Visual LAMP Method for Detection of Vibrio vulnificus in Aquatic Products and Environmental Water

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Abstract

Background A visualized, rapid, simple method was developed based on loop-mediated isothermal amplification (LAMP) assay to detect *Vibrio vulnificus* in aquatic products and aquaculture waters.

Results Genomic DNA was extracted from *Vibrio vulnificus* using the boiling and column extraction methods and optimized primers were used to detect the *gyrB* gene using a visual LAMP method. The sensitivity of the assay was 10 fg/μL, and the results were stable and reliable. Of 655 aquatic product samples and 558 aquaculture waters samples, the positive rates of *Vibrio vulnificus* detection were 9.01% and 8.60%, respectively, which is markedly higher than that of the traditional culture identification methods.

Conclusion The relatively simple technical requirements, low equipment costs, and rapid detection time make the visual LAMP method for detection of *Vibrio vulnificus* a convenient choice for field diagnosis in the aquaculture industry.

Background

Vibrio species are the most dominant bacteria in the marine environment and are widely distributed in estuaries, bays and coastal waters, as well as the body surface and intestinal tract of marine organisms [1]. Human infections with *Vibrio spp* caused by consumption of fish, shellfish, shrimp, crab and other aquatic products have become a worldwide concern [2,3]. At least 12 pathogenic *Vibrio* species have been reported, which are not only a public health issue, but also cause huge economic losses to the aquaculture industry [1-4]. *V. vulnificus* is responsible for more than 50% of infectious diseases in aquaculture [5-6] and has the highest fatality rate of any foodborne pathogen [7-9].

V. vulnificus infection can be caused by eating raw or uncooked oysters [10]. *V. vulnificus* infections are characterized by acute onset, severe disease and high mortality, with 50% of patients dying as a result of multiple organ failure within 48 h after onset [11], increasing to 100% if patients are not treated within 72 h [12]. *V. vulnificus* infections tend to increase with the increase with climate warming and offshore activities. The risk of infection is also increased by risk factors such as alcohol consumption, liver disease, cardiovascular disease and diabetes [13-14].

The detection of *V. vulnificus* in aquatic products is a challenge because it is difficult to isolate and grow under laboratory conditions and is readily inhibited by other *Vibrio* species. The technology used for biochemical identification of *Vibrio* is complex and not easily applied in aquaculture farms. Traditional techniques are time-consuming and often require professional technicians. Thus, rapid, accurate and sensitive techniques for the detection of pathogenic *Vibrio* species on-site. Loop-mediated isothermal amplification (LAMP) is a simple and rapid technique for gene amplification that was developed by Notomi et al. (2000) [15]. With the advantages of high specificity, efficiency and simple technical requirements, LAMP has been widely used for the detection of pathogens [16-18]. In this study, we developed a visual LAMP-based method for the detection of *V. vulnificus* in aquatic products and

environmental water samples with high specificity, sensitivity and reproducibility by targeting the *gyrB* gene.

Methods

Vibrio species

The following *Vibrio* species were used in this study: *V. vulnificus* ATCC 27562, *V. splendidus* ATCC 33125, *V. mimicus* CICC 21613. *V. metschnikovii* ATCC 27562, *V. furnissii* IQCC 12309, *V. fluvialis* CICC 21612, *V. alginolyticus* ATCC17749, *V. parahaemolyticus* ATCC 17802, and *V. anguillarum* CICC 10475. All *Vibrio* species were stored by Dalian Customs Technology Center and were identified using biochemical methods and were stored at -80°C.

Sample preparation and DNA extraction

Environmental water samples (500 mL) were collected from rivers (upper, central, and lower parts) and the sea. For each sample, 1 mL water was added to a tube containing 9 mL alkaline peptone broth (APB) with 3% NaCl. For marine shellfish, the shells were washed with running water, sterilized with 70% alcohol and approximately 20 g was homogenized in 50 mL 0.85% sterile normal saline. Infected fresh water or marine fish were sterilized with 70% alcohol before the liver, spleen, kidney and ulcerative lesions were removed. Approximately 20 g of each tissue was homogenized. Shrimp and crab were sterilized with 70% alcohol before samples (20 g) were homogenized and 1 mL of the homogenate was added to 9 mL APB with 3% NaCl. The samples were then incubated overnight at 37°C to amplify the bacteria. Subsequently, 1 mL of the culture was centrifuged at 12,000 rpm for 2 min, and the supernatant was collected for DNA extraction. The culture mixture was used to inoculate TCBS agar plates using a sterilized loop and incubated overnight at 37℃ for identification of *Vibrio* using the most-probable-number (MPN) method as previously described [19].

