL sterilized water. Seed extraction suspension was serial diluted in sterilized water and each dilution was plated on PF and mEBB media for bacterial isolation (Schaad 1999). Plates were incubated at 28°C for three to four days and suspicious colonies were confirmed with PCR. The bacterial amount in seed sample was calculated as: (Number of confirmed colonies/Plating volume)×Dilution factor×Total volume. The undiluted seed extraction suspension was divided into two sub-samples: One sub-sample was stored at –20°C for molecular-based assays, the other sub-sample was used for DNA extraction.

2.4. DNA extraction of bacteria and seed extraction

DNA from the pure bacterial cultures and seed extracts was extracted and purified using EasyPure Bacteria Genomic DNA Kit (Transgen, Beijing). The DNA of pure bacterial culture was diluted to concentrations of 20 ng μL^{-1}, 2 ng μL^{-1}, 200 pg μL^{-1}, 20 pg μL^{-1}, 2 pg μL^{-1}, 200 fg μL^{-1}, 20 fg μL^{-1}, and 2 fg μL^{-1}, and then stored at –20°C until analysis.

2.5. Multiplex PCR assay

In PCR-based assays, tested samples included bacterial suspension, bacterial DNA, seed extraction, and seed extraction DNA. Negative control included sterilized water, and healthy seed extract. Samples were set up in duplicates as technical replications. Multiplex PCR using two sets of primers WFB 1/2 and SEQ 4/5 was performed on T100™ Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA). Primer and probe information were listed in Table 1. The PCR cycling program was set up for 5 min at 94°C, followed by 30 s at 94°C, 30 s at 55°C, and 40 s at 72°C, and finally ended with 5 min at 72°C (Walcott and Gitaitis 2000; Walcott *et al*. 2003). PCR products were checked by agarose gel electrophoresis, and the presence of a 360-bp band (primer WFB 1/2) or 246-bp band (primer SEQ 4/5) was recorded for positive.

2.6. qPCR assay

Same templates used for PCR were used for qPCR assay. qPCR reactions were performed on LightCycler® 480 System (Roche, Basel, Switzerland) with cycling conditions: 3 min at 95°C, followed by 45 cycles of 15 s at 95°C, and 20 s at 60°C, and final 5 min at 40°C, using standard temperature ramping mode. The reaction volumes of 25 µL contained 0.35 µL primer BoxAACF (20 µmol L^{-1}), 0.35 µL primer BoxAACR2 (20 µmol L^{-1}), 0.3 µL 6-FAM and BHQ-1 labeled probes (20 µmol L^{-1}), 12.5 µL 2× qPCR master mix and 1 µL sample (Ha *et al*, 2009). No template control and standard curve constructed for serials dilutions of bacteria suspensions were included in all runs and every sample was measured in replicates.

2.7. Droplet digital PCR assay

ddPCR was performed with QX200 Droplet Digital PCR System and T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). The QX200 Droplet Digital PCR System consists of two instruments, the QX200 droplet generator and reader, and QuantaSoft for data analysis.

To optimize the ddPCR reaction conditions, eight gradient annealing temperatures and three different primer/probe concentrations were tested and compared. The ddPCR reaction volumes of 20 µL contained 0.3 µL primer BoxAACF (20 µmol L^{-1}), 0.3 µL primer BoxAACR2 (20 µmol L^{-1}), 0.3 µL 6-FAM and BHQ-1 labeled probes (20 µmol L^{-1}), 10 µL 2×ddPCR Supermix for probes and 1 µL sample. After droplet generation, 40 µL of the generated droplet emulsion was transferred to a new 96-well PCR plate (Eppendorf) and amplified in T100™ Thermal Cycler. The temperature ramp rate was set to 2.5°C s^{-1} according to the Bio-Rad ddPCR recommendation.

Table 1 List of primers and Taqman probe used for detecting *Acidovorax citrulli* in this study

Primer	Sequence (5´→3´)	Amplicon		Reference
		Size (bp)	Copy number	
SEQ 4	CCTCCACCAACCAATACGCT	246	3	Walcott *et al*. (2003)
SEQ 5	GTCATTACTGAATTTCAACA			
WFB 1	GACCAGCCACACTGGGAC	360	3	Walcott and Gitaitis (2007)
WFB 2	CTGCCGTACTCCAGCGAT			
BoxAACF	GCGTATGAGTCCCGA AGA AAT	121	1	Ha *et al*. (2009)
BoxAACR2	GCA TGCCTTGTATTCAGCTAT			
AACPROBE	6-FAM-CCGAAATCCGTATTGGACGGATCGAA-BHQ-1			

To access the performance of ddPCR in detecting bacterial and seed samples, same templates used for PCR were used for ddPCR assay. After the thermal cycling, the plate was transferred to a droplet reader (Bio-Rad). The software package provided with the Droplet Digital PCR (ddPCR™) System (QuantaSoft 1.7, Bio-Rad) was used for data analysis.

