

Full Length Research Paper

Establishment of universal loop-mediated isothermal amplification method (LAMP) for rapid detection of pathogenic *Vibrio* spp. in aquatic organisms

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Pathogenic *Vibrio* spp. are regarded as the causative agent of prevalent vibriosis in aquatic organisms and food-borne zoonotic diseases of human, therefore, a rapid and effective detection method is useful for confirming early stages of the infection when the bacterial infection is at a relatively low level, which would significantly reduce the economic loss in aquaculture and seafood industry. Loop-mediated isothermal amplification (LAMP) is a convenient diagnostic method that amplifies DNA with high specificity and rapidity under isothermal conditions. In this study, using the LAMP method, a universal diagnostic protocol was successfully developed for the detection of *Vibrio* spp. in aquatic organisms. Based on 16S rRNA gene conservative sequence of genus *Vibrio*, a set of four primers was designed. The reaction time and temperature were optimized for 60 min at 62°C, respectively. The detection limit using the universal LAMP method was up to 10³ cfu/ml and it was 100 times higher than the detection limit of the standard PCR method. The amplification products were detected by visual inspection using SYBR Green I. The specificity of the LAMP was evaluated by using 30 strains of nine *Vibrio* species and 21 related non-*Vibrio* micro-organism strains as controls. The target 30 *Vibrio* strains were all amplified, and no cross-reaction was found with all the non-*Vibrio* micro-organism strains. In conclusion, the universal LAMP assay should be a potential tool with high convenience, rapidity, sensitivity and specificity for the detection of *Vibrio* spp. in infected aquatic organisms, thereby facilitating surveillance of vibriosis.

Key words: Loop-mediated isothermal amplification (LAMP), pathogenic *Vibrio* spp., detection, aquatic organisms, China.

INTRODUCTION

Vibrio species are opportunistic pathogens that are widely distributed in marine aquaculture environment, causing infections to commercially important species of crustaceans (Sung et al., 2001; Soto-Rodriguez et al., 2010; Alagappan et al., 2010; Phumkhachorn et al., 2010; Ji et al., 2011; Raissy et al., 2011), bivalves

(Tubiash et al., 1970; Beaz-Hidalgo et al., 2010), fish (Toranzo et al., 2005; Won and Park 2008; Pal et al., 2010; Frans et al., 2011), and even human by the reason of sea food poisoning (Sack et al., 2004; Tracz et al., 2007). These bacteria can therefore cause large economic losses to aquaculture and severe complications for human health. Consequently, the rapid detection of *Vibrio* spp. would be needed to provide long-term financial stability for the aquaculture industry and ensure a steady supply of seafood that is safe for consumers.

The treatments against pathogenic *Vibrio* species infections in aquaculture are basically similar, for example, antimicrobial therapy. When infections occurred, farmers' most concern is whether the cultured organisms are infected by pathogenic *Vibrio* spp. or not,

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Abbreviations: LAMP, Loop-mediated isothermal amplification method; PCR, polymerase chain reaction; UNG, uracil-N-glycosylase; dUTP, 2'-Deoxyuridine 5'-Triphosphate.

but less likely to care for differentiating *Vibrio* species. Therefore, it is essential to develop a rapid universal detection method of pathogenic *Vibrio* spp. for their diagnosis and improving industrial aquaculture production. Traditionally, detection of *Vibrio* spp. has consisted of isolation on selective agar medium followed by biochemical and serological testing (Harwood et al., 2004), which is time-consuming and laborious, requiring more than three days. In the last few years, molecular techniques for the detection of *Vibrio* spp. have been described, including oligonucleotide probes (Wright et al., 1993; Raghunath et al., 2007), DNA microarray technologies (Panicker et al., 2004a; Wang et al., 2011) and PCR-based methods (Panicker et al., 2004b; Han, 2010a; Cao et al., 2010; Balboa et al., 2011; Izumiya et al., 2011; Zhao et al., 2011). These molecular techniques offer more sophisticated approaches to detect pathogenic *Vibrio* spp. than conventional biochemical-based assays, but they require expensive equipment and also too tedious, time-consuming and not yet widely available.