Bacterial genomic DNA was extracted using boiling and column extraction methods. For the boiling method, the samples (10mg) were mixed with 100 µL lysis buffer, vortexed and heated at 95℃ for 10 min before centrifugation at 12,000 rpm for 5 min as described previously. The supernatant containing the genomic DNA was transferred to a new microtube and stored at -20℃ for downstream applications. For column extraction, DNA was extracted using a commercial bacterial genomic DNA extraction kit (Cat No. TDP302 Tiangen, Beijing, China) according to manufacturer's instruction.

Primer design and synthesis

Homology analysis of *gyrB* gene (GenBank ID: MN540397.1) was performed by DNAStar software. Six LAMP primers were designed based on the *gyrB* gene using the Primer Explorer V4 software (Eiken Chemical Co., Ltd., Japan) (Table 1). These primers were synthesized by TaKaRa (Dalian, China).

LAMP reaction system and conditions

LAMP assays were performed in a 25-μL reaction volume containing 12.5 μL 2× RM reaction solution, 1.0 μL *Bst* DNA polymerase, 0.5 μL SYTO-9 fluorescent dye (or 1.0 μL of visual $MnCl_2$-calcein stock solution), 1.0 μL of each primer (final concentrations: 0.4–1.6 μM for inner primers and 0.1–0.2 μM for outer primers, and 0.1–0.8 μM for loop primers), 25 μL DNA template and ddH_2O.

The CFX96 Touch Real-Time PCR Detection System (Bio-Rad) or the Loopamp Realtime Turbidimeter LA-500 (Eiken Genome, Tokyo, Japan) was used to observe the fluorescence amplification curve (with SYTO-9 fluorescent dye) using the following reaction conditions: 63℃ for 15 s, followed by 45 cycles at 63℃ for 45 s.

For the visual LAMP assay, $MnCl_2$-calcein was added to the reaction mixture, which was incubated at 65℃ for 30 min. After termination of the reaction at 95℃ for 2 min, the samples were placed on ice and observed under UV light (240–260 nm or 350–370 nm). Samples that turned green were considered positive for *V. vulnificus*, while samples that remained orange were considered negative.

Assay specificity and sensitivity

To verify the specificity, the LAMP assay was performed as described above the using genomic DNA from *V. vulnificus*, *V. splendid*, *V. mimicis*, *V. metschnikovii, V. fischeri, V. fluvibrio, V. algolyticus* and *V. parahaemolyticus.* The sensitivity of the LAMP assay was determined by amplification of 10-fold serial dilutions of *V. vulnificus* genomic DNA (1 ng/μL, 100 pg/μL, 10 pg/μL, 1 pg/μL, 100 fg/μL, 10 fg/μL, 1 fg/μL, and 0.1 fg/μL); the assay was repeated twice for each dilution.

Detection of aquatic products and aquaculture waters

Samples of aquatic products [n = 655] obtained from restaurants (n = 155), supermarkets (n = 189), farmers' markets (n = 206), and online stores (n = 105)] and environmental water samples [n = 558; sea water (n = 440), river water (n = 98) and 20 aquaculture sea water (n = 20)] were also analyzed using the visualized LAMP detection method. Positive samples were isolated and cultured for biochemical identification of *V. vulnificus* [19].

Statistical analysis

SPSS (Statistical Product and Service Solutions, IBM) software was used to perform chi-squared (χ^2) tests, and Mann–Whitney tests was used to evaluate the significance of the difference between the results obtained using the two detection methods. $P < 0.05$ was considered to indicate statistical significance.

Results

Optimized method for extraction of *Vibrio* genomic DNA

The genomic DNA of *V. vulnificus*, *V. splendidus*, *V. parahaemolyticus*, and *V. angularis* was extracted using the boiling and column extraction methods. The purity and concentration of each sample were evaluated using an ultra-micro spectrophotometer (Table 2). As shown in Figure 1, nucleic acid purity index values (A_{260}/A_{280}) of ³1.5 or above were achieved by both boiling and column extraction, with no significant difference between the two methods ($P > 0.05$, Mann–Whitney test).

LAMP fluorescence amplification curves were generated for the extracted DNA after the addition of the SYTO-9 fluorescent dye (Figure 2), and changes in fluorescence intensity of the product were observed under UV light after the addition of $MnCl_2$-calcein (Figure 3). Typical LAMP fluorescence amplification curves were generated using *V. vulnificus* DNA extracted by both methods (Figure 2), with no difference between the Ct values obtained for each group. Typical changes in the fluorescence intensity were also observed using the visual dye method (Figure 3). These findings indicate that the residual carbohydrates produced in the sample extracted using the boiling DNA cleavage method do not affect the LAMP reaction. Furthermore, this method has the advantages of rapid extraction, low cost and convenience. Therefore, we selected the boiling method for extraction of *Vibrio* genomic DNA in this study.

