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One Real-Time Fluorescent Loop-Mediated Isothermal Amplification Combined with Propidium Monoazide for Detection of Viable *Vibrio Parahaemolyticus* in Seafood

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Abstract: *Vibrio parahaemolyticus* is one of the crucial foodborne pathogens in the world. At present, the rapid detection methods for *V. parahaemolyticus* cannot distinguish between dead and viable cells and false positive results may occur. In this study, one rapid and accurate method that combines Propidium Monoazide (PMA) with real-time fluorescent loop-mediated isothermal amplification (LAMP) using SYTO-16 dye was developed to detect viable cells of *V. parahaemolyticus*. LAMP amplification was performed on specific primers designed for *toxR* gene of *V. parahaemolyticus* and the concentration of PMA and the maximum concentration of dead cells were optimized. The results showed that the concentration of PMA was 10 µM and PMA could efficiently treat dead cells up to 4.5×10^5 CFU/mL. The detection sensitivity of real-time fluorescent PMA-LAMP were 4.5×10^0 CFU/mL and PMA-qPCR were 4.5×10^1 CFU/mL. In addition, the correlation coefficients (R^2) is 0.9992, indicating that SYTO-16 dyed real-time fluorescent PMA-LAMP assay can be used for quantification with high sensitivity. This method exhibits high specificity and sensitivity, can be used as an effective tool for rapid detection of *V. parahaemolyticus* and a scientific basis to follow the effect of the pathogen infection on growth of cultured seafood.

Keywords: *V. parahaemolyticus*, Propidium Monoazide, Real-Time Fluorescent Loop-Mediated Isothermal Amplification, Seafood

Introduction

Vibrio parahaemolyticus, a kind of halophilic Gram-negative bacterium, has been considered as the leading causes of seafood-borne illness in the world (Nair *et al*., 2007; Guo *et al*., 2010). *V. parahaemolyticus* was first isolated from a food poisoning incident in Osaka and was generally widely distributed in areas such as coastal areas or estuary of rivers (Su and Liu, 2007). It is reported that seafood infected with *V. parahaemolyticus* can lead to nausea, vomiting, diarrhea, abdominal cramps, low fever, chills and even gastroenteritis and septicemia (Zhong *et al*., 2016). In addition, the *Vibrio* species have been highly resistant to most commercially available antibiotics (Stalin and Srinivasan, 2016). Therefore, rapid and sensitive detection of *V. parahaemolyticus* is significant to prevent the disease.

Most traditional biochemical culture detection methods, including detection of *V. parahaemolyticus* by the gold standard method Bacteriological Analytical Manual (BAM), are time-consuming and laborious, which are not conducive to timely identification of pathogens, elimination of safety hazards or prompt treatment (Zhi *et al*., 2017). In recent years, molecular diagnostic techniques have been developed for *V. parahaemolyticus* detection, such as PCR and real-time PCR, but both of which require complicated instruments and professionals (Davis *et al*., 2004; Cai *et al*., 2006; Bunpa *et al*., 2018; Xu *et al*., 2018). These drawbacks can be compensated by using Loop-Mediated Isothermal Amplification (LAMP) method, which can utilize *Bst* DNA polymerase and a set of four primers targeted six distinct regions for efficient amplification of the target gene under isothermal condition (60-65 °C) within 60 min (Notomi *et al*., 2000). Additionally, loop primers could be added to the assay reaction which was designed

according to the four primers set to enhance efficiency and increase specificity of the assay (Nagamine *et al.*, 2002). Moreover, the quantitative detection of LAMP products can be realized in real time by using fluorescent dyes and evaluating the amplification curve of fluorescent signal (Oscorbin *et al.*, 2016). In our study, fluorescence dye SYTO-16 was used to visualize the LAMP assay with excitation wavelength of 488 nm and emission wavelength of 518 nm. SYTO-16 is a kind of cell permeant nucleic acid stain that shows a large fluorescence enhancement upon binding nucleic acids (Udovich *et al.*, 2010).

However, none of the above methods can distinguish between dead and viable cells, which often result in false positive results. Ethidium Bromide Monoazide (EMA) and propidium monoazide (PMA) are often applied to eliminate the interference of dead cells (Wang and Mustapha, 2010; Zhu *et al.*, 2012; Saiyudthong and Trevanich, 2013). PMA was less toxic than EMA and had higher specificity to dead cells (Fittipaldi *et al.*, 2012).

In this study, we report the use of SYTO-16 dyed real-time LAMP with six target-specific primers for the detection of *V. parahaemolyticus* in seafood samples. We also used PMA treatment prior to real-time LAMP screening to quantitatively detect viable *V. parahaemolyticus*.