2.8. Data analysis

The qPCR Cq values were generated by Light Cycler® 480 Software (Roche, Basel, Switzerland). For ddPCR, positive and negative droplets were discriminated by applying a fluorescence amplitude threshold with the QuantaSoft version 1.7 (Bio-Rad, Hercules, CA, USA). The copy concentrations were automatically reported as copies μL^{-1} of the final 1× ddPCR reaction by QuantaSoft software. Linear regression analyses of the template starting concentrations with Cq values from qPCR and copy number concentrations from ddPCR were performed using Minitab/Regression/Fitted Line plot (version 18).

3. Results

3.1. Optimization of the ddPCR assay

To determine the optimum annealing temperature for the ddPCR assay, temperature gradient range from 50 to 61°C was set on the thermal cycler. As showed in Fig. 1-A, the largest difference in fluorescence intensity between negative and positive droplets were obtained at 60.1°C. Therefore, the optimized ddPCR conditions was 10 min at 95°C, followed by 45 cycles of a two-step thermal profile of 30 s at 94°C for denaturation, and 60 s at 60°C for annealing and extension, followed by a final hold of 10 min at 98°C for droplet stabilization and cooling to 4°C.

The concentrations of primers and probe also affected ddPCR amplification efficiency. Of the three different concentration combinations tested, primers and probe at 0.5 μmol L^{-1}/0.5 μmol L^{-1} showed the largest difference in florescence signals between positive and negative droplets (Fig. 1-B). Thus, the final reaction volume of 20 μL mix contained 10 μL of 2× ddPCR Supermix for probes (Bio-Rad), 1 μL template, 0.5 μmol L^{-1} of each primer, and 0.5 μmol L^{-1} of probe.

3.2. Comparison of analytical sensitivity, linearity and dynamic range of PCR, qPCR and ddPCR assays

To compare the analytical sensitivity and reliability of PCR, qPCR and ddPCR assays, serial diluted bacterial suspension and DNA samples of *A. citrulli* were tested and analyzed.

Analytical sensitivity (AS) of an assay was the lowest concentration that showed positive with all the five replicates, whereas the lowest concentration with at least one positive replicate was defined as the lowest concentration detected (LCD). The analytical sensitivity of multiplex PCR with two