Screening of LAMP primers

Six primers designed for specific detection of the *gyrB* gene sequence of *V. vulnificus* (Table 1) were screened by LAMP amplification curve analysis (with the SYTO-9 fluorescent dye) using *V. vulnificus*, and *V. parahaemolyticus* as positive and negative controls, respectively (Figure 4). The results showed that *V.vulnificus* and *V.parahaemolyticus* were amplified with the first, second, third and sixth primer sets (Figure 4A, 4B, 4C and 4F). *V.vulnificus* were not amplified in the fourth primer (FIG. 4D). The peak time of *V.vulnificus* with the fifth primer (FIG. 4E) was 9min, and the S-type amplification curve was typical, while *V.parahemolyticus* was not amplified. Therefore, based these results, we selected the fifth set of primers for detection of *V. vulnificus* due to its earlier peak Ct value and high specificity for subsequent experiments.

LAMP assay specificity

The specificity of the selected primers was then evaluated for detection of eight *Vibrio* strains (*V. vulnificus*, *V. splendidus, V. mimicus*, *V. metschnikovii*, *V. furnissii*, *V. fluvialis*, *V. alginolyticus*, and *V. parahemolyticus*) using the fluorescence amplification curve (SYTO-9 fluorescent dye) and color change ($MnCl_2$-calcein) methods of LAMP amplification as shown in Figures 5 and 6, respectively. *V. vulnificus* was amplified specifically, while no amplification of the other *Vibrio* strains was detected. Furthermore, the results obtained using the two detection methods were consistent. These findings indicate that these primers allow specific detection of *V. vulnificus* using the LAMP method.

LAMP assay sensitivity

The sensitivity of the LAMP assay for detection of *V. vulnificus* using the optimized primers was evaluated using serial dilutions of the bacterial genomic DNA as templates. Using the LAMP reaction

fluorescence amplification curve (with SYTO-9) method, the fluorescence amplification curves were consistent were stable at concentrations of *V. vulnificus* genomic DNA ³10 fg/μL, while the amplification was inconsistent and unstable at concentrations of £1 fg/μL (Figure 7). Using the color change ($MnCl_2$-calcein) method, *V. vulnificus* amplification products were detected at concentrations of genomic DNA ³10 fg/μL, but not at concentrations of £1 fg/μL (Figure 8). Thus, both LAMP methods can be used to detect *V. vulnificus* with a sensitivity of 10 fg/μL.

Analysis of actual samples

The LAMP assay established in this study was then evaluated for the analysis of aquatic product samples and water samples. Among 655 samples of aquatic products, 59 samples (9.01%) were positive for *V. vulnificus* (Table 3). Among 558 environmental water samples, 48 samples (8.60%) were positive for *V. vulnificus* (Table 4). Furthermore, consistent results for the detection of *V. vulnificus* in aquatic product and environmental water samples were obtained using the fluorescence amplification curve (with SYTO-9 fluorescent dye) and color change (with $MnCl_2$-calcein) methods.

Validation of the LAMP results by real-time fluorescent PCR [4] revealed 100% consistency between the two methods. Furthermore, *V. vulnificus* samples cultured in vitro were detected with 83.76% positivity (P = 0.00002). The results of this study showed that the rate of *V. vulnificus* detection in aquatic products and environmental water samples using biochemical methods was significantly lower than that of achieved using the LAMP method. This discrepancy can be accounted for by the slow growth of many *Vibrio* isolates in vitro, which limits detection using biochemical methods.

Detection of *V. vulnificus* in different kinds of samples

We also analyzed the detection *V. vulnificus* rates of 655 aquatic product samples comprised of pools of DNA obtained from different numbers of biological samples using the LAMP assay (Table 3). The number of positive samples was The highest positive detection rate was obtained for the pool of 35 shellfish samples (18.52%; 35/189), indicating that *V. vulnificus* is enriched in shellfish. Furthermore, the positive detection rate of *V. vulnificus* in shellfish samples was significantly higher than that in seawater fish samples (χ^2 =10.461, P < 0.01), freshwater fish samples (χ^2 = 9.221, P < 0.01) and freshwater shrimp and crab samples (χ^2 =7.895, P < 0.01). There was no significant difference in the positive detection rates of cephalopod samples (χ^2 = 21.271, P < 0.01), and sea shrimp and crab samples (χ^2 =1.524, P > 0.05).

Similar analysis of the 558 environmental water samples (Table 5) showed that the positive *V. vulnificus* detection rates for seawater, river water and aquaculture water were 10.23%, 2.04%and 5.00%, respectively. Furthermore, the positive rate of *V. vulnificus* detection in seawater samples was significantly higher than that in river water samples (χ^2 = 6.737, P < 0.01), whereas there was no significant difference in the positive rate between the aquaculture and river water samples (P > 0.05).