Recently, LAMP assay is an alternative method of rapid DNA amplification within one reaction tube under isothermal conditions (Notomi et al., 2000), and it has already been applied for the detection of viral, bacterial, fungal, and parasitic agents (Savan et al., 2005). The method requires a specially designed primer set that recognizes at least six independent regions of the target gene, which increases the specificity as well as the rapidity of the reaction (Notomi et al., 2000). The amplification of products can be detected by agarose gel electrophoresis, by a visual assessment of turbidity, by the use of a turbidometer, or by the addition of fluorescent reagents such as SYBR green I (Iwamoto et al., 2003). Also, LAMP allows one-step detection of gene amplification without specialized equipment, and requires only a simple incubator, such as a heat block providing a constant temperature. Thus LAMP assay is faster and easier to perform than the above-mentioned detection methods, as well as being more specific. LAMP of conserved regions of the bacterial genome, in particular the 16S rRNA gene, is a well-established technique for the identification of bacterial pathogens. The main advantage of targeting the *Vibrio* genus-specific 16S rRNA sequences is that the broad range of *Vibrio* species can be detected (DeLong et al., 1989; Weisburg et al., 1991; Dorsch et al., 1992; Ruimy et al., 1994; Rantakokko-Jalava et al., 2000). Although current LAMP assays for the identification of a single species of pathogenic *Vibrio* genus have been developed independently (Yamazaki, et al., 2008a, b; Ren, et al., 2009; Srisuk et al., 2010; Han and Ge, 2010b; Li et al., 2010; Yamazaki et al., 2011; Han et al., 2011), no assay for the universal detection of multiple pathogenic *Vibrio* spp. has been described.

In the present study, we described a sensitive, rapid and simple universal LAMP assay based on the highly conserved region of the genus *Vibrio* 16S rRNA gene fragments and assessed its viability as a potential

diagnostic tool when compared with the standard PCR method for the detection of *V. alginolyticus* in tissues from infected aquatic organisms under laboratory conditions.

MATERIALS AND METHODS

Bacteria strains

V. alginolyticus (ATCC 33840), *V. parahaemolyticus* (ATCC 33847), *V. harveyi* (ATCC 33867) and *V. fluvialis* (ATCC 33812) were purchased from Institute of Microbiology Chinese Academy of Science, used as positive controls; *Aeromonas hydrophila* purchased from National Pathogen Collection Centre for Aquatic Animals, China; *Escherichia coli* and *Salmonella* spp. were freely provided by the Veterinary microbiology laboratory in Zhejiang University, China; *Aeromonas* spp. (laboratory isolate), *Bacillus* spp. (laboratory isolate), *Pseudomonas* spp. (laboratory isolate), *Acinetobacter* spp. (laboratory isolate) were used as negative controls.

Other bacterial stains used in this study were isolated originally from aquaculture environment or cultured aquatic organisms in China and identified with a battery of biochemical tests over a 10-year period. All strains were retrieved from -80°C stock and cultured in Luria-Bertani broth (per liter: 10 g Bacto-tryptone, 5 g NaCl, 5 g yeast extract) at 28°C . A list of the isolates used in this study is given in Table 1. After overnight incubation, bacterial cells were collected by centrifugation and subjected to genomic DNA extraction.

Extraction of DNA from bacterial cultures

Bacterial DNA for the LAMP assay was extracted by boiling (Queipo-Ortuno, et al., 2008). In brief, one milliliter liquid medium with pure culture of bacteria was centrifuge in 12 000 rpm for 5 min, added with 100 μl of sterile water, mixed and bathed at 100°C for 10 min. After ice bathed for 2 min and 12 000 rpm centrifuged for 5 min, the supernatant was extracted as a template for next LAMP and PCR amplification. The extracted DNA was stored at -20°C until use.

LAMP Primer design

After sequences alignment of 16S rRNA gene of *Vibrio* spp. was performed, a set of genus-specific LAMP primers was designed based on their conserved gene sequences, including two outer primers (F3, B3), and two inner primers (FIP, BIP) (as shown in Table 2). Another set of primer was designed for PCR targeted the upstream and downstream of the LAMP target fragments (as shown in Table 2). All of the primers were synthesized by Invitrogen.