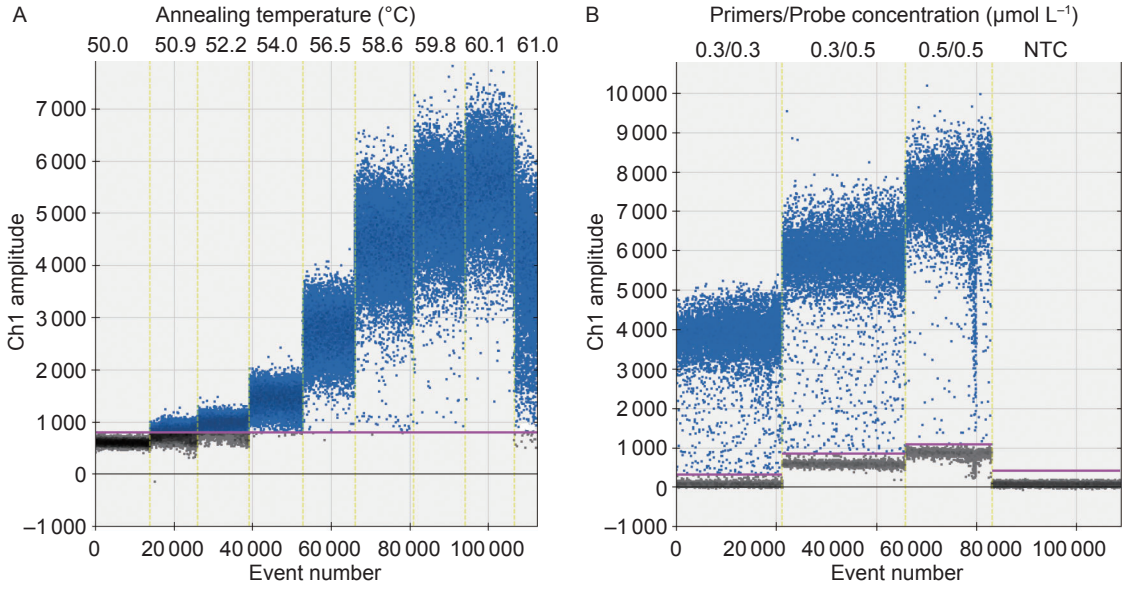

Fig. 1 Optimization droplet digital PCR (ddPCR) assay for detecting *Acidovorax citrulli*. A, fluorescence amplitude plotted against temperature gradients. B, primer/probe concentrations. The pink line is the threshold automatically identified by QuantaSoft, above which are the positive droplets (blue) with PCR amplification and below which are the negative droplets (gray) without any amplification. Each ddPCR reaction is divided by vertical dotted yellow line. NTC, no template control.

sets of primers WFB 1/2 and SEQ 4/5 in detecting bacterial cells were consistent and both up to 10^5 CFU mL^{-1} (Table 2). Compared to multiplex PCR, qPCR and ddPCR increased 10-fold and 100-fold in analytical sensitivity, respectively. The LCD of multiplex PCR, qPCR and ddPCR were 10^4, 10^3, and 10^2 CFU mL^{-1}, respectively. Similar trend was found in detection sensitivity using serial diluted DNA as template. The lowest concentration of ddPCR with reliable positive result was 20 fg μL^{-1}, and it was 10- and 10 000-fold lower than that of qPCR and PCR, respectively (Table 3). In addition, there was one positive droplet with 5 893 fluorescence units detected at 2 fg μL^{-1} in one out of five replicates by ddPCR assay.

As expected, regression analysis indicated a negative linear relationship between bacterial cells concentration and Cq values in qPCR assay, higher correlation efficacy (R^2=98.6%) was obtained with *A. citrulli* concentration range of 10^4 to 10^8 CFU mL^{-1} (Fig. 2-A). Whereas, in ddPCR assay, bacterial cell concentration and ddPCR copy numbers were positive corelated and optimal range of quantification ranged from 10^3 to 10^7 CFU mL^{-1} with R^2=99.9% (Fig. 2-B). Good correlation regressions were also obtained with DNA concentrations in both ddPCR (R^2=99.8%) and qPCR assays (R^2=99.7%) (Fig. 2-C and D).

3.3. Comparison of PCR, qPCR and ddPCR assays in testing artificially infested seeds

With regards to the sensitivity of seed sample detection, artificially infested watermelon and melon seeds at different infestation levels were prepared and tested by molecular methods and dilution plating method to confirm the accuracy of each detection method. The average inoculum on each watermelon and melon seed was 2.6×10^5 and 1.2×10^5 CFU respectively, confirmed by dilution plating method.

Samples at all infestation levels were detectable by ddPCR assay using either seed extraction or extracted DNA as template. The detection sensitivity was 1/1000 infestation level for both melon and watermelon seeds. Surprisingly, multiplex PCR only detected 1/1 infested seed

Table 2 Performance of PCR, real-time PCR and droplet digital PCR (ddPCR) in detecting *Acidovorax citrulli* cells

Starting concentration (CFU mL^{-1})	Positive/All replicates			
	PCR primer		Real-time PCR (Cq)	ddPCR (lg(copies μL^{-1}))
	WFB 1/2	SEQ 4/5		
2.67×10^8	5/5	5/5	5/5 (18)	5/5 (4.05)
2.67×10^7	5/5	5/5	5/5 (20)	5/5 (4.01)
2.67×10^6	5/5	5/5	5/5 (25)	5/5 (3.01)
2.67×10^5	5/5	5/5	5/5 (30)	5/5 (1.96)
2.67×10^4	3/5	3/5	5/5 (32)	5/5 (0.96)
2.67×10^3	Neg	Neg	3/5 (35)	5/5 (0.11)
2.67×10^2	Neg	Neg	Neg	3/5 (0.06)
2.67×10	Neg	Neg	Neg	Neg
Analytical sensitivity[1)]	lg5	lg5	lg4	lg3
Lowest concentration detected[2)]	lg4	lg4	lg3	lg2

[1)] Analytical sensitivity, the lowest bacterial cell concentration that all five replicates were positive.
[2)] Lowest concentration detected, the lowest bacterial cell concentration that at least one replicate was positive.
Neg, five replicates were all negative.