Detection of *V. vulnificus* in samples collected at different times of year

Studies have shown that the positive detection rate of *V. vulnificus*, which is a thermophilic bacterium, increases as the water temperature rises throughout the year, with the highest detection rate in summer [20]. In our analysis of samples collected at different times of year, the highest positive *V. vulnificus* detection rate (29.79%) was observed between June and August, which was 29.79% (Table 5).

Detection of *V. vulnificus* in samples obtained at different stages of the sales process

Most farmers' markets in China operate based on open management and sales models. Compared with farmers' markets, the conditions in supermarkets will be more standardized, with better sanitation and less cross-contamination between goods. In accordance with this, we found that the average rate of *V. vulnificus* contamination of samples from farmers' markets was higher (30.01%; 68/206) than that in supermarkets (7.41%; 14/189) (Table 6).

Discussion

In this study, we established a sensitive, specific and reproducible LAMP-based method for detection of *V. vulnificus* in aquatic products (9.01%) and environmental water samples (8.60%) in different seasons and from different commercial sources such as farmers' markets and supermarkets. This technique provides an important resource to ensure the safety of edible aquatic products and environmental water [21].

The detection of *Vibrio* species in aquatic products is challenging since many are difficult to isolate and culture in vitro. Visual LAMP-based techniques can be used to overcome this obstacle and are ideal for rapid, convenient and low-cost detection of these species in the field and aquaculture industry. Previous studies on this issue have focused on PCR methods for the detection of pathogenic *Vibrio* species such as *V. cholerae* [22], *V. parahaemolyticus* [23], *V. alginolyticus* [24,25], *V. fluvibrio* [26], and *V. furnissii* [27] in aquatic products. Multiple PCR [28-32] and LAMP-based methods for detection of *V. vulnificus* have also been studied [12,33,34], although visual LAMP-based detection method have not previously been studied.

In a study of 105 samples of seafood randomly collected in Beijing markets, Wang et al. [4] reported accuracy of *V. vulnificus* detection in 100% and 67.50% of samples by real-time fluorescent PCR and VITEK methods, respectively. In this study, we established a visual LAMP-based method for detection of *V. vulnificus* and confirmed the applicability of this approach for aquaculture field monitoring by analyzing 655 aquatic product samples and 558 environmental water samples. We found that the coincidence rate of results obtained using the visual LAMP-based and real-time PCR methods was 100%, while the coincidence rate between this method and classical biochemical culture identification was 83.76% (P = 0.00002). Furthermore, the positive *V. vulnificus* detection rate of the visual LAMP-based detection method was significantly higher than that that of the of classical isolation and culture identification method.

Conclusions

In this study, we established a LAMP-based method for the rapid (within 30 min) detection of *V. vulnificus* in aquatic products and environmental water. In particular, the color change ($MnCl_2$-calcein) methods in which results can be obtained by visual inspection provides a simple, rapid and economic technique that can be applied in the field and will be important in the prevention and control of *V. vulnificus* infections in aquaculture.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

Zhuo Tian and Lili Yang carried out the sample preparation, experiments and wrote the manuscript. Dejing Shang and Jijuan Cao gave practical guidance and carried out the study design. Qiuyue Zheng and Xin Qi help sample preparation and data analysis. All authors reviewed and approved the final manuscript.

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Conflict of interests

The authors declare that they have no conflict of interests.

Availability of data and materials

All the data required is included in the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The author declares that they have no competing interests.

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Tables

Table 1 Sequences of primers for LAMP of the *gyrB* gene of *Vibrio vulnificus*

Group	Primers	Sequences (5'-3')
Set 1	VV1-OF	CGCTGATCACTGCACTAG
	VV1-OB	ACAATCTTCGGTGAGAACAG
	VV1-IF	CGGTAGAAGAAGGTCAACAGTAGCAACTGCGTTACCACAACAT
	VV1-IB	GGATCGTATGAGCCGTCGTTACACCAGTTGCTCTGTCCA
	VV1-LF	GTACGGATGTGCGAACCA
	VV1-LB	GCCGATCAAGCAGCAATG
Set 2	VV2-OF	TAACCGTAAGAACCAAGCAAT
	VV2-OB	ACAATCTTCGGTGAGAACAG
	VV2-IF	TACGGATGTGCGAACCATCGCGCTGATCACTGCACTAG
	VV2-IB	GGATCGTATGAGCCGTCGTTACACCAGTTGCTCTGTCCA
	VV2-LF	GATGATGTTGTGGTAACGCAG
	VV2-LB	GCCGATCAAGCAGCAATG
Set 3	VV3-OF	TTCTGTTAGCGATGATGGC
	VV3-OB	GCCACTTCAACCGCAA
	VV3-IF	CACCTACACCGTGAAGACCGCTGCTGCCGAAGTTATCAT
	VV3-IB	CCTGAACTCTGGCGTGTCGTTGTTGCGGTTCAAGTGA
	VV3-LF	ACTTACCACCAGCGTGC
	VV3-LB	CATGTATGAAGGTGGTATTCAAGC
Set 4	VV4-OF	CTGCTGCCGAAGTTATCAT
	VV4-OB	CACCGTCACGCTGTG
	VV4-IF	CATCAGGCACACCATGACGATCTTCACGGTGTAGGTGT
	VV4-IB	TGTATGAAGGTGGTATTCAAGCGTTGCCACTTCAACCGCAA
	VV4-LF	GGTATGACCACCACGATGAA
	VV4-LB	TCACTCACTTGAACCGCAA
Set 5	VV5-OF	GCTTGCTATCATCGGTGAT
	VV5-OB	CACCGTCACGCTGTG
	VV5-IF	AACGCTTGAATACCACCTTCATACAATCCTAGCGAAGCGTCT
	VV5-IB	TCACTCACTTGAACCGCAACAGCCACTTCAACCGCAA

