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Rapid Detection of Viable Salmonellae in Produce by Coupling Propidium Monoazide with Loop-Mediated Isothermal Amplification▽

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Recent outbreaks linked to *Salmonella*-contaminated produce heightened the need to develop simple, rapid, and accurate detection methods, particularly those capable of determining cell viability. In this study, we examined a novel strategy for the rapid detection and quantification of viable salmonellae in produce by coupling a simple propidium monoazide sample treatment with loop-mediated isothermal amplification (PMA-LAMP). We first designed and optimized a LAMP assay targeting *Salmonella*. Second, the performance of PMA-LAMP for detecting and quantifying viable salmonellae was determined. Finally, the assay was evaluated in experimentally contaminated produce items (cantaloupe, spinach, and tomato). Under the optimized condition, PMA-LAMP consistently gave negative results for heat-killed *Salmonella* cells with concentrations up to 10^8 CFU/ml (or CFU/g in produce). The detection limits of PMA-LAMP were 3.4 to 34 viable *Salmonella* cells in pure culture and 6.1×10^3 to 6.1×10^4 CFU/g in spiked produce samples. In comparison, PMA-PCR was up to 100-fold less sensitive. The correlation between LAMP time threshold (T_T) values and viable *Salmonella* cell numbers was high ($R^2 = 0.949$ to 0.993), with a quantification range (10^2 to 10^5 CFU/reaction in pure culture and 10^4 to 10^7 CFU/g in produce) comparable to that of PMA in combination with quantitative real-time PCR (PMA-qPCR). The complete PMA-LAMP assay took about 3 h to complete when testing produce samples. In conclusion, this rapid, accurate, and simple method to detect and quantify viable *Salmonella* cells in produce may present a useful tool for the produce industry to better control potential microbial hazards in produce.

Nontyphoidal *Salmonella* is a leading cause of food-borne illness worldwide, with an estimated 1.03 million cases, 19,336 hospitalizations, and 378 deaths occurring in the United States each year (40). According to the U.S. Centers for Disease Control and Prevention (CDC), in 2009, *Salmonella* was responsible for over 40% of the total laboratory-confirmed infections from 10 bacterial/parasitic enteric agents under FoodNet surveillance (4). Furthermore, in recent years, an increasing number of *Salmonella* outbreaks linked to produce has been observed, implicating a wide variety of items such as melons, tomatoes, sprouts, mangoes, and peppers (3, 13). Therefore, multifaceted approaches are needed to better ensure produce safety, among which simple, rapid, and accurate detection methods that determine cell viability are especially needed in order to identify potential live *Salmonella* contamination problems throughout the production, processing, and distribution of produce.

Traditional culture-based methods for detecting *Salmonella* are reliable but labor-intensive and time-consuming, demanding several days for a definitive result (1). Immunoassays such as enzyme-linked immunosorbent assay (ELISA) have been developed for *Salmonella* detection (22). However, low specificity has limited their use (6). Recently, molecular biology-based methods such as PCR and real-time quantitative PCR (qPCR) have been used widely to detect *Salmonella* (21, 36). Although they are rapid and sensitive, a sophisticated thermal cycling instrument is an essential requirement for these techniques. More recently, loop-mediated isothermal amplification (LAMP) (32) has emerged as a promising alternative to detecting food-borne bacterial and viral agents. LAMP uses four specially designed primers and a strand-displacing *Bst* DNA polymerase to produce a target-specific stem-loop DNA structure during initial assay steps, followed by quasiexponential amplification of this structure under isothermal conditions (60 to 65°C), resulting in 10^9 copies of target DNA within an hour (32). The addition of one to two loop primers accelerates the LAMP reaction by hybridizing to stem-loop DNAs and facilitating strand displacement and amplification (25). Since it is isothermal, LAMP can be performed in much simpler instruments such as a heater or water bath. To date, LAMP assays have been developed for *Campylobacter* spp. (47), Shiga toxin-producing *Escherichia coli* (14), norovirus (50), *Staphylococcus aureus* (9), *Vibrio parahaemolyticus* (5, 26), and *Vibrio vulnificus* (10, 12), as well as *Salmonella* (15, 19, 20, 33, 34, 45, 48). Although LAMP was reported to be rapid, specific, and sensitive, several of the *Salmonella* LAMP studies (33, 34, 48) targeted only one specific *Salmonella* serovar or serogroup, and none of the studies evaluated the quantification of salmonellae in produce samples by LAMP. Additionally, a major drawback associated with DNA-based molecular detection assays, LAMP included, is the inability to distinguish viable from dead cells.

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TABLE 1. Bacterial strains used in this study

Group/genus and species	Strain ID and serotype	Origin and reference/source
Salmonella (n = 28)	H9812; Braenderup	Unknown
	LT2, UMD 373; Typhimurium	Unknown
	S133, S134; Agona	Chicken, retail, Louisiana (18)
	S32, S33, S61, S62; Braenderup	
	S49, S50; Enteritidis	
	S37, S38, S98, S99; Hadar	
	S67, S68, S70, S71, S127, S128; Kentucky	
	S16, S46, S47; Mbandaka	
	S8, S9; Montevideo	
	S25, S26; Thompson	
Non-*Salmonella* (n = 25)		
Campylobacter jejuni	81-176	Human
Campylobacter jejuni	ATCC 33560	Bovine feces
Citrobacter freundii	ATCC 8090	Unknown
Enterobacter aerogenes	ATCC 13048	Sputum, South Carolina
Enterococcus faecalis	ATCC 29212	Urine
Escherichia coli	ATCC 25922	Human
Listeria monocytogenes	ATCC 13932; 4b	Spinal fluid, Germany
Litonella anguillarum	ATCC 19264	Ulcerous lesion in cod, United Kingdom
Pseudomonas aeruginosa	ATCC 27853	Human blood
Shigella flexneri	ATCC 12022; 2b	Unknown
Shigella sonnei	ATCC 25931	Human feces, Panama
Staphylococcus aureus	ATCC 29213	Wound
Streptococcus pneumoniae	ATCC 49619; type 59	Sputum, Arizona
Vibrio alginolyticus	ATCC 17749	Spoiled horse mackerel, Japan
	ATCC 33787	Seawater, Hawaii
Vibrio cholerae	ATCC 14035; O1	NCTC, United Kingdom
Vibrio cincinnatiensis	ATCC 35912	Blood/cerebrospinal fluid, Ohio
Vibrio fluvialis	ATCC 33809	Human feces, Bangladesh
Vibrio harveyi	ATCC 14126	Dead amphipod, Massachusetts
	ATCC 35084	Brown shark, Maryland
Vibrio mimicus	ATCC 33653	Human ear, North Carolina
	ATCC 33655	Feces, Tennessee
Vibrio natriegens	ATCC 14048	Salt marsh mud, Georgia
Vibrio parahaemolyticus	ATCC 17802; O1:K1	Shirasu food poisoning, Japan
Vibrio vulnificus	ATCC 27562	Blood, Florida