Materials and Methods

Preparation of Bacterial Strains

V. parahaemolyticus ATCC 17082 was used for real-time fluorescent PMA-LAMP assay optimization and sensitivity test. Two additional *V. parahaemolyticus* strains (H4-3 and FJ14A) isolated from seafood and a total of 12 non-*V. parahaemolyticus* strains including *Vibrio cholerae*, *Salmonella enteritidis*, *Staphylococcus*

aureus, *Escherichia coli*, *Listeria monocytogenes*, *Shigella*, *Staphylococcus epidermidis*, *Vibrio hollisae*, *Vibrio mimicus*, *Vibrio damsela*, *Vibrio vulnificus*, *Vibrio fluvialis* were employed for the specificity test (Table 1). 25 g of seafood samples were dissolved in 225 mL 3% Nacl APW. *V. parahaemolyticus* strains were cultured 18 h on TCBS agar plate (Hopebio, China) at 37 °C, showing a round, opaque, green or bluish colony on TCBS agar plate with a diameter of 2-3 mm. A single colony was transferred to alkaline saline peptone water of 3% sodium chloride and grown at 37 °C overnight. The bacterial culture was used for the extraction of genome, PMA treatment or plate counting. The non-*V. parahaemolyticus* strains were cultured in Luria-Bertani medium (LB, Sangon, China) at 37°C overnight. All experiments with bacteria culture were carried out in a biosafety level 2 laboratory.

Preparation of Dna Template

The DNA template of Gram-negative bacteria, including *V. parahaemolyticus* was prepared by the method described previously (Zhi *et al.*, 2017; Fang *et al.*, 2018). The bacteria cells were collected by centrifugation at $10000 \times g$ for 5 min. The pellets were washed and resuspended in 50 µL Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The suspension was frozen at -20 °C for 10 min and heated immediately at 100 °C for 10 min. After centrifugation at $12000 \times g$ for 5 min, the supernatant containing bacteria DNA was used as the DNA template. The DNA template of Gram-positive bacteria, spiked samples and practical samples were extracted by a bacterial DNA extraction kit according to the instruction (Sangon, China).

Table 1: Information of bacterial strains used for specificity tests in the study

Species	ID of strains	Result of LAMP	Result of qPCR
Vibrio parahaemolyticus	ATCC 17802	+	+
	H4-3[b]	+	+
	FJ14A[b]	+	+
Vibrio cholerae	GIMCC 1.449	-	-
Salmonella enteritidis	GIMCC 1.345	-	-
Staphylococcus aureus	GIMCC 1.142	-	-
Escherichia coli	GIMCC 1.201	-	-
Listeria monocytogenes	ATCC 19115	-	-
Shigella	GIMCC 1.424	-	-
Staphylococcus epidermidis[a]	/	-	-
Vibrio hollisae[a]	/	-	-
Vibrio mimicus[a]	/	-	-
Vibrio damsela[a]	/	-	-
Vibrio vulnificus[a]	/	-	-
Vibrio fluvialis[a]	/	-	-

GIMCC: Guangdong Microbiology Culture Center, ATCC: American Type Culture Collection
[a]Afforded by Zhoushan Entry-Exit Inspection and Quarantine Bureau without marks
[b]Afforded by Zhoushan Entry-Exit Inspection and Quarantine Bureau with marks

Table 2: Sequences of *Vibrio parahaemolyticus* LAMP primers and qPCR primers/probe

Primers	Sequence (5'-3')	Target gene	Fragment length (bp)
	LAMP Primers	*toxR*	192
VP -F3	TAATTCGCTCGCAGACCA		
VP -B3	CGCAGAGTAGAAATCGCT		
VP -FIP	TCTCCGCCAACATCATTTTGGAGTAGGCAACGAAGTTGTACGA		
VP -BIP	TAACCCGTAACGTTTTAGCTTCACGTCAGGCTTGAGTCATCCA		
VP -LF	ACGGCTTTCGTTGCTTCC		
VP -LB	TGTTTGGCGTGAGCAAGG		
	qPCR Primers		213
VP -F	GTGCAGGTTTATGTGTTC		
VP -R	CCTGAATACGGGATTAGAC		
VP-Probe	*FAM*-CGCTGATGGCACAGATCACAG-*TAMRA*		

F refers to forward and R refers to reverse

PMA Treatments and Optimization of PMA Working Concentration

To obtain the optimal concentration of PMA treatment, dead cells of *V. parahaemolyticus* ATCC 17082 were prepared by boiling at 95 °C for 15 min according to the method described previously (Zhi *et al.*, 2017; Fang *et al.*, 2018). To confirm all the bacteria were killed, the cells after treated were cultured on TCBS at 37 °C for 48 h. Then 20 mM PMA (Biotium Inc., Hayward, CA, USA) was added at final concentration of 0, 5, 10, 15, 20, 40 and 80 μM to 500 μL suspension containing 4.5×10^5 CFU/mL dead *V. parahaemolyticus* ATCC 17082 in 1.5 mL eppendorf tubes and solution was mixed up and down for several times to make the sample fully in contact with the PMA dye and left in the dark for 5 min. Then the tubes placed on ice were transferred to 60 W PMA-Lite Photolysis Device (Biotium) and were illuminated for 30 min by LED lights to activate the PMA. In order to obtain better concentration of dead cells, the copy number of dead cells to be processed was set as 4.5×10^8, 4.5×10^7, 4.5×10^6, 4.5×10^5, 4.5×10^4 CFU/mL. Three control groups were set at the same time, which were 4.5×10^5 CFU/mL pure viable cells that were not treated by PMA, pure dead cells treated by PMA and negative control. The results were determined by whether the DNA amplification of dead cells could be completely suppressed.