LAMP reaction

The LAMP was carried out in 50 μl of a mixture containing 2 μl DNA template, 2 μl (40 pmol) of each of the FIP and BIP primers, 1 μl (5 pmol) of each the F3 and B3 primers, 2 μl (16 units) of *Bst* DNA polymerase (New England Biolabs), 5 μl 10 \times reaction buffer (200 mM Tris-HCl (pH 8.8 @ 25°C), 100 mM KCl, 100 mM $(\text{NH}_4)_2\text{SO}_4$, 20 mM MgSO_4 , 1% Triton X-100), 10 μl (0.8 M) betaine (Sigma-Aldrich), and 2 μl (2.5 mM of each) dNTPs and 23 μl double-distilled water. Incubation was then carried out at 58, 60, 62, 64 and 66°C

Table 1. Bacterial strains used for determining specificity of LAMP.

Classification	Name of organism	Numbers of strains	Source		
Vibrio spp.	<i>V. alginolyticus</i> ATCC 33840	1	IMCAS		
	<i>V. parahaemolyticus</i> ATCC 33847	1	IMCAS		
	<i>V. harveyi</i> ATCC 33867	1	IMCAS		
	<i>V. fluvialis</i> ATCC 33812	1	IMCAS		
	<i>V. parahaemolyticus</i>		2	Shrimp	
			2	Crab	
			3	Fish	
	<i>V. vulnificus</i>		3	Fish	
		<i>V. harveyi</i>	1	Shrimp	
			2	Fish	
	1		Shellfish		
	<i>V. alginolyticus</i>		1	Shrimp	
			3	Fish	
			1	AE	
		<i>V. fluvialis</i>		1	AE
				3	Fish
				1	Shrimp
				1	AE
	<i>V. cincinnatiensis</i>	1	AE		
Non- Vibrio spp.	<i>A. hydrophila</i>	1	NPCCAA		
	<i>E. coli</i>	1	ZJU		
	<i>Salmonella</i> spp.	1	ZJU		
	<i>Pseudomonas</i> spp.	3	AE		
	<i>Bacillus</i> spp.	4	AE		
	<i>Aeromonas</i> spp.		2	Fish	
			2	Shrimp	
			3	AE	
	<i>Acinetobacter</i> spp.	4	AE		

Note: IMCAS: Institute of Microbiology Chinese Academy of Science; AE: aquaculture environment; ZJU: Veterinary microbiology laboratory in Zhejiang University; NPCCAA: National Pathogen Collection Centre for Aquatic Animals, China.

for 60 min, and the reaction was terminated by heating at 80°C for 5 min. To find the optimum time, five different reaction times including 30, 40, 50, 60 and 70 min were used separately in the LAMP reaction. The products (2.5 µl) were electrophoresed on 2% agarose gels to determine the optimal conditions. Added with SYBR Green I, the color change of the reaction tube can be observed by the naked eyes.

Sensitivity of LAMP

To find the sensitivity of LAMP assay, a 10-fold serial dilution of an initial culture of *V. alginolyticus* (1.8 × 10⁷ cfu/ml) was performed and DNA extracted from the respective dilutions of the initial

inoculums was used for LAMP following the predetermined conditions as determined above. To compare the detection sensitivity of LAMP, standard PCR assay was performed. DNA extracted from the respective dilutions was subjected to thermal cycling using the Va-Pf and Va-Pr primers, which amplified a 474-bp product. The PCR reaction was carried out for 35 cycles, each of which consisted of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 30 s.

Specificity of LAMP

To determine the specificity of detection, LAMP assays were carried out with the DNA templates of 30 strains of nine *Vibrio* species and

Table 2. LAMP and PCR Primers used in this study.

Method	Primer	Sequence
LAMP	FIP	5'-CGGCTGCTGGCACGGAGT-TTTT-GCATTATTTGACGTTAGCGACAGAAG-3'
	BIP	5'-GAGCGTTAATCGGAATTACTGGGC-TTTT-CCGGGCTTTTACATCTGACTTAAC-3'
	F3	5'-CAGTCGTGAGGAAGGTGGTGT-3'
	B3	5'-CTAGTCTGCCAGTTTCAAATGCT-3'
PCR	Va-Pf	5'-AGACACGGTCCAGACTCCTAC-3'
	Va-Pr	5'-AGGGTATCTAATCCTGTTTGCT-3'