	VV5-LF	CGACACGCCAGAGTTCA
	VV5-LB	TTAATGCCGAGCGTGAAGA
Set 6	VV6-OF	CGTCATGGTGTGCCTG
	VV6-OB	CACCGTCACGCTGTG
	VV6-IF	AACGCTTGAATACCACCTTCATACAACATCCTAGCGAAGCGT
	VV6-IB	TCACTCACTTGAACCGCAACAGCCACTTCAACCGCAA
	VV6-LF	CGACACGCCAGAGTTCA
	VV6-LB	TTAATGCCGAGCGTGAAGA

Table 2 Absorption and concentration of genomic DNA extracted from four *Vibrio* strains

Vibrio strain	DNA extraction method	Mean±standard deviation	
		A_{260}/A_{280}	Concentration（μg/mL）
V. vulnificus	Boiling	1.594±0.034	679.950±16.193
	Centrifugation	1.498±0.023	18.650±0.566
V. splendidus	Boiling	1.491±0.036	356.800±7.990
	Centrifugation	1.671±0.009	41.450±1.768
V. parahemolyticus	Boiling	1.642±0.046	399.500±10.607
	Centrifugation	1.679±0.025	22.750±0.495
V. anguillarum	Boiling	1.696±0.023	422.050±3.182
	Centrifugation	1.875±0.015	42.650±0.071

Table 3 LAMP detection of *Vibrio vulnificus* in different aquatic products

Sample type	Sampling quantity (pieces)	Number of positive results of *V. Vulnificus*, n / number of total samples, %		
		the results of the fluorescence amplification curve	the results of color change	Positive rate (%)
Freshwater shrimp and crab	36	0	0	0
Freshwater fish	105	6	6	5.71
Seawater shrimp and crab	53	6	6	11.32
Seawater fish	171	12	12	7.02
Shellfish	189	35	35	18.52
Cephalopods	101	0	0	0
Total	655	59	59	9.01

Table 4 LAMP detection of *Vibrio vulnificus* in environmental water samples

Sample	Sampling quantity (pieces)	Number of positive results of *V. Vulnificus*, n / number of total samples, %		
		the results of the fluorescence amplification curve	the results of color change	Positive rate (%)
Sea water	440	45	45	10.23
River water	98	2	2	2.04
Aquaculture water	20	1	1	5.00
Total	558	48	48	8.60

Table 5 LAMP detection of *Vibrio vulnificus* in samples collected at different times of year

Sampling time	Sampling quantity (pieces)	Number of positive results of *V. Vulnificus*, n / number of total samples, %		
		the results of the fluorescence amplification curve	the results of color change	Positive rate (%)
March–May	174	18	18	10.34
June–August	235	70	70	29.79
September–November	132	17	17	28.03
December–February	114	2	2	1.75
Total	655	107	107	16.34

Table 6 LAMP detection of *Vibrio vulnificus* in samples from different sampling links

Sampling link	Sampling quantity (pieces)	Number of positive results of *V. Vulnificus*, n / number of total samples, %		
		the results of the fluorescence amplification curve	the results of color change	Positive rate (%)
Café	155	18	18	11.61
Supermarket	189	14	14	7.41
Farmers' market	206	68	68	33.01
Online store	105	7	7	6.67
Total	655	107	107	16.34