Several strategies have been used in molecular detection assays to differentiate viable/dead cells. First, as bacterial mRNA degrades more rapidly than DNA after cell death, assays targeting mRNA such as reverse transcriptase PCR, nucleic acid sequence-based amplification (NASBA), and reverse transcriptase LAMP have greater potential to detect only viable cells (8, 41, 42). However, working with RNA is technically demanding and some mRNA molecules can persist in dead cells for extended periods, leading to false-positive results (27). More recently, ethidium monoazide (EMA) or propidium monoazide (PMA) sample treatment has been combined with qPCR to distinguish viable from dead cells in *Campylobacter*, *E. coli* O157:H7, *Listeria monocytogenes*, *Salmonella*, and *V. vulnificus* (17, 28, 31, 35, 38, 39, 44, 46). These DNA binding dyes selectively penetrate compromised membranes of dead cells but not intact membranes of viable cells and intercalate into DNA once inside the cell membrane (28). Upon exposure to intense visible light, the photoreactive azide group on the dye is converted to a highly reactive nitrene radical that cross-links with dead cell DNAs, making them unavailable for subsequent qPCR amplifications (28, 31). Both EMA (31, 44) and PMA (29) sample treatments in combination with qPCR have been previously tested in *Salmonella*. Very recently, EMA coupled with LAMP was examined to detect viable *Salmonella* cells (20). However, EMA has been reported previously to compromise EMA/qPCR results due to insufficient differentiation of live and dead bacterial cells (7), and PMA was demonstrated to be advantageous over EMA in terms of dead cell exclusivity (28). Additionally, in that EMA-LAMP study (20), no loop primers were designed, and the quantitative capability of LAMP and the application of the assay in foods were not examined.

Subsequently, the objectives of this study were 2-fold. First, we aimed to develop and evaluate a LAMP assay targeting *Salmonella invA*. Second, we examined the novel PMA-LAMP combination for the rapid and specific detection and quantification of viable *Salmonella* in spiked produce samples.

MATERIALS AND METHODS

Bacterial strains and DNA template preparation. *Salmonella enterica* serovar Typhimurium LT2 was used for assay optimization and sensitivity testing. An additional 27 *Salmonella* strains of 10 serovars and 25 non-*Salmonella* strains (Table 1) were used to evaluate assay specificity. *Salmonella* strains were cultured using Trypticase soy agar or broth (TSA or TSB, respectively; BD Diagnostic Systems, Sparks, MD) at 37°C overnight. Non-*Salmonella* strains were grown on TSA or blood agar (BD Diagnostic Systems), and *Campylobacter* strains were grown under microaerophilic conditions (85% N_2, 10% CO_2, and 5% O_2).

To make DNA templates for specificity testing, several single colonies were suspended in 500 μl of TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA; Sigma-

TABLE 2. LAMP and PCR primers used in this study to detect *Salmonella* by targeting the *invA* gene

Primer name	Sequence (5′–3′)	Position[a]	Amplicon size (bp)	Reference
LAMP primers				
F3	CGGCCCGATTTTCTCTGG	503–520	Ladder-like bands with variable sizes	This study
B3	CGGCAATAGCGTCACCTT	665–682		
FIP	GCGCGGCATCCGCATCAATA-TGCCCGGTAAACAGATGAGT	573–592 (F1c), 527–546 (F2)		
BIP	GCGAACGGCGAAGCGTACTG-TCGCACCGTCAAAGGAAC	593–612 (B1c), 635–652 (B2)		
Loop-F	GGCCTTCAAATCGGCATCAAT	547–567		
Loop-B	GAAAGGGAAAGCCAGCTTTACG	613–634		
PCR and qPCR primers				
139	GTGAAATTATCGCCACGTTCGGGCAA	371–396	285	36
141	TCATCGCACCGTCAAAGGAACC	634–655		

[a] The positions are numbered based on the coding sequence of *Salmonella* serovar Typhimurium *invA* gene (GenBank accession number M90846).

FIG. 1. Comparison of LAMP amplification judgment graphs obtained when running the assay under optimized or prototypic conditions. Samples 1 to 3 and 4 to 6 are run under the optimized and prototypic conditions, respectively, and sample 7 is water.

Aldrich, St. Louis, MO) and heated at 95°C for 10 min in a dry heating block. After centrifugation at 12,000 × *g* for 2 min, the supernatants were stored at −20°C until use. For sensitivity testing, overnight *S.* Typhimurium LT2 culture was diluted 100-fold in fresh TSB and grown for 8 h to achieve mid-log phase (optical density at 600 nm [OD_{600}] = 1; approximately 10^9 CFU/ml). The culture was 10-fold serially diluted in TSB, and the exact cell number was determined by standard plate counting. To test LAMP sensitivity, aliquots (500 μl) of each dilution were used to prepare DNA templates similarly by the heating method. To test PMA-LAMP sensitivity in pure culture and spiked produce samples, aliquots (500 μl) of each dilution (representing viable cells) were mixed with equal volumes of 3.8×10^5 CFU/ml of heat-killed salmonellae (incubated in boiling water bath for 10 min, representing dead cells), and the mixtures were treated with PMA as described below.

LAMP primer design and reaction conditions. The *Salmonella* invasion gene (*invA*; GenBank accession number M90846) was used as the target for designing LAMP primers. A set of six primers (Table 2), two outer (F3 and B3), two inner (FIP and BIP), and two loop (Loop-F and Loop-B), which recognized eight distinct regions of the target sequence, were designed by the PrimerExplorer 4 software (Fujitsu Limited, Japan).