Table 3 Performance of PCR, real-time PCR and droplet digital PCR (ddPCR) in detecting *Acidovorax citrulli* DNA

Starting concentration (ng μL^{-1})	Positive/All replicates			
	PCR primer		Real-time PCR (Cq)	ddPCR (lg(copies μL^{-1}))
	WFB 1/2	SEQ 4/5		
2	5/5	5/5	5/5 (24.8)	5/5 (3.79)
2×10^{-1}	5/5	5/5	5/5 (27.7)	5/5 (2.85)
2×10^{-2}	Neg	Neg	5/5 (30.5)	5/5 (1.98)
2×10^{-3}	Neg	Neg	5/5 (32.6)	5/5 (1.08)
2×10^{-4}	Neg	Neg	5/5 (35.9)	5/5 (0.34)
2×10^{-5}	Neg	Neg	2/5 (36.5)	5/5 (0.23)
2×10^{-6}	Neg	Neg	Neg	1/5 (0.08)
Analytical sensitivity[1)]	200 pg μL^{-1}	200 pg μL^{-1}	200 fg μL^{-1}	20 fg μL^{-1}
Lowest concentration detected[2)]	200 pg μL^{-1}	200 pg μL^{-1}	20 fg μL^{-1}	2 fg μL^{-1}

[1)] Analytical sensitivity, the lowest bacterial cell concentration that all five replicates were positive.
[2)] Lowest concentration detected, the lowest bacterial cell concentration that at least one replicate was positive.
Neg, five replicates were all negative.