Figures

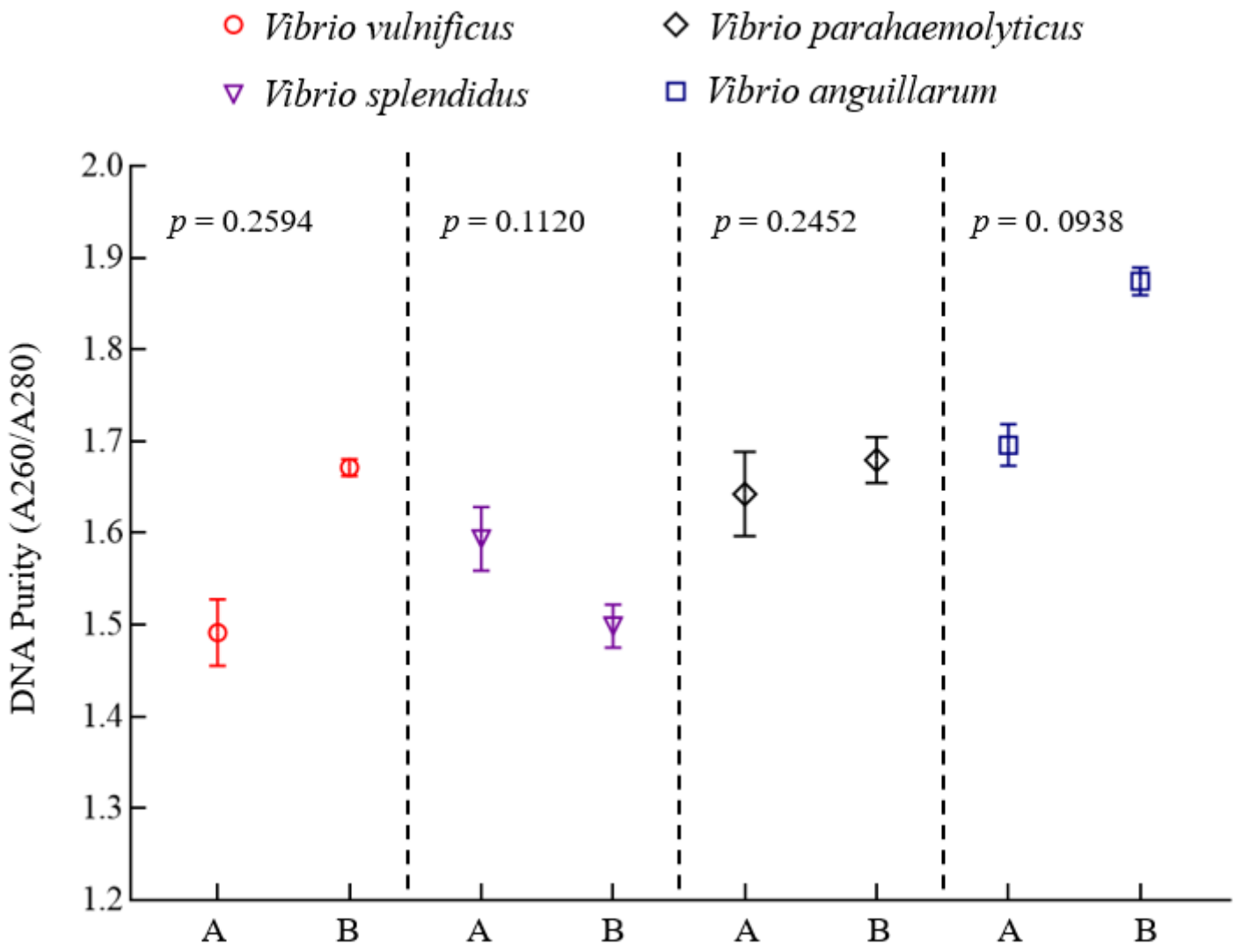

Figure 1

Genomic DNA purity indexes (A260/A280) obtained from samples using the boiling and column extraction methods. A: Column extraction method; B: Boiling method