LAMP Primers and Establishment of Real-time Fluorescent LAMP Assay

The sequence of *toxR* gene were obtained from GenBank in NCBI database (accession numbers: GQ228073.1). The specific regions were selected as the target fragment after aligning by using Clustal W software. Based on the detailed analysis and comparison, specific primers (Table 2) of LAMP including two loop-primers were designed with the online tool Primer Explorer V5.0 (http://primerexplorer.jp/e/), which was

supplied by Eiken Chemical (Tokyo, Japan). The DNA template of 2.5 μL was added to the amplification reagent (total volume 25 μL) containing 0.4 μM outer primer F3 and B3, 3.2 μM inner primer FIP and BIP, 1.6 μM loop primer LF and LB, together with 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 8 mM MgSO$_4$, 10 mM (NH$_4$)$_2$SO$_4$, 0.1% Tween20 (Sigma-Aldrich), 0.8 M betaine, 0.5 mM MnCl$_2$, 1.4 mM dNTPs (Thermo Scientific, USA), 10 μM SYTO-16 (Thermo Scientific, USA) and 0.32 U/μL *Bst* 2.0 DNA polymerase (NEB, USA). No-template control reactions contained distilled water instead of template DNA. The amplification program was: 65 °C for 1 min, followed by 40 cycles of 65 °C for 60 sec. Fluorescence signals were collected using the SYBR channel by using the ABI Step One Plus™. Fluorescence data were analyzed after amplification using the ABI software.

qPCR Primers and Establishment of qPCR Assay

A TaqMan-based qPCR assay was also established by targeting the *toxR* gene. Primers and the TaqMan probe were designed by Beacon Designer 7.9 (Table 2) and were synthesized by Invitrogen Biotechnology Co. Ltd. The reaction mix contained 2.5 μL DNA template, 0.2 μL RoxII, 0.8 μM of primer sets, 0.1 μM of probe and 10 μL 2 × Premix Ex Taq (Takara, Japan) and ddH$_2$O to a final volume of 20 μL. The thermal cycle program was: 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 sec and 60 °C for 30 sec.

Specificity and Sensitivity of Real-time Fluorescent PMA-LAMP and PMA-qPCR Assays

To determine assay specificity, three viable and three heat-killed dead *V. parahaemolyticusSSS* strains and 12 non-*V. parahaemolyticus* strains including *Vibrio cholerae*, *Salmonella enteritidis*, *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes*, *Shigella*, *Staphylococcus epidermidis*, *Vibrio hollisae*, *Vibrio mimicus*, *Vibrio damsela*, *Vibrio vulnificus*, *Vibrio fluvialis* (Table 1) were treated with PMA and DNA was extracted.

To determine assay sensitivity, *V. parahaemolyticus* ATCC 17082 was grown to the mid-exponential growth phase and serially diluted 10-fold in alkaline saline peptone water of 3% sodium chloride. The initial cell number was quantified by plate counting on plate count agar (Hopebio, China) using BAM methods. Concentrations of viable *V. parahaemolyticus* ATCC 17082 between 4.5×10^8 and 4.5×10^0 CFU/mL mixed with 4.5×10^5 CFU/mL dead *V. parahaemolyticus* ATCC 17082 cells were treated with the optimal concentration of PMA. Finally, DNA was extracted for sensitivity testing. Both tests were repeated three times. The amplification performance of real-time fluorescent PMA-LAMP was compared with real-time PMA-PCR. The threshold time was plotted against the log values of the detected molecules to generate an amplification standard curve.

Evaluation of Real-Time Fluorescence PMA-LAMP Assay with Spiked Samples

Fresh shrimp, sleevefish and cod samples were purchased from the local market and confirmed to be negative for *V. parahaemolyticus* using BAM methods. Each sample weighed 50 g, homogenized and was added to 450 mL of alkaline saline peptone water of 3% sodium chloride under sterile conditions. The homogenates were contaminated with *V. parahaemolyticus* suspension which containing 4.5×10^5 to 4.5×10^0 CFU/mL viable *V. parahaemolyticus* ATCC 17082 cells and 4.5×10^5 CFU/mL dead cells. DNA extraction after PMA treatment were performed as the above description. Each sample was analyzed for the presence of *V. parahaemolyticus* using the real-time fluorescence PMA-LAMP, PMA-qPCR and BAM methods.

Evaluation of Real-Time Fluorescence PMA-LAMP Assay with Practical Samples

A total of 139 practical samples (shrimp, sleevefish, trichiurus lepturus, cod, meretrix, grilled fish, grilled yellow croaker and dried squid) which purchased from local markets and afforded by Zhoushan Entry-exit Inspection and Quarantine Bureau were detected by the SYTO16-dyed real-time fluorescence PMA-LAMP and PMA-qPCR assays. After a pre-enrichment step at 37 °C for 4 h, 2000 × g, 50 g of each samples were diluted 1000-fold in alkaline saline peptone water of 3% sodium chloride and treated with PMA. DNA was extracted for real-time fluorescence PMA-LAMP and PMA-qPCR assays, with BAM method used as control.

Data analysis

Data collected from the real-time fluorescence PMA-LAMP and PMA-qPCR assays including standard curves were analyzed by ABI Step One Plus™ and Microsoft Excel software (Microsoft Inc., USA). Results of qLAMP and qPCR were judged as positive ones when the C_t value ≤ 35. The coefficients of variation (CV) of Ct values of the sensitivity and artificial contamination testing were statistically analyzed for the repeatability (CV value should be less than 10). In addition, the detection rate of PMA-LAMP, PMA-qPCR assay and BAM method for tests of practical samples was used to compare the coincidence rate between the three methods.