Based on the prototypic condition recommended by the manufacturer (Eiken Chemical Co., Ltd., Tokyo, Japan), the LAMP reagent mix and reaction condition were optimized by varying each parameter one at a time. Upon optimization, the final LAMP reagent mix in a total volume of 25 μl contained 1× ThermoPol reaction buffer (New England BioLabs, Ipswich, MA), 6 mM $MgSO_4$, 1.2 mM each deoxynucleoside triphosphate (dNTP), 0.1 μM F3 and B3, 1.8 μM FIP and BIP, 1 μM Loop-F and Loop-B, 10 U of *Bst* DNA polymerase (New England BioLabs), and 2 μl of DNA template. The reaction was carried out at 63°C for 40 min and terminated at 80°C for 5 min in a real-time turbidimeter (LA-320C; Eiken Chemical Co., Ltd.), which acquired the turbidity readings at 650 nm every 6 s. The time threshold (T_T; min) values were obtained when the turbidity increase measurements (the differential value of the moving average of turbidity) exceeded a threshold of 0.1, as shown in turbidity judgment graphs (Fig. 1). The net turbidity values during amplification were monitored in turbidity amplification graphs (Fig. 2A and 3A). Additionally, to facilitate future field applications, detection of LAMP products was also performed by adding 1 μl of 1:10-diluted original SYBR green I dye (Invitrogen, Carlsbad, CA) and observed immediately visually for color change (from orange to green or greenish yellow).

To confirm specific amplification of the *Salmonella invA* gene by LAMP, LAMP products were digested with 10 U of the restriction enzyme AluI (New England BioLabs). Digested and undigested LAMP products were analyzed side by side using electrophoresis on a 2% agarose gel containing ethidium bromide and visualized under UV light.

PCR and qPCR. As comparison, a PCR assay targeting the *Salmonella invA* gene was performed side by side with LAMP using primers (Table 2) and conditions described previously (36). In addition, a SYBR green I-based qPCR assay was also carried out in parallel. The qPCR reagent mix (25 μl) consisted of 1× FastStart SYBR green Master Mix (Roche Applied Science, Indianapolis, IN), 0.3 μM (each) primer (36), and 2 μl of DNA template. The qPCRs were conducted using 40 cycles of denaturation at 95°C for 20 s, annealing at 64°C for 30 s, and extension at 72°C for 25 s in a SmartCycler II System (Cepheid, Sunnyvale, CA). Fluorescence readings were obtained using the 6-carboxyfluorescein (FAM) channel followed by melting curve analysis from 72°C to 94°C

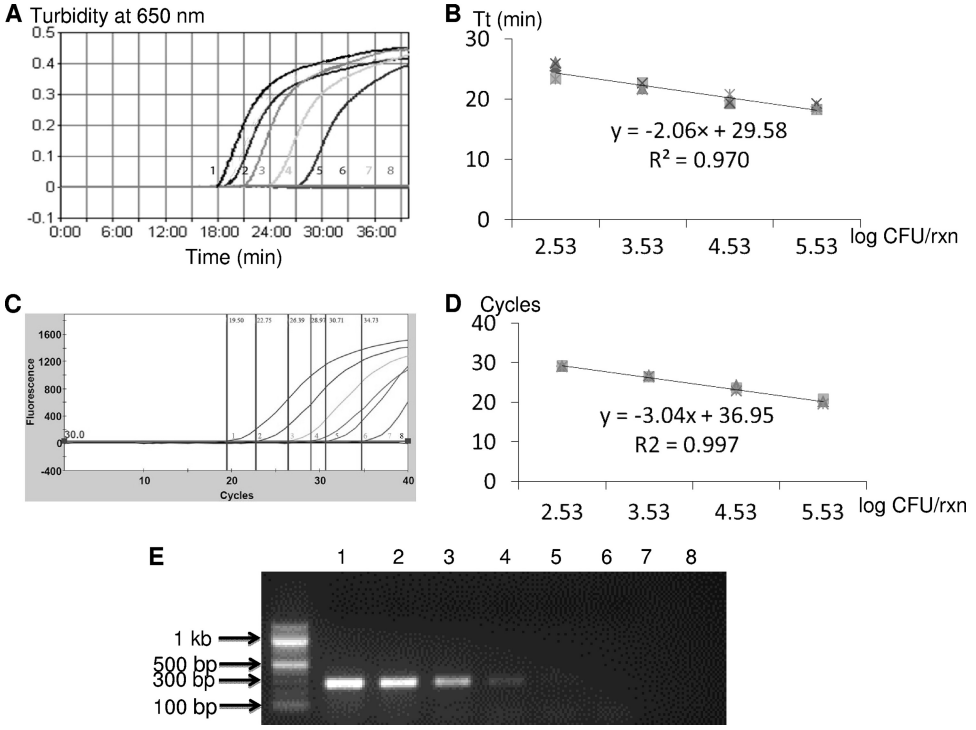

FIG. 2. Comparison of sensitivities and quantitative capabilities of PMA-LAMP and PMA-qPCR assays when testing 10-fold serially diluted viable *Salmonella enterica* serovar Typhimurium LT2 in the background of 3.8×10^5 CFU/ml of dead *Salmonella* cells in pure culture. (A) Representative PMA-LAMP amplification graph. (B) Standard curve generated based on four independent repeats of PMA-LAMP. (C) Representative qPCR optical graph. (D) Standard curve generated based on four independent repeats of PMA-qPCR. (E) Representative gel image generated by PMA-PCR. Samples 1 to 7 correspond to 10-fold serial dilutions of viable *S.* Typhimurium LT2 cells ranging from 3.4×10^5 to 3.4×10^{-1} CFU/reaction; sample 8 is water.

with increments of 0.2°C per second. The cycle threshold (C_T) values were obtained when the fluorescence reading exceeded a threshold of 30 units.

LAMP specificity and sensitivity. Fifty-three bacterial strains (Table 1) were used to determine LAMP specificity. Aliquots (2 μl) of each DNA template as prepared above were subjected to both LAMP and PCR/qPCR amplifications. Specificity tests were repeated twice.