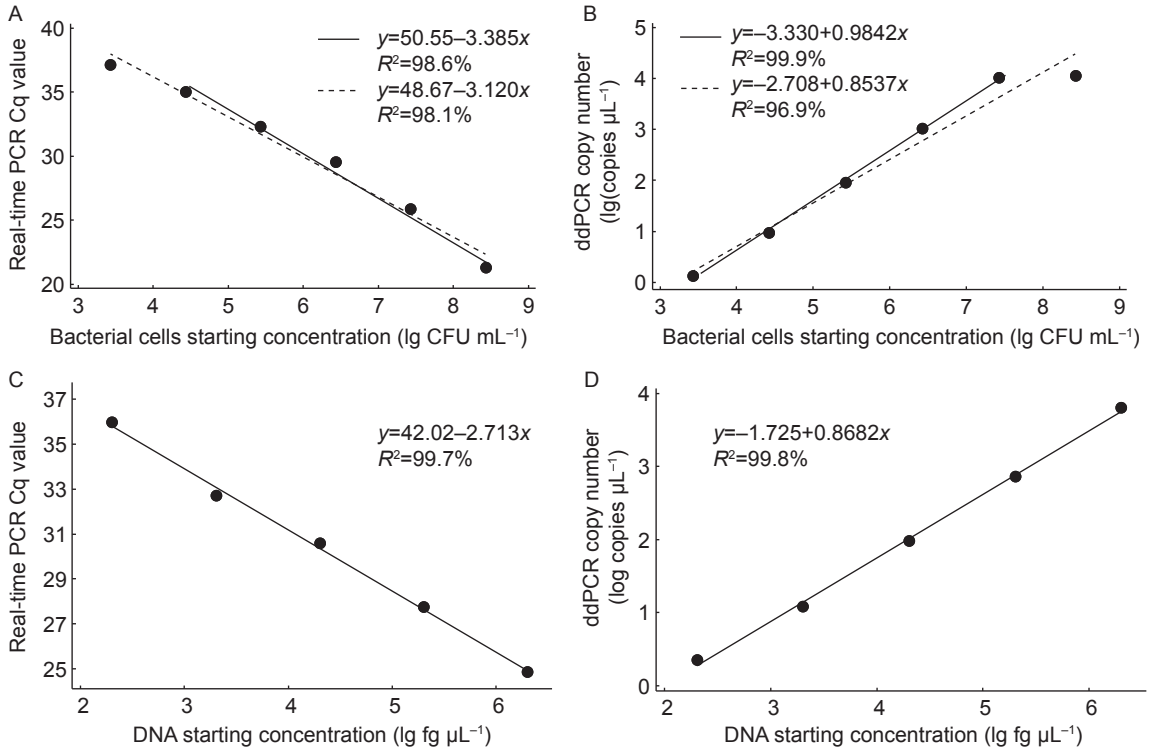

Fig. 2 Liner regression of real-time PCR assay Cq values and droplet digital PCR (ddPCR) copy numbers *vs*. bacterial cell (A and B) and genomic DNA (C and D) at different starting concentrations generated by ten-fold serial dilution. Two linear regression lines were drawn for bacterial cells at different concentrations *vs*. Cq values (A) and *vs*. the copy number (B) to compare the difference in quantification ranges of real-time PCR and ddPCR assay. In the detection of bacteria cells, six concentrations were tested (broken line in A and B), the data showed that the quantification range were obviously affected between the two detection assays. When tested by real-time PCR assays, the best quantification range was obtained as 10^4 to 10^8 CFU mL^{-1} (A, unbroken line). Whereas in ddPCR detection, the best quantification range was 10^3–10^7 CFU mL^{-1} (B, unbroken line).

(Table 4). The result of PCR detection showed the lowest sensitivity in detecting artificially infested seed samples. There were only three out of five samples and two out of five samples detected by PCR at 1/1 infestation level on detection of watermelon and melon seeds, respectively, whereas the result of samples at lower infestation levels were all negative. It was also unsuccessful in detecting DNA samples extracted from the suspension as template. qPCR was more sensitive compared with PCR. Most of the samples at 1/100 infestation level had been detected in qPCR detection, whereas samples at 1/500 infestation level were also detectable using qPCR for both watermelon (one out of five detected) and melon samples (one out of five detected). Interestingly, the results showed the same trend that using seed extract directly as the template could identify more positive samples compared with using DNA obtained from seed extract as the template by all the PCR-based molecular assays. It was suspected that the target DNA was lost during the DNA extraction process, because in the case of low concentration of target bacteria of the seed extraction, the DNA could not be detected.

3.4. Comparison of PCR, qPCR and ddPCR assays in testing commercial seed samples

A total of 201 commercial seed samples were tested with three PCR-based methods, and dilution plating assay. Four patterns of results were obtained. Majority samples (n=175) showed negative results by all the four assays (Pattern 1). Sixteen samples were positive by ddPCR assay but negative by other assays (Pattern 2). Seven samples were detected as positive by both ddPCR and dilution plating (Pattern 3) and only three samples were positive by qPCR, ddPCR and dilution plating (Pattern 4). No positive sample was identified by multiplex PCR. As expected, the highest positive sample number was identified by ddPCR assay (Table 5). Samples in Pattern 2 (only positive by ddPCR assay) were further tested by seedling grow-out assays and no symptomatic seedlings were observed. As ddPCR was a very sensitive molecular assay that detected very low titer of bacterial DNA, it was highly suspected that the *A. citrulli* titers in seed were too low to induce disease or bacterial cells were dead after seed treatments in those 16 samples.

Table 4 Comparation of different methods in detecting *Acidovorax citrulli* in artificially infested melon and watermelon seeds

Sample	Infestation level[1)]	Positive/All replicates				
		PCR primer		Real-time PCR (Cq)	ddPCR (lg(copies μL^{-1}))	Dilution plating (titer)
		WFB 1/2	SEQ 4/5			
Watermelon						
Seed extract	1/1	3/5	3/5	5/5 (28)	5/5 (1.78)	5/5 (2.6×10^5 CFU)
	1/100	Neg	Neg	4/5 (32)	5/5 (1.26)	5/5
	1/500	Neg	Neg	1/5 (35)	4/5 (1.08)	3/5
	1/1 000	Neg	Neg	Neg	2/5 (0.62)	1/5
DNA of seed extract	1/1	Neg	Neg	3/5 (34)	5/5 (1.02)	–
	1/100	Neg	Neg	1/5 (35)	3/5 (0.83)	–
	1/500	Neg	Neg	Neg	2/5 (0.52)	–
	1/1 000	Neg	Neg	Neg	2/5 (0.20)	–
Melon						
Seed extract	1/1	2/5	2/5	5/5 (30)	5/5 (1.99)	5/5 (1.2×10^5 CFU)
	1/100	Neg	Neg	3/5 (35)	5/5 (1.88)	5/5 (1.1×10^4 CFU)
	1/500	Neg	Neg	1/5 (35)	4/5 (1.08)	3/5 (2.4×10^3 CFU)
	1/1 000	Neg	Neg	Neg	2/5 (0.58)	1/5
DNA of seed extract	1/1	Neg	Neg	2/5 (36)	5/5 (1.01)	–
	1/100	Neg	Neg	1/5 (36)	4/5 (0.72)	–
	1/500	Neg	Neg	Neg	3/5 (0.41)	–
	1/1 000	Neg	Neg	Neg	2/5 (0.18)	–

[1)] Artificially infested seed samples, 1 infested seed was mixed with 100, 500 or 1 000 seeds to generate different infestation levels. Neg, all replications were negative; –, sample was DNA, not applicable for dilution plating method.