Results

The proper concentration of PMA working concentration and the maximum treatable concentration of dead *V. parahaemolyticus* cells

PMA, a kind of DNA binding dye, was applied prior to the DNA amplification assay to differentiate viable cells form dead cells by LED light treatment. The proper concentration is important that not only the amplification of dead cell DNA cannot be completely suppressed, but also had no toxicity to viable cells (Zhi *et al*., 2017; Fang *et al*., 2018). To obtain the proper concentration of PMA, an amount of 4.5×10^5 CFU/mL heat-killed *V. parahaemolyticus* dead cells or viable cells were treated with different concentration of PMA from 0 to 80 µM (Fig. 1). The amplification of dead cells was suppressed fully by 10 µM to 80 µM PMA treatment. In the meanwhile, when the concentration of PMA is 15 µM or more, the amplification of viable cells was suppressed somewhat. Therefore, 10 µM was considered to be the proper concentration of PMA.

The heat-killed *V. parahaemolyticus* dead cells were serially diluted from 4.5×10^8 CFU/mL to 4.5×10^4 CFU/mL. DNA was extracted for real-time fluorescent PMA-LAMP and PMA-qPCR after treated with 10 µM PMA. The results indicated that the optimal 10 µM PMA was effective for suppression *V. parahaemolyticus* dead cells lower than 4.5×10^5 CFU/mL (Fig. 2). The results is consistent with the previous report (Zhi *et al*., 2017; Fang *et al*., 2018).

Specificity of Real-Time Fluorescent PMA-LAMP and PMA-qPCR

The specificity of the real-time fluorescent PMA-LAMP and PMA-qPCR assay was determined using three viable *V. parahaemolyticus* cells, three heat-killed dead *V. parahaemolyticus* cells and 12 non-*V. parahaemolyticus* strains including *Vibrio cholerae*, *Salmonella enteritidis*, *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes*, *Shigella*, *Staphylococcus epidermidis*, *Vibrio hollisae*, *Vibrio mimicus*, *Vibrio damsela*, *Vibrio vulnificus*, *Vibrio fluvialis*. The two developed methods were performed specifically that only three viable *V. parahaemolyticus* cells were observed to show positive results (Fig. 3).
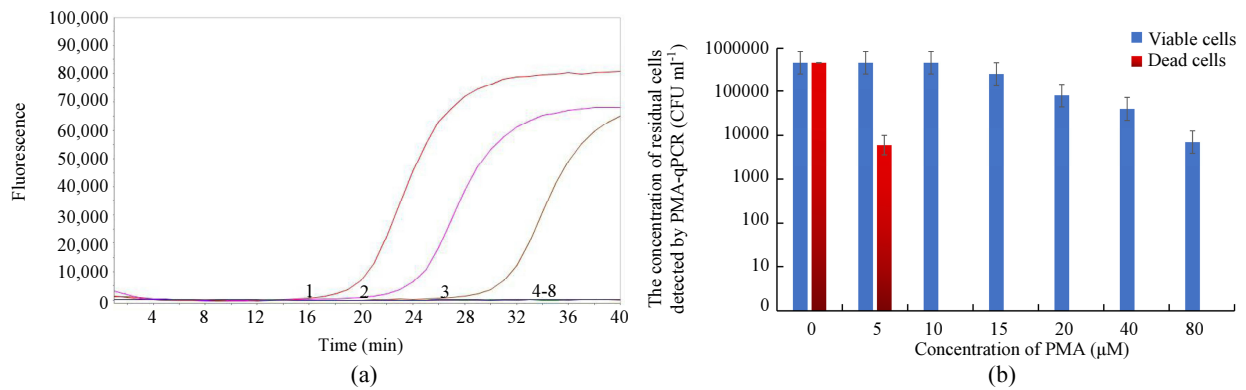
Fig. 1: The optimal concentration of propidium monoazide (PMA) for viable *V. parahaemolyticus* detection. (a) Heat-killed *V. parahaemolyticus* cells treated with different PMA concentrations and analysed by real-time fluorescent LAMP assay. 1: positive control (untreated *V. parahaemolyticus*); 2-8: *V. parahaemolyticus* treated with 0, 5, 10, 15, 20, 40 and 80 µM PMA. (b) Concentration of residual heat-killed and viable *V. parahaemolyticus* cells detected by qPCR after PMA treatment
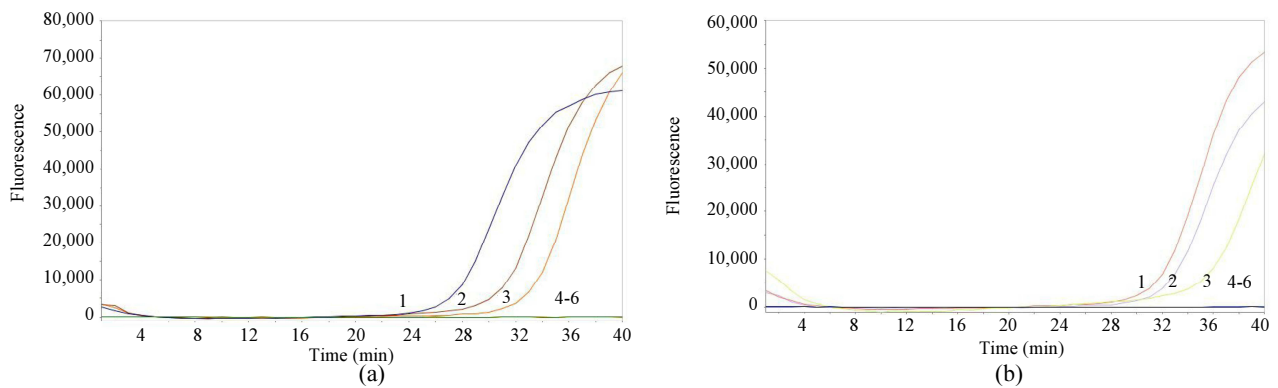