To determine LAMP sensitivity (limit of detection), aliquots (2 μl) of the 10-fold serially diluted *S.* Typhimurium LT2 sensitivity templates prepared above were subjected to both LAMP and PCR/qPCR amplifications. Sensitivity tests were repeated four times.

PMA sample treatment and DNA extraction. Freshly thawed PMA stock solution (20 mM in 20% dimethyl sulfoxide; Biotium Inc., Hayward, CA) was added to 1 ml *Salmonella* cell suspension (viable, dead, or viable/dead mix) to a final concentration of 100 μM. The mixture was incubated in a 2-ml light-transparent microcentrifuge tube in the dark for 5 min with periodic mixing. After the dark incubation, the tube was placed horizontally on ice and exposed for 2 min to a 650-W halogen lamp (FCW 120V; GE Lighting, Cleveland, OH) at a distance of 20 cm. During light exposure, the tube was gently shaken to ensure complete cross-linkage of dead cell DNA and photolysis of unbound PMA. *Salmonella* DNA was isolated from PMA-treated samples using a microbial DNA isolation kit (Mo Bio Laboratories Inc., Carlsbad, CA) according to the manufacturer's instructions. The extracted DNA was finally eluted with 100 μl elution buffer by centrifugation.

Specificity (for viable cells) and sensitivity of PMA-LAMP. To determine whether PMA-LAMP could specifically detect only viable *Salmonella* cells but not dead ones, aliquots (2 μl) of the 10-fold serially diluted *S.* Typhimurium LT2 sensitivity DNA templates prepared above (dead cells) were subjected to PMA treatment and DNA extraction followed by both LAMP and PCR/qPCR amplifications. Specificity tests were repeated three times.

To determine the sensitivity of PMA-LAMP in detecting viable *Salmonella* in the background of dead *Salmonella* cells, mixtures of 10-fold serially diluted *S.* Typhimurium LT2 culture (viable cells) with 10^5 CFU/ml of heat-killed *Salmonella* (dead cells) were subjected to PMA treatment and DNA extraction followed by both LAMP and PCR/qPCR amplifications. Sensitivity tests were repeated four times.

Quantification of viable *Salmonella* in spiked produce. Three replicate samples of each produce item (cantaloupe, spinach, and tomato) were obtained from local grocery stores and analyzed within 4 h of purchase. To facilitate homogenization, spinach leaves were cut into 4-cm^2 squares using sterile scissors, and cantaloupe and tomato samples were sliced into fresh-cut-size pieces (2.5-cm^3 cubes and 1/8 fruit wedge, respectively) using a sterile knife. Each sample (10 g) was mixed with 90 ml of buffered peptone water (BPW; BD Diagnostic Systems) and homogenized for 2 min in a food stomacher (Model 400; Tekmar Company, Cincinnati, OH) to produce 1:10 produce-BPW homogenate. The homogenate was analyzed for the presence/absence of endogenous *Salmonella* according to methods described previously (18).

Confirmed *Salmonella*-negative produce homogenates were spiked with mixtures of *Salmonella* viable/dead cells as described above and analyzed immediately. Briefly, 100 μl of *Salmonella* cell suspension (dead or viable/dead mix) was added to 900 μl of 1:10 produce-BPW homogenate, mixed thoroughly, and centrifuged at $900 \times g$ for 3 min to remove large produce tissues. The supernatant was subjected to PMA treatment and DNA extraction as described above. Aliquots (2 μl) of the extracted DNA were used for both LAMP and PCR/qPCR amplifications. In addition to direct testing, enrichment was also performed by incubating the *Salmonella*-spiked produce homogenate at 37°C for 4 h. After enrichment, the homogenate was processed similarly as described above for direct testing. The produce tests were repeated three times.

Data analysis. For specificity data, means and standard deviations of T_T or C_T values were calculated by using Microsoft Excel software (Microsoft, Seattle, WA). For sensitivity data, means and standard deviations of T_T or C_T values for detecting 10-fold serially diluted *S.* Typhimurium LT2 in pure culture and spiked produce samples were calculated similarly using Microsoft Excel. The detection limits (CFU/reaction in pure culture or CFU/g in spiked produce) were presented as the lowest number of cells that could be detected by the assays. In spiked produce samples, CFU/reaction was calculated by using CFU/g $\times$ 0.09 g/ml $\times$ 10 $\times$ 2 $\times$ 10^{-3}, i.e., CFU/g $\times$ 1.8 $\times$ 10^{-3}. Standard curves to quantify