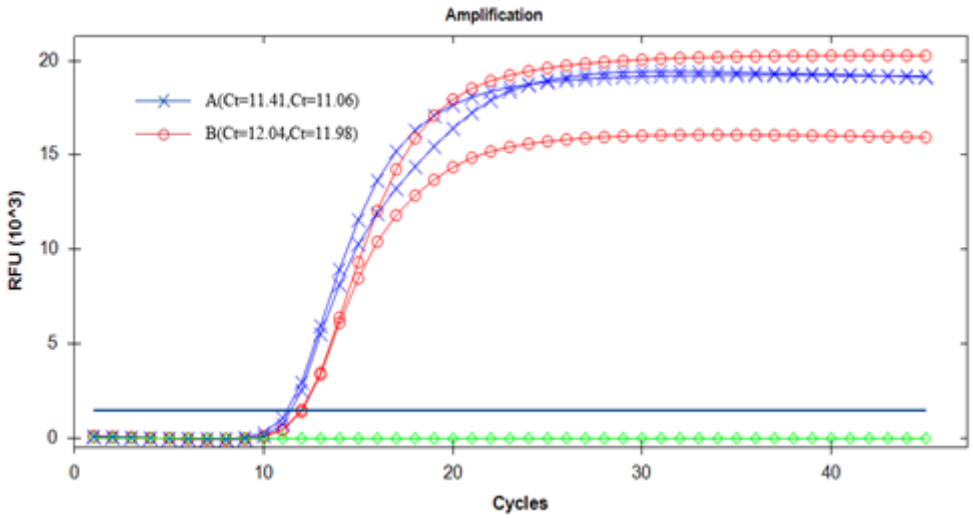

Figure 2

LAMP fluorescence amplification curve of genomic DNA isolated from V. vulnificus using different extraction methods. A: Column extraction method; B: Boiling method

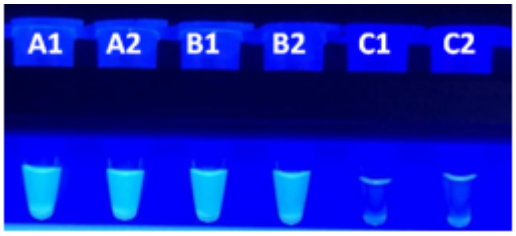

Figure 3

Visual detection of LAMP amplification products in samples of genomic DNA isolated from V. vulnificus using different extraction methods. A1, A2: Column extraction method; B1, B2: Boiling method; C1, C2: Blank control

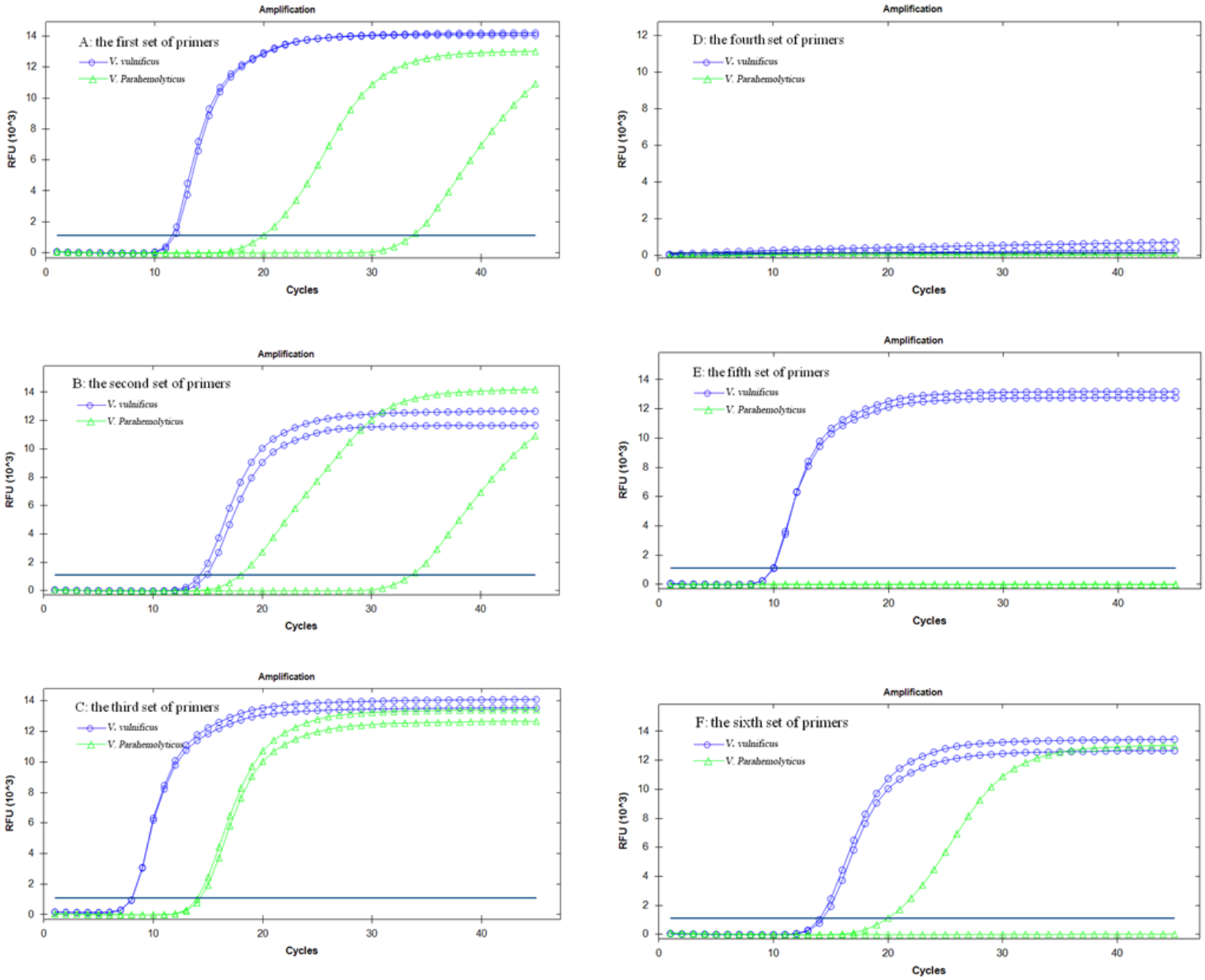

Figure 4

Screening of six primers for LAMP amplification of the gyrB gene of Vibrio vulnificus