Fig. 2: Maximum heat-killed dead cell concentrations for propidium monoazide (PMA) treatment. (a) real-time fluorescent LAMP assay results. (b) real-time qPCR assay results. 1-5: 4.5×10^8, 4.5×10^7, 4.5×10^6, 4.5×10^5, 4.5×10^4 CFU/mL; 6: No template control
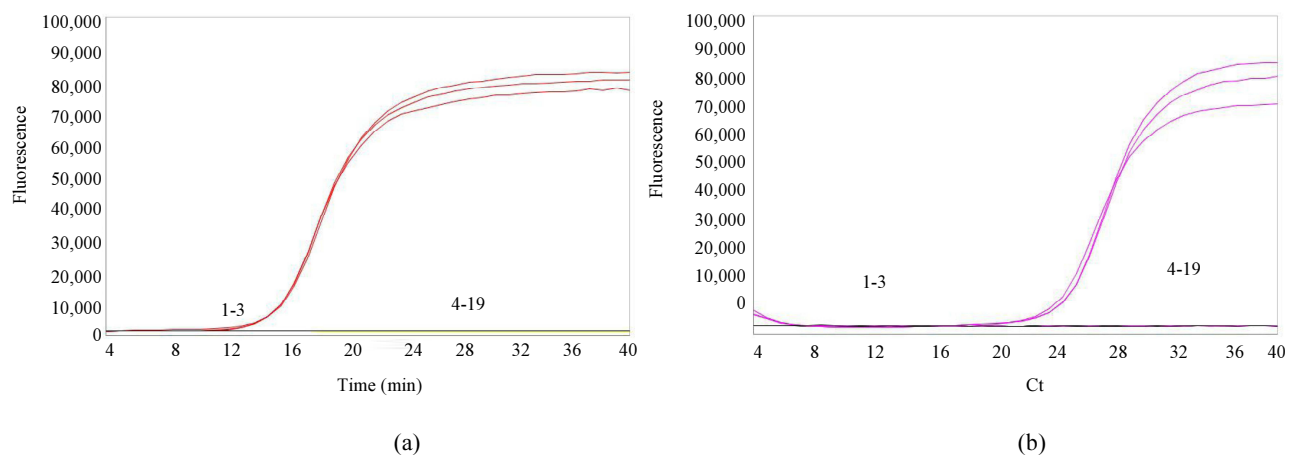