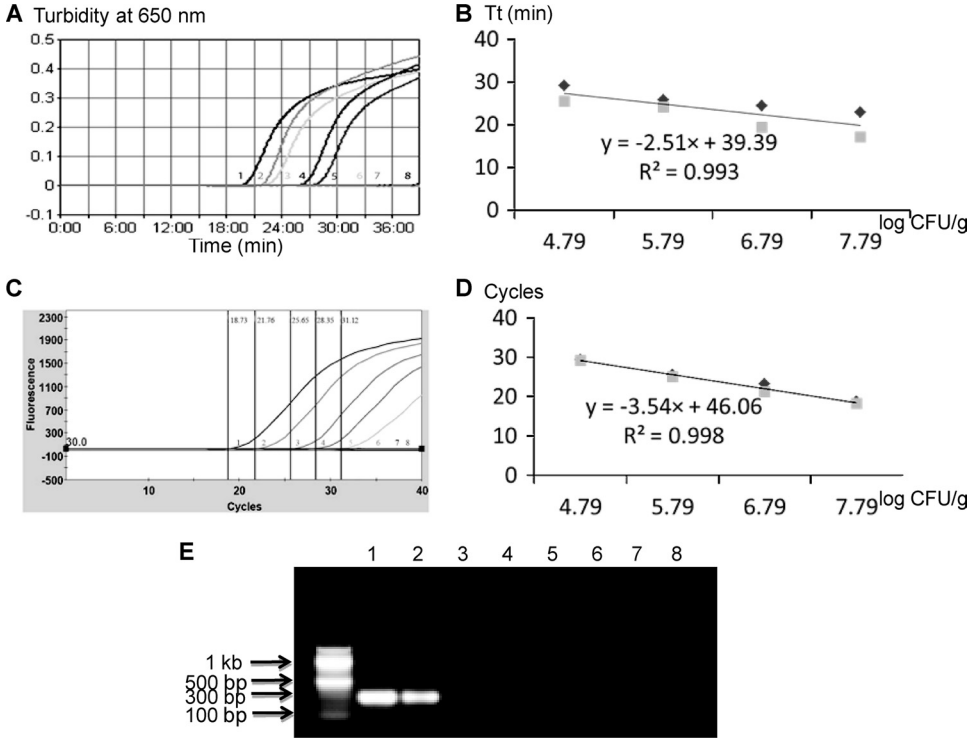

FIG. 3. Quantitative detection of *Salmonella enterica* serovar Typhimurium LT2 in spiked cantaloupe samples by PMA-LAMP and PMA-qPCR. Three sets of independent spiking experiments were performed, and the LAMP reactions were repeated two times for each set of inoculations. (A) Representative PMA-LAMP amplification graph. (B) Standard curve generated for PMA-LAMP. (C) Representative PMA-qPCR optical graph. (D) Standard curve generated for PMA-qPCR. (E) Representative gel image generated by PMA-PCR. Samples 1 to 7 correspond to spiked cantaloupe samples containing 10-fold serially diluted viable *Salmonella* cells ranging from 6.1×10^7 to 61 CFU/g in the background of dead *Salmonella* at 4.2×10^6 CFU/g; sample 8 is water.

Salmonella in pure culture and spiked produce were generated by plotting T_T or C_T values against log CFU/reaction or log CFU/g, and linear regression was calculated using Microsoft Excel. Quantitative capabilities of the assays were derived based on the correlation coefficient (R^2) values from the standard curves.

RESULTS

The optimized LAMP assay. Besides completely eliminating betaine (0.8 M in the prototype), the optimized reagent mix contained slightly modified concentrations of dNTP, primers (inner, outer, and loop), and *Bst* DNA polymerase compared to those in the prototype. Additionally, a reaction temperature of 63°C was found to be optimal in the present study. Figure 1 shows the turbidity judgment graphs generated using the optimized condition in comparison with those using the prototypic one. Besides decreasing T_T values (15 min using the optimized condition versus 19.3 min using the prototypic one), the turbidity increase measurements were also strikingly greater under the optimized condition, with maximum values of 0.26 and 0.11 under optimized and prototypic conditions, respectively.

LAMP specificity. Among 53 bacterial strains (Table 1) used to evaluate the specificity of the *invA*-based LAMP assay, no false-positive or false-negative results were observed. For the 28 *Salmonella* strains of 10 serotypes, the T_T values ranged from 15 to 17.8 min with an average of 16.3 ± 0.4 min. For the 25 non-*Salmonella* strains, no T_T value was obtained, indicating negative results for LAMP. Similarly, PCR and qPCR assays included as comparisons successfully detected 28 *Salmonella* strains while showing negative results for 25 non-*Salmonella* strains (data not shown).

Additionally, digestion of the LAMP products with AluI yielded the expected fragment of 165 bp (data not shown), indicating the specific amplification of the target *invA* sequence by LAMP.

LAMP sensitivity and quantitative capability. Table 3 summarizes the sensitivity and quantitative capability of the *invA*-based LAMP when testing 10-fold serially diluted *S.* Typhimurium LT2 DNA templates in comparison with those of PCR and qPCR. For pure culture templates ranging from 1.3×10^5 to 13 CFU/reaction, the average T_T values for LAMP based on four repeats fell between 20.4 and 27.3 min (data not shown). In one out of four repeats, amplification of the 1.3-CFU template occurred, yielding a T_T value of 37.7 min. Therefore, the detection limit for the *invA*-based LAMP assay was 1.3 to 13 CFU/reaction. Similarly, the *invA*-based qPCR had a detection limit of 1.3 CFU/reaction with C_T values averaging between 23.3 and 35.3 cycles and melting temperatures consistently falling at around 81°C (data not shown). In contrast, the *invA*-based PCR had a detection limit of 130 CFU/reaction, up to 100-fold less sensitive than that of LAMP or qPCR. Additionally, the correlation coefficients (R^2) of LAMP and qPCR were calculated to be 0.983 and 0.997, respectively, indicating excellent linear relationship between *Salmonella* cell numbers

TABLE 3. Comparison of sensitivities and quantitative capabilities of LAMP, PCR, and qPCR assays alone or in combination with PMA when testing serially diluted *Salmonella enterica* serovar Typhimurium LT2 viable cells in pure culture and spiked produce samples

Sample type[a]	Method[b]	Produce type	Detection limit (CFU/reaction or CFU/g)	Quantification equation[g]	Linear R^{2g}
Pure culture	LAMP	NA[h]	1.3–13[c]	$y = -0.85x + 24.82$	0.983
	PCR	NA	130	NA	NA
	qPCR	NA	1.3	$y = -1.82x + 32.72$	0.997
	PMA-LAMP	NA	3.4–34[d]	$y = -2.06x + 29.58$	0.970
	PMA-PCR	NA	340	NA	NA
	PMA-qPCR	NA	3.4–34[e]	$y = -3.04x + 36.95$	0.997
Spiked produce	PMA-LAMP	Cantaloupe	6.1×10^3	$y = -2.51x + 39.39$	0.993
		Spinach	6.1×10^4	$y = -4.63x + 55.47$	0.977
		Tomato	6.1×10^4	$y = -1.78x + 36.57$	0.949
	PMA-PCR	Cantaloupe	6.1×10^5	NA	NA
		Spinach	6.1×10^5	NA	NA
		Tomato	6.1×10^5	NA	NA
	PMA-qPCR	Cantaloupe	6.1×10^3	$y = -3.54x + 46.06$	0.998
		Spinach	6.1×10^2–6.1×10^3[f]	$y = -3.50x + 46.08$	0.993
		Tomato	6.1×10^2	$y = -3.47x + 46.03$	0.987

[a] Four independent repeats were conducted for pure culture testing, and three repeats were conducted for spiked produce testing.
[b] For testing involving PMA, dead *Salmonella* cells were present at a level of 3.8×10^3 CFU/reaction (or 2.1×10^5 CFU/g).
[c] One out of four repeats was positive for the 1.3-CFU/reaction level.
[d] One out of four repeats was positive for the 3.4-CFU/reaction level.
[e] Three out of four repeats were positive for the 3.4-CFU/reaction level.
[f] One out of two repeats was positive for the 6.1×10^2-CFU/g level.
[g] Quantitative equation and R^2 were calculated based on the linear relationship of average T_T or C_T values and log CFU/reaction between viable cell levels ranging from 10^2 to 10^5 CFU/reaction for pure culture and between 10^4 and 10^7 CFU/g for spiked produce samples.
[h] NA, not applicable.