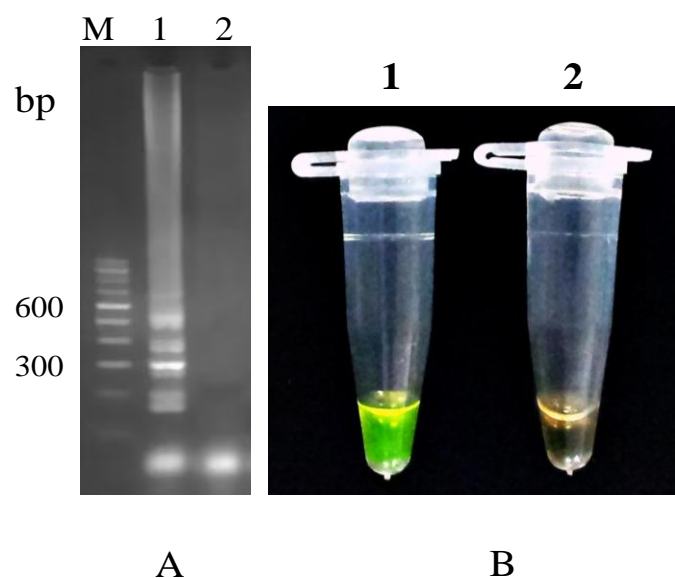


Figure 1. Detection of *Vibrio* spp. by electrophoretic analysis (A) and visual inspection (B) in the LAMP assay. (A) amplified products detected by agarose gel electrophoresis. Lane M, 100 bp DNA ladder; lane 1, detection of *V. alginolyticus*; lane 2, negative control (distilled water). (B) amplified products detected by visual inspection. Tube 1, LAMP for *V. alginolyticus*. Tube 2, negative control.

21 related non-*Vibrio* micro-organism strains as controls (as shown in Table 1).

Bacterial challenge and LAMP detection

Living banded grouper (*Epinephelus awoara*) (body weight 500 g), greasyback shrimp (*Metapenaeus ensis*) (body weight 8 g) and manila clam (*Ruditapes philippinarum*) (body weight 15 g) were purchased at a local fish market in Hangzhou, China, and acclimatized under the laboratory conditions for one week before bacterial challenge. The groupers, shrimps and clams were divided randomly into two groups respectively ($n=15$ each). Each grouper in test group was injected intraperitoneally with 0.3 ml of *V. alginolyticus* suspension at a density 10^7 cfu/ml, and each shrimp was injected intramuscularly with 0.01 ml of the bacteria suspension in the second abdominal segment, while each of the controls were injected with the same volume of sterile saline. Both groups of

clams were placed in two tanks with one liter of sterile seawater separately, and the bacterial suspension was added to the seawater of the test group, giving a final concentration of 1×10^7 cfu/ml. Two hundred milligrams of liver tissues of fish, gill tissues of shrimps and viscera tissues of clams were collected and homogenized separately. Then 300 μ l sterile distilled water was added and the mixture was incubated at 100°C for 10 min; followed by 2 min in ice, and then centrifuged by 12 000 rpm for 5 min. These supernatants were used for LAMP test. The tissues also run the classical microbiological examinations of *V. alginolyticus* as the method control.

RESULTS

LAMP method for detection

The 16S rRNA gene of *Vibrio* spp. was amplified successfully by LAMP with the set of genus-specific primers designed. The electrophoresis results were shown in Figure 1A. Ladder-like bands appeared in *V. alginolyticus* lane, but not in the negative control with double distilled water. The green color, produced by the adding with fluorescent dye SYBR Green I, was observed in the positive LAMP reactions, while an orange color was obvious in the negative control (Figure 1B).

Determination of conditions for *Vibrio* spp. detection by LAMP

The LAMP was carried out using *V. alginolyticus* DNA as template to determine the optimal temperature and time of reaction. LAMP products were formed at 58, 60, 62, 64 and 66°C. However, 62°C was considered the optimal temperature due to the clarity of the bands (Figure 2). The LAMP amplicons could be observed at 30 min. However, at 60 min, the intensity of LAMP amplicons was stronger and clearly (Figure 3). Hence, the condition for LAMP assay was optimized for 60 min at 62°C.

Sensitivity for *Vibrio* spp. detection by LAMP

Based on initial inoculums of *V. alginolyticus* (1.8×10^7 cfu/ml), a 10-fold serial dilution of the culture was used