Fig. 3: Specificity of the real-time fluorescent PMA-LAMP and PMA-qPCR detection. Three standard strains of *V. parahaemolyticus* and 12 non-*V. parahaemolyticus* strains were amplified by real-time fluorescent PMA-LAMP assay (a) and PMA-qPCR assay (b). 1-3: *V. parahaemolyticus* positive strain, 4-6: heat-killed *V. parahaemolyticus* cells, 7-18: *Vibrio cholerae*, *Salmonella enteritidis*, *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes*, *Shigella*, *Staphylococcus epidermidis*, *Vibrio hollisae*, *Vibrio mimicus*, *Vibrio damsela*, *Vibrio vulnificus*, *Vibrio fluvialis*, 19: No template control
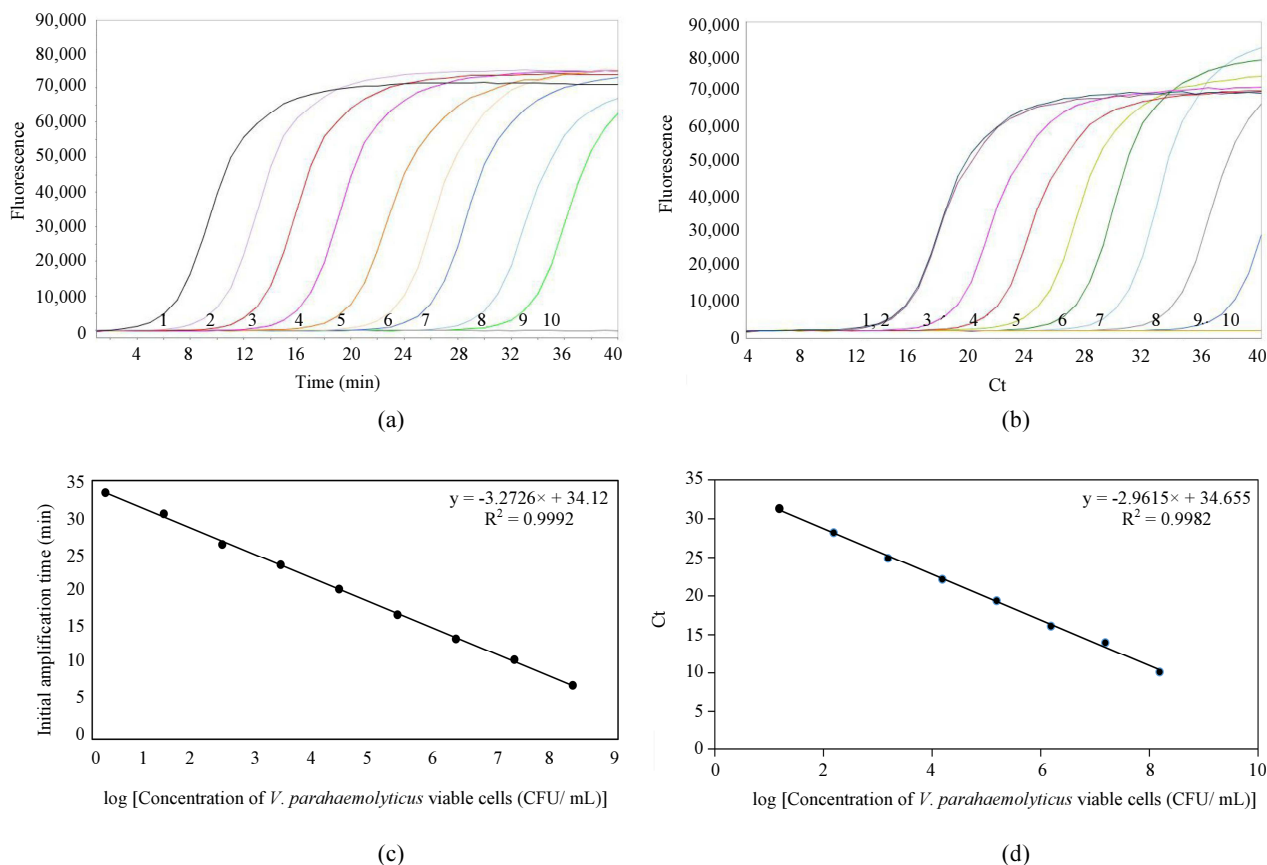
(a)

(b)

(c)

(d)

Fig. 4: Sensitivity of real-time fluorescent PMA-LAMP and PMA-qPCR assay for *V. parahaemolyticus*. The amplification curve of real-time fluorescent PMA-LAMP assay (a) and PMA-qPCR assay (b). The standard curves of the real-time fluorescent PMA-LAMP assay (c) and PMA-qPCR assay (d) were generated from the sample of *V. parahaemolyticus* bacteria solution, ranged from 10^8 to 10^0 CFU/mL, with the ABI Step One Plus™. 1-9: 4.5×10^8, 4.5×10^7, 4.5×10^6, 4.5×10^5, 4.5×10^4, 4.5×10^3, 4.5×10^2, 4.5×10^1, 4.5×10^0 CFU/mL, 10: No template control

Therefore, it confirmed that the real-time fluorescent PMA-LAMP and PMA-qPCR assay for detection of viable *V. parahaemolyticus* cells were highly specific.

Sensitivity of Real-Time Fluorescent PMA-LAMP and PMA-qPCR

The real-time fluorescent PMA-LAMP and PMA-qPCR detection methods for *V. parahaemolyticus* were established based on PMA treatment. The results showed that the detection limit of real-time fluorescence PMA-LAMP for viable *V. parahaemolyticus* was 4.5×10^0 CFU/mL, which was 10 times higher than that of PMA-qPCR (Fig. 4). The correlation coefficients (R^2) of the real-time fluorescent PMA-LAMP and PMA-qPCR assays were 0.9992 and 0.9982, respectively. In addition, the CV values of initial amplification time and Ct values of PMA-LAMP and PMA-qPCR for sensitivity analysis were all lower than 10.0%. It indicated that SYTO-16 dyed real-time fluorescent PMA-LAMP assay had high repeatability and can be used for quantification with high sensitivity.

Artificial Contamination Testing

Before artificial contamination, all samples purchase from supermarket were confirmed to be *V. parahaemolyticus* negative by BAM methods. As shown in Table 3, three different seafood samples, containing 4.5×10^5 to 4.5×10^0 CFU/mL viable *V. parahaemolyticus* ATCC 17082 cells and 4.5×10^5 CFU/mL dead cells, were assessed by real-time fluorescent PMA-LAMP, PMA-qPCR and BAM assays (Table 3). The CV values of PMA-LAMP and PMA-qPCR for three different seafood samples were between 0.49% to 7.88% and 0.47% to 4.38%, respectively, which demonstrated high repeatability. Furthermore, the results indicated that 4.5×10^5 CFU/mL of the dead cells of *V. parahaemolyticus* did not interfere with the developed real-time fluorescent PMA-LAMP method.

Tests of Practical Samples

The applicability of fluorescence-quantitative PMA-LAMP assay for 139 blind samples was demonstrated.