(log CFU/reaction) and the amplification signals (T_T or C_T). PCR, on the other hand, is not quantitative.

Specificity of PMA-LAMP for viable *Salmonella* cells. The testing of 10-fold serially diluted dead *Salmonella* cells by PMA-LAMP, PMA-PCR, and PMA-qPCR indicated that after PMA treatment, LAMP consistently gave negative results for dead *Salmonella* cells ranging in concentration from 3.8×10^2 to 3.8×10^8 CFU/ml (i.e., 7.5×10^1 to 7.5×10^6 CFU/reaction). Amplification occurred at the 10^9-CFU/ml level (i.e., 7.5×10^7 CFU/reaction) with an average T_T value of 29.6 min (data not shown). With qPCR, amplifications of dead *Salmonella* cells at both 10^8- and 10^9-CFU/ml levels occurred with average C_T values of 33 and 33.4 cycles, respectively, and melting temperatures at around 81°C, suggesting false-positive (i.e., viable cells) results for dead *Salmonella* cells. On the other hand, PCR consistently gave negative results for dead *Salmonella* cells at up to 10^9 CFU/ml.

Sensitivity and quantitative capability of PMA-LAMP. Table 3 shows the sensitivity and quantitative capability of PMA-LAMP when testing 10-fold serially diluted *S.* Typhimurium LT2 viable culture in the presence of 3.8×10^5 CFU/ml (i.e., 3.8×10^3 CFU/reaction) of dead *Salmonella* cells. For viable *Salmonella* cells between 3.4×10^5 and 34 CFU/reaction, after PMA treatment, consistent LAMP positive results were obtained with average T_T values ranging from 19.3 to 29.6 min (data not shown). In one out of four repeats, amplification (T_T = 29.7 min) also occurred for the sample with 3.4 CFU/reaction of viable *Salmonella* cells. No amplification was observed for the reaction tube containing 0.34 viable *Salmonella* cells and 3.8×10^3 dead ones. Therefore, the detection limit of PMA-LAMP was determined to be 3.4 to 34 CFU/reaction (Table 3). A similar sensitivity was observed for qPCR following PMA treatment (PMA-qPCR) with average C_T values ranging from 20 to 33.4 cycles for samples containing 3.4×10^5 to 3.4 viable *Salmonella* cells/reaction (data not shown). In contrast, PMA-PCR had a detection limit of 340 CFU/reaction, up to 100-fold less sensitive than that of PMA-LAMP or PMA-qPCR (Table 3).

Figure 2 shows typical amplification graphs and standard curves generated when testing 10-fold serially diluted *S.* Typhimurium LT2 viable culture in the presence of 3.8×10^5 CFU/ml of dead *Salmonella* cells by PMA-LAMP (Fig. 2A and B) and PMA-qPCR (Fig. 2C and D), as well as a PCR gel (Fig. 2E). The correlation coefficients (R^2) for PMA-LAMP and PMA-qPCR were calculated to be 0.970 and 0.997, respectively.

Rapid and specific quantification of viable *Salmonella* in produce by PMA-LAMP. For produce samples spiked only with 10-fold serially diluted dead *S.* Typhimurium LT2 cells, PMA-LAMP consistently gave negative results for samples with dead cell concentrations ranging from 4.2×10^2 to 4.2×10^8 CFU/g (equivalent to 0.75×10^1 to 7.5×10^5 CFU/reaction). However, amplifications for produce samples containing 4.2×10^9 CFU/g (7.5×10^6 CFU/reaction) of dead *Salmonella* cells occurred with average T_T values of 28.5, 38.8 and 25.2 min for cantaloupe, spinach, and tomato, respectively. In comparison, neither PMA-qPCR nor PMA-PCR showed amplifications for dead *Salmonella* cells up to 4.2×10^9 CFU/g, which was equivalent to 7.5×10^6 CFU in the reaction tube.

The sensitivities and quantitative capabilities of PMA-LAMP, PMA-PCR, and PMA-qPCR in detecting 10-fold serially diluted viable *S.* Typhimurium in the presence of 2.1×10^5 CFU/g (i.e., 3.8×10^2 CFU/reaction) of dead *Salmonella* cells are also summarized in Table 3. In three independent spiking experiments, PMA-LAMP consistently detected viable *Salmonella* cells down to 6.1×10^3 CFU/g (11 CFU/reaction)

in cantaloupe samples with average T_T values ranging from 20.1 to 30.9 min, whereas for spinach and tomato samples, the detection limit for both was at 6.1×10^4 CFU/g with average T_T values of 19.9 to 34.2 min and 22.2 to 27.7 min, respectively. In comparison, PMA-qPCR could detect viable *Salmonella* cells down to 6.1×10^3 CFU/g in cantaloupe and 6.1×10^2 CFU/g (110 CFU/reaction) in spinach and tomato samples. The average C_T values ranged between 18.5 and 31.8 cycles, 18.6 and 33.1 cycles, and 18.9 and 31.5 cycles for cantaloupe, spinach, and tomato samples, respectively. For PMA-PCR, the detection limits of viable *Salmonella* were 6.1×10^5 CFU/g in all three produce items, up to 100- and even 1,000-fold less sensitive than those of PMA-LAMP or PMA-qPCR. The R^2 values ranged from 0.949 to 0.993 for PMA-LAMP and 0.987 to 0.998 for PMA-qPCR (Fig. 3).