Table 5 Results of detecting *Acidovorax citrulli* in commercial melon and watermelon seed samples by different assays

Result pattern	PCR	Real-time PCR	ddPCR	Dilution plating	No. of samples
Pattern 1	–	–	–	–	175
Pattern 2	–	–	+	–	16
Pattern 3	–	–	+	+	7
Pattern 4	–	+	+	+	3
No. of positive sample	0	3	26	10	

–, negative result; +, positive result.

4. Discussion

This is the first study on application of ddPCR in detecting bacterial pathogens in seed. The lowest concentration of *A. citrulli* DNA and bacterial suspension detected by ddPCR was 2 fg μL^{-1} and 10^2 CFU mL^{-1}, respectively, which was both ten times lower than that detected by qPCR. These results were consistent with previous research reported that the detection threshold value of qPCR on *A. citrulli* DNA and cells was 100 fg μL^{-1} and 10^2 CFU mL^{-1}, respectively (Ha *et al*. 2009). Other molecular-based method, such as padlock-probe-based assay showed similar detection sensitivity in testing *A. citrulli* DNA with qPCR (Zhang *et al*. 2013).

The genome size of *A. citrulli* was about 5 Mb (GenBank accession NC_008752), thus the weight of *A. citrulli* genome DNA was approximately 5 fg. Since only 1-μL template was added in each reaction for each PCR-based assay, it made sense that DNA template at 2 fg μL^{-1} with approximately half genome was detectable in one out of five replicates by ddPCR assay. Considering there was only one copy of amplified target by primers BOXAACF/R2 in both qPCR and ddPCR (Table 1), the analytical sensitivity might be improved using primers that amplifies target with higher copy number in genome.

The LCD of ddPCR assay on infected seed was 1/1 000 infestation level for both melon and watermelon seeds, and that was 1/500 and 1/1 for both melon and watermelon by qPCR and PCR assays, respectively. Whereas the DNA extraction of seed extract would not improve detection limit, on the contrary, the target DNA might be lost during the DNA extraction procedure and led to a negative result. Previous study by Ha *et al*. (2009) also tested artificially infested watermelon seeds with qPCR, and one out of four samples could be detected at the infestation rate of 0.1%. However, the template amount used in Ha's study (Ha *et al*. 2009) was 5 μL, which was five times of the amount tested in this study. In parallel comparison using only 1 μL of seed extraction as the template, ddPCR showed obvious higher detection sensitivity than qPCR assay in all the sample types

including DNA, bacterial whole cells, infested seed extract.

The generally accepted seed testing method for *A. citrulli* is seedling grow-out assay, which is time-consuming and labor intensive. Seedling grow-out detection requires 10 000–30 000 seeds grow out in the greenhouse and observation by experienced inspector for two–three weeks. The PCR-based methods have also been widely used for seed health detection, however, the inhibitory compounds released from seed and saprophytes interfere with the detection and may lead to false negative (Feng 2013). The newly released International Seed Health Initiative (ISIH) recommended method for *A. citrulli* detection added qPCR as a pre-screen option for testing seed extract (ISF 2018). Positive samples identified in pre-screen will be subject to grow out assay and negative samples can be regarded as healthy seed. As shown in this study with testing the commercial seed samples, qPCR had false negative results compared with ddPCR and dilution plating. Therefore, application of ddPCR for seed extract pre-screen would be more sensitive and reliable. Though the cost for each reaction of ddPCR is much higher than that of qPCR, seed detection by ddPCR assay will reduce subsample numbers by increasing subsample size for its higher detection sensitivity. For molecular pre-screening of large account of seed samples, ddPCR could be economically comparable to qPCR with less false negative.

5. Conclusion

A new *A. citrulli* seed detection method based on ddPCR technique were established. The feasibility of the ddPCR assay were evaluated by comparing with the detection sensitivity of other seed detection methods. The results demonstrated the ddPCR assay was a reliable alternative method with higher sensitivity, accuracy in seed detection of *A. citrulli*. Moreover, higher detection sensitivity achieved by ddPCR demonstrated a promising direction for improving seed-borne pathogen detection sensitivity in the future.

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