Table 3: Detection of *Vibrio parahaemolyticus* PMA-LAMP, PMA-qPCR assays, and BAM in artificially contaminated produce commodities

Samples (n = 6 each)	Strain	Spiked level (CFU/mL)	Detection method PMA-LAMP (min)		PMA-qPCR(Ct)[a]		BAM
			Mean	CV (%)[b]	Mean	CV (%)[b]	
Shrimp	*Vibrio parahaemolyticus* ATCC 17802	4.5×10^5 (dead cells)	—		—		—
		4.5×10^5	+ (16.56±0.43)	2.6	+ (19.03±0.40)	2.1	+
		4.5×10^4	+ (19.68±1.55)	7.88	+ (22.94±0.58)	2.53	+
		4.5×10^3	+ (22.03±0.28)	1.27	+ (25.75±0.12)	0.47	+
		4.5×10^2	+ (25.03±0.40)	1.6	+ (29.92±1.28)	4.38	+
		4.5×10^1	+ (28.94±0.58)	2.01	+ (32.63±0.40)	1.22	+
		4.5×10^0	+ (31.94±0.18)	0.56	—	—	+
Sleevefish		4.5×10^5 (dead cells)	—		—		—
		4.5×10^5	+ (17.22±0.21)	1.22	+ (18.53±0.41)	2.21	+
		4.5×10^4	+ (19.78±0.22)	1.11	+ (22.04±0.58)	2.63	+
		4.5×10^3	+ (23.03±0.78)	3.39	+ (26.75±0.62)	2.32	+
		4.5×10^2	+ (26.76±1.18)	4.41	+ (29.92±0.88)	2.94	+
		4.5×10^1	+ (30.88±0.15)	0.49	+(32.54± 1.40)	4.3	+
		4.5×10^0	+ (33.22±0.21)	0.63	—	—	+
Cod		4.5×10^5 (dead cells)	—		—		—
		4.5×10^5	+ (17.76±0.99)	5.56	+ (19.55±0.81)	4.14	+
		4.5×10^4	+ (20.62±0.12)	0.58	+ (22.24±0.85)	3.82	+
		4.5×10^3	+ (24.19±0.35)	1.45	+ (24.75±0.26)	1.05	+
		4.5×10^2	+ (27.03±0.63)	2.33	+ (27.92±0.88)	3.14	+
		4.5×10^1	+ (30.67±0.64)	2.09	+(31.54± 0.74)	2.35	+
		4.5×10^0	+ (33.38±1.73)	5.18	—	—	+

C_t[a] values are given where the fluorescence signal was higher than the background.
CV (%)[b] are Coefficient of variation.
+, *Vibrio parahaemolyticus* positive by the method. -, *Vibrio parahaemolyticus* negative by the method

Table 4: Detection of practical samples by real-time fluorescent PMA-LAMP assay compared with PMA-qPCR and BAM method

Samples	Number of samples	Positive number		
		PMA-LAMP	PMA-qPCR	BAM method
Shrimp[a]	33	1	1	1
Sleeve-fish[a]	12	0	0	0
Trichiurus lepturus[a]	11	0	0	0
Cod[a]	14	1	1	1
Meretrix[a]	12	0	0	0
Grilled fish[a]	18	0	0	0
Grilled yellow croaker[a]	10	1	1	1
Dried squid[a]	12	0	0	0
Dried squid[b]	17	0	0	0
Total	139	3	3	3
Positive detection rate (%)	-	2.16	2.16	2.16

[a] Purchased from local market.
[b] Afforded by Zhoushan Entry-Exit Inspection and Quarantine Bureau

All the samples were also analyzed by PMA-qPCR assay and BAM method. The results showed that two of the above three methods were detected in 139 samples, with a detection rate of 2.16%. The concordance between real-time fluorescent PMA-LAMP and PMA-qPCR tests in 139 blind samples for detection of the *V. parahaemolyticus* were shown (Table 4). The results of the two methods are consistent with the rate of 100%.

Discussion

V. parahaemolyticus has been reported to be the causative agent in 50-70% from all prevalent food poisoning outbreaks as a widely distributed halophile pathogenic in coastal and estuarine environments (Wang *et al*., 2012; Ye *et al*., 2014). In the past decades, there were many several reliable and powerful technologies available for the detection of *V.*

parahaemolyticus genomic DNA (Yang *et al.*, 2018). The most widely used method for detection of *V. parahaemolyticus* infection was based on the molecular level, such as polymerase chain reaction (PCR). However, due to the need for complex instruments, complex laboratory procedures, expensive reagents and trained personnel, this method may not be suitable for use in developing countries or in the field (Chen and Ge, 2010; Li *et al.*, 2017). Therefore, an ever-expanding array of novel nucleic acid amplification techniques have been developed to meet the challenge of performing diagnostics without well-equipped equipment in low-resource environments (Fang *et al.*, 2018). On this basis, a variety of nucleic acid detection methods were developed under isothermal conditions. Some of these methods have been shown to detect small amounts of nucleic acid copies (Kanayama *et al.*, 2008; Li *et al.*, 2018; Peng *et al.*, 2018). Among these isothermal methods, LAMP assay is an excellent diagnostic tool. It offers an attractive option because it is simple, cost effective, efficient and specific. In isothermal conditions, the test can be performed using a water bath or a heating block, which are readily available under electrified conditions (Cao *et al.*, 2010).