After 4 h of enrichment, both PMA-LAMP and PMA-qPCR were able to detect an initial spiking of 40 viable *Salmonella* cells per gram of cantaloupe, spinach, or tomato, up to 1,000-fold more sensitive compared to direct testing without enrichment (data not shown).

DISCUSSION

The *Salmonella invA*-based LAMP assay developed in the present study was rapid (15 to 40 min), specific (no false-positive or false-negative results for 53 strains tested), sensitive (1.3 to 13 CFU/reaction), and quantitative ($R^2 = 0.983$). When coupled with a simple PMA sample treatment, PMA-LAMP demonstrated good dead cell exclusivity (up to 10^8 CFU/ml in pure culture and 10^8 CFU/g in spiked produce), viable cell sensitivity (3.4 to 34 CFU/reaction in pure culture and 6.1×10^3 to 6.1×10^4 CFU/g in spiked produce), and quantitative capability ($R^2 = 0.949$ to 0.993). To our knowledge, this is the first report examining the novel combination of PMA and LAMP in detecting and quantifying viable bacterial cells.

We chose the *Salmonella invA* gene as the target for designing LAMP primers. Previously, *invA*-based molecular detection assays using multiple platforms such as PCR, qPCR, and LAMP have been designed to accurately detect *Salmonella* with a broad specificity for more than 100 *Salmonella* serovars while demonstrating excellent exclusivity for non-*Salmonella* strains (8, 15, 36, 45). Findings of this study corroborated with these previous reports on the high specificity of *invA*-based molecular detection assays for *Salmonella*. A closer examination of primer sequences in this study and previously published *invA*-based LAMP studies (15, 20, 45) showed that the region (5′ end of F3 and 3′ end of B3) covered by our primers (503 to 682 bp) and those of Hara-Kudo et al. (225 to 468 bp) (15) overlapped with that (371 to 655 bp) targeted by the widely used *Salmonella invA* PCR primers (36). Primers reported in the other two studies (20, 45) were essentially the same with only one nucleotide deletion at the 3′ end of each FIP and BIP primer in the EMA-LAMP study (20), and the region (672 to 912 bp) covered was downstream of the *invA* PCR primers without any overlap. In addition, two loop primers were each incorporated in this study and the study by Hara-Kudo et al. (15), while the other two studies had no loop primers (20, 45).

It is noteworthy that the optimized LAMP reagent mix in the present study differed from that described in many previous LAMP publications (5, 10, 15, 19, 34), which essentially followed the formulation of the LoopAmp DNA amplification kit (Eiken Chemical Co., Ltd.). Comparing the turbidity judgment graphs (Fig. 1) clearly indicated that under the optimized condition, the LAMP reaction progressed faster and the turbidity signals increased markedly more speedily. A major deviation of the optimized conditions from the prototype was the elimination of betaine. Our recent study optimizing a LAMP assay for potentially virulent *V. vulnificus* also indicated that betaine had no beneficial effect on LAMP amplification (12). Several previous studies, however, suggested that higher betaine concentration resulted in elevated LAMP amplification efficiency and increased target selectivity (32, 49). Betaine was capable of isostabilizing DNA and preventing secondary structure formation in GC-rich region, thus reducing base stacking and promoting DNA amplification (16, 37). However, unlike LAMP, betaine has not been used routinely in PCRs. Therefore, our data indicate that when amplifying non-GC-rich target sequences, eliminating betaine may be preferable in order to increase LAMP amplification efficiency.

The LAMP assay developed in this study was capable of detecting 1.3 to 13 *Salmonella* cells per reaction in pure culture. This level of sensitivity was comparable to that of qPCR but up to 100-fold more sensitive than that of PCR run in parallel. The first published *invA*-based LAMP for *Salmonella* detection had a sensitivity of 2.2 CFU/test tube (15), whereas a more recent one reported a detection limit of 100 fg DNA/tube (45), approximately 20 CFU/tube (23). In both studies, LAMP was found to be 10-fold more sensitive than PCR for all serotypes tested (15, 45). Additionally, another LAMP assay for *Salmonella* detection that targeted the *phoP* gene was able to detect down to 35 CFU per reaction (19), and two LAMP assays specific for *Salmonella* O4 or O9 groups possessed a detection limit of 10^3 CFU/ml (equivalent to 10^0 CFU/tube), 100-fold more sensitive than PCR (33, 34). A very recent study compared the sensitivity of LAMP and TaqMan qPCR in detecting *Salmonella enterica* serovar Enteritidis and reported a detection limit of 4 copies per reaction by both assays (48). Therefore, the sensitivities of current LAMP assays for *Salmonella*, including the one developed in the present study, fell between 10^0 and 10^1 CFU/reaction, 10- to 100-fold more sensitive than PCR but similar to qPCR. This improved sensitivity (at least 10-fold) of LAMP compared to PCR has also been reported in previous LAMP studies on the detection of other food-borne pathogens (5, 11, 14). It is noteworthy that LAMP is markedly faster than qPCR by at least 10 min.

LAMP amplicons were commonly detected by gel electrophoresis, naked eye observation of turbidity or color change, and real-time turbidimeter monitoring (5, 10, 11, 15, 19, 24, 42, 45, 48). Since LAMP synthesizes a large amount of DNA (10 to 20 μg/25-μl reaction mixture), open-tube procedures after amplification such as gel electrophoresis potentially act as a significant source of cross-contamination, whereas a closed-tube procedure such as monitoring with a real-time turbidimeter is preferred (11). In addition, among these LAMP amplicon detection methods, real-time turbidimeter monitoring is the only one that is potentially quantitative. However, very few studies have examined the quantitative capability of LAMP. One study monitoring ammonia-oxidizing bacteria using LAMP reported that it possessed good quantitative capability between 10^4 and 10^{10} DNA copies (2). Two other studies

demonstrated strong linear correlation coefficients ($R^2 = 0.94$ to 0.99) of LAMP in detecting *V. parahaemolyticus* and *V. vulnificus* in spiked oysters (5, 11). In the present study, the R^2 values were found to be 0.983 for cell concentrations ranging between 10^2 and 10^5 CFU/reaction in pure culture and 0.949 to 0.993 for viable *Salmonella* cells ranging from 10^4 to 10^7 CFU/g in spiked produce samples, suggesting an excellent quantitative capability.