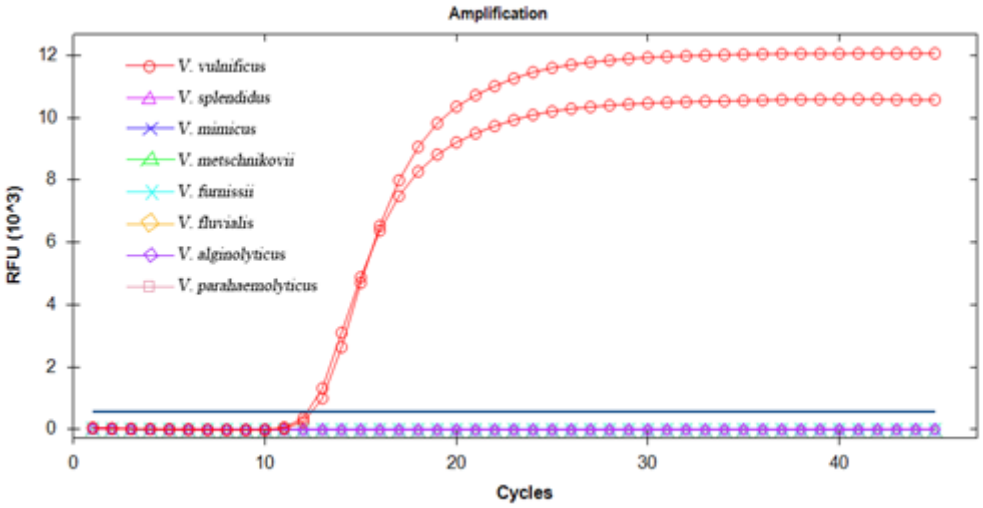

Figure 5

Specificity of the LAMP fluorescence assay for detection of V. vulnificus.

Figure 6

Specificity of the visual LAMP assay for detection of V. vulnificus under UV light. A: V. vulnificus; B:V. splendidus ; C: V. mimicus; D:V. metschnikovii; E:V. furnissii; F:V. fluvialis; G:V. alginolyticus; H:V.parahaemolyticus

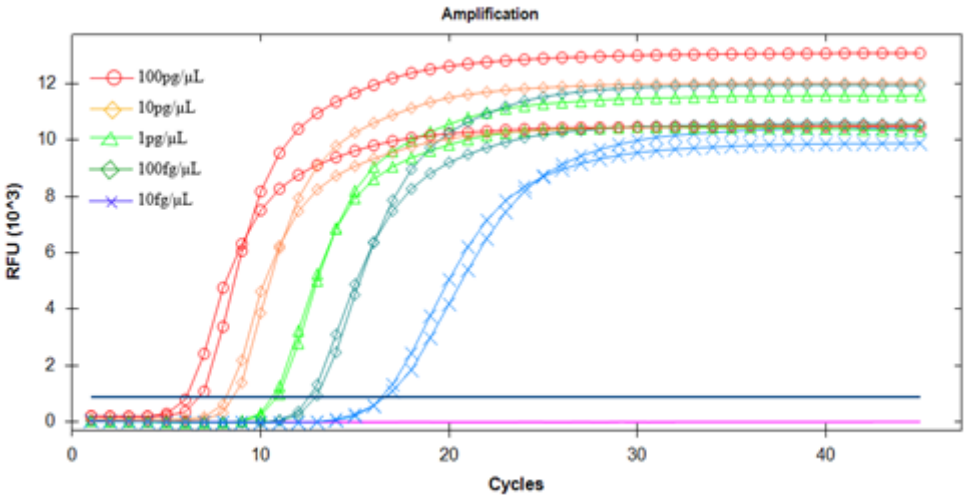

Figure 7

Sensitivity of the of LAMP fluorescence method for the detection of V. vulnificus.

Figure 8

Sensitivity of the of visual LAMP method for the detection of V. vulnificus. A:1ng/μL; B: 100pg/μL; C: 10pg/μL; D: 1pg/μL; E: 100 fg/μL; F: 10 fg/μL; G: 1fg/μL; H: 0.1fg/μL; I: Blank control