Although this method has significant specificity, its disadvantage is that once the test tube is opened, there will be contamination after culture. In order to reduce the risk of pollution and eliminate the need to open the tubes, the fluorescence dyes offer greater flexibility and less cost. Fluorescence-based detection helps prevent false negatives that may result from base pair mismatches when template sequences change (Seyrig *et al.*, 2015). As long as the optimum concentration of fluorescent dye is determined, the method gave the best results of short initial amplification time, high fluorescence intensity, low inhibition rate and best effect. Signal-to-noise ratio is an important characteristic of the ratio of signal intensity to noise intensity. High SNR means high reliability of signal and low probability of false positive result. In previous studies, the signal-to-noise ratio was calculated by comparing the reaction performed by DNA template with the fluorescence ratio of the non-template control (Oscorbin *et al.*, 2016). According to the rule, SYTO-16 demonstrated the best SNR at appropriate concentrations tested and gave better results than other fluorescence dyes.

Traditional LAMP detection method could not distinguish the dead and viable cells of *V. parahaemolyticus*, while PMA-LAMP and EMA -LAMP method made up this defect. It has been reported that the toxicity of PMA dyes is far less than that of EMA dyes. Under the same conditions, the working concentration of PMA is always higher than that of EMA (Nagamine *et al.*, 2002). Therefore, PMA-based LAMP method has more advantages in the rapid detection of *V. parahaemolyticus*. Zhong *et al.* (2016) employed SYTO-9 dye to develop a

PMA-based LAMP for rapid detection of *V. parahaemolyticus*. The amplification was confirmed in 7 min (7cycles) and the detection limit was 6.8×10^{0} copies.

In this study, the PMA-based LAMP method was established by using a fluorescent dye SYTO-16 with higher SNR and amplification efficiency for the detection of *V. parahaemolyticus*. It was simple and effective for specific detection of the *toxR* gene under optimized conditions at 65 °C for 40 min. The initial amplification time was defined as the moment of produced fluorescence. Based on this, the amplification was observed from 5 min, the complete reaction duration was optimized to 40 min. The fluorescence dye concentration was optimized because these can affect the previously described signal-to-noise ratio. The results showed that 10 μM Syto16 was the best combination of time-threshold and SNR in LAMP reaction. In addition, betaine did not affect the amplification of the short target fragment (<300 bp) and reduced the non-specific amplification (Suebsing *et al.*, 2013).

The PMA-LAMP method was able to detect *V. parahaemolyticus* in as little as 4.5×10^{0} CFU/mL DNA template extracted from infected seafood, which was consistent with the results of PMA-qPCR. Meanwhile, the number of genomes in the PMA-LAMP method was linearly correlated with the initial amplification time. In these experiments, the time required to form a visual fluorescence value unit is inversely proportional to the number of templates, indicating that the number of signals is proportional to the abundance of templates (Burbulis *et al.*, 2015). The result of the standard curves indicated that there were significant correlations between the initial amplification time and the template concentration ($R^{2} = 0.9992$). In addition, no cross-amplification with other pathogens was found, indicating that the assay was highly specific for the detection of *V. parahaemolyticus*. The applicability of the fluorescence-quantitative LAMP assay was assessed with field samples and the results were compared with those obtained from the qPCR method. It showed that in 139 blind samples, the positive detection rates were the same (2.16%) by using the fluorescence-quantitative PMA-LAMP and PMA-qPCR, which were in agreement with the BAM method. The results of the two methods are consistent with each other. This method, combined with a simple and rapid DNA extraction method, can be used for the identification of *V. parahaemolyticus* live cells in field and laboratory, saving time and cost. However, this developed method should be applied and validated by using more natural seafood samples in the future.

The LAMP assay was capable of amplifying a DNA target under isothermal conditions and the amplification products can be quantitatively detected. In addition, PMA-

based LAMP method made up for the inability to identify the dead and live cells. The assay could be performed in less than 40 min without special equipment. Compared with PMA-qPCR method, it has greater advantages in sensitivity and practical sample detection. In conclusion, this method provides great advantages for the rapid field diagnostics of *V. parahaemolyticus* infection in aquatic products. It can be considered as a powerful technology for the monitoring/tracing of *V. parahaemolyticus*, which was rapid, accurate, simple and economic, with good sensitivity and specificity.

Conclusion

The LAMP assay was capable of amplifying a DNA target under isothermal conditions and the amplification products can be quantitatively detected. In addition, PMA-based LAMP method made up for the inability to identify the dead and live cells. The assay could be performed in less than 40 min without special equipment. Compared with PMA-qPCR method, it has greater advantages in sensitivity and practical sample detection. In conclusion, this method provides great advantages for the rapid field diagnostics of *V. parahaemolyticus* infection in aquatic products. It can be considered as a powerful technology for the monitoring/tracing of *V. parahaemolyticus*, which was rapid, accurate, simple and economic, with good sensitivity and specificity.

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Author's Contributions

Mingzhou Zhang: Has guided the project, determined the research framework, data analysis and involved in paper writing

Huanteng Yu: Has contributed to the experimental works, data analysis and paper writing

Jiehong Fang: Has contributed in the data analysis and paper writing

Biao Ma: Has contributed in the data analysis and paper writing

Jiali Li: Has contributed in the experimental works and data analysis

Ethics

This article does not contain any studies with human participants or animals performed by any of the authors.

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