From a public health perspective, determining cell viability is a critical requirement for pathogen testing methods in foods in order to accurately assess the potential risks (17). This is the first report examining the novel combination of PMA and LAMP in detecting viable bacterial cells. Dead *Salmonella* cells, up to 3.8×10^8 CFU/ml (4.2×10^8 CFU/g in spiked produce), were not detected by PMA-LAMP, illustrating excellent dead cell exclusivity. In comparison, PMA-PCR had 10-fold-better dead cell exclusivity, which was possibly due to the lower sensitivity associated with PCR compared to LAMP. On the other hand, PMA-qPCR gave positive signals for *Salmonella* dead cells at 3.8×10^8 CFU/ml, likely attributable to the high sensitivity of qPCR (Table 3). Previous studies of EMA or PMA in combination with qPCR found that qPCR consistently gave late signals for samples containing only dead bacterial cells due to its superior sensitivity, implying a great potential to generate false-positive results when detecting viable bacteria in the background of high concentrations of dead cells (29, 30, 43). Nonetheless, this level of dead cell concentration (10^7 to 10^9 CFU/g) is not commonly encountered in an agriculture field or in produce samples. Additionally, optimizing PMA treatment parameters, including final concentration, PMA incubation time, and light exposure time, may be able to further improve the dead cell exclusivity of PMA-LAMP.

In pure culture testing, PMA-LAMP possessed a similar sensitivity as PMA-qPCR, i.e., 3.4 to 34 viable *Salmonella* cells, which was comparable to the detection limit of 1.3 to 13 *Salmonella* cells obtained by LAMP alone in pure culture. Additionally, the R^2 value of PMA-LAMP was 0.970 for viable *Salmonella* cell concentrations ranging from 10^2 to 10^5 CFU/reaction, indicating a comparable quantitative capability to that obtained using LAMP alone. This suggested that PMA did not have significant inhibitory effect on the overall assay sensitivity and quantitative ability, contrary to findings reported in a recent study (43). The recent EMA-LAMP study (20) reported the same level of sensitivity as that of LAMP (45), i.e., 100 fg DNA of *Salmonella* cells, suggesting EMA had no inhibitory effect on LAMP. In terms of sensitivity comparison, a recent study detecting viable *Salmonella* using a TaqMan-based reverse transcriptase qPCR assay targeting *invA* mRNA reported a detection limit of ca. 120 viable *Salmonella* cells at mid-exponential growth stage (8). Another study using reverse transcriptase LAMP had 10^5 CFU/ml by visual observation and 10^1 CFU/ml by gel electrophoresis for unenriched *Salmonella* overnight culture (42). However, a sensitivity of 10^1 CFU/ml would be equivalent to 0.05 CFU (0.05 DNA copy) per LAMP reaction tube, theoretically unattainable by molecular detection assays due to the absence of template DNA. The use of overnight culture where a high proportion of cells do not produce CFU was used to partly explain the detection limit of less than one cell (e.g., 0.1 cell) in a LAMP assay designed for virulent *V. parahaemolyticus* (26). In addition, in that reverse transcriptase LAMP study (42), the big discrepancy of sensitivity (4 logs) between visual observation and gel electrophoresis reported was rather uncommon. In the present study, we observed a general agreement between visual observation of color change after adding SYBR green I and real-time turbidity monitoring (data not shown).

Without enrichment, the detection limits of PMA-LAMP for viable *Salmonella* in spiked produce samples ranged from 6.1×10^3 CFU/g (11 CFU/reaction) in cantaloupe to 6.1×10^4 CFU/g in spinach and tomato, up to 100-fold more sensitive than those of PMA-PCR but less sensitive than PMA-qPCR. The differences observed in sensitivity for different produce items may be due to inherent factors such as pH and minerals and warrant further evaluations. Adding 4 h of enrichment, PMA-LAMP could detect an initial spiking of 40 CFU/g of viable *Salmonella* cells, comparable to that obtained by PMA-qPCR. This short-term enrichment procedure allowed for sample processing and LAMP confirmation within an 8-h working day. In comparison, Gonzalez-Escalona et al. (8) demonstrated a sensitivity of 2 viable *Salmonella* cells per 25 g of bagged spinach by reverse transcriptase qPCR after 24 h of preenrichment. Techathuvanan et al. (42) used reverse transcriptase LAMP to detect *S.* Typhimurium from pork and reported detection limits of 10^2 CFU/25 g with 10 h of enrichment and 10^6 CFU/25 g without enrichment. Neither study included dead *Salmonella* cells in the background to ascertain that the assay did not detect dead *Salmonella* DNAs. On the other hand, a recent study using PMA-qPCR to quantify viable *Campylobacter* cells on chicken carcasses reported a detection limit of 100 CFU/ml of chicken carcass rinse (17). Again, no dead *Campylobacter* cells were added in the background to ascertain that the assay did not detect dead *Campylobacter* cell DNAs.

In conclusion, the overall advantages of the PMA-LAMP assay were well demonstrated in terms of sensitivity, quantitative capability, rapidity, and simplicity. First, PMA-LAMP had comparable sensitivity to PMA-qPCR but up to 100-fold more sensitivity than PMA-PCR in both pure culture and produce samples. Second, PMA-LAMP showed excellent quantitative capabilities ($R^2 = 0.949$ to 0.993) comparable to PMA-qPCR. Third, the total assay time for PMA-LAMP in produce without enrichment was 3 h, faster than either PMA-qPCR or PMA-PCR. Furthermore, PMA-LAMP is technically simpler than PMA-PCR as it eliminated gel electrophoresis. However, one limitation of this study was that log-phase cells were used to inoculate produce samples in order to obtain accurate counts of live *Salmonella* cells. In naturally contaminated produce samples, *Salmonella* cells are unlikely to be in this active physiological state; therefore, a longer enrichment step may be necessary. It is also helpful to apply this PMA-LAMP to examine the survival and persistence of *Salmonella* in produce samples with conditions mimicking industry practices. Therefore, upon further evaluation, this rapid, accurate, and simple method to detect and quantify viable *Salmonella* in produce may present a valuable tool for the produce industry to better control potential microbial hazards in produce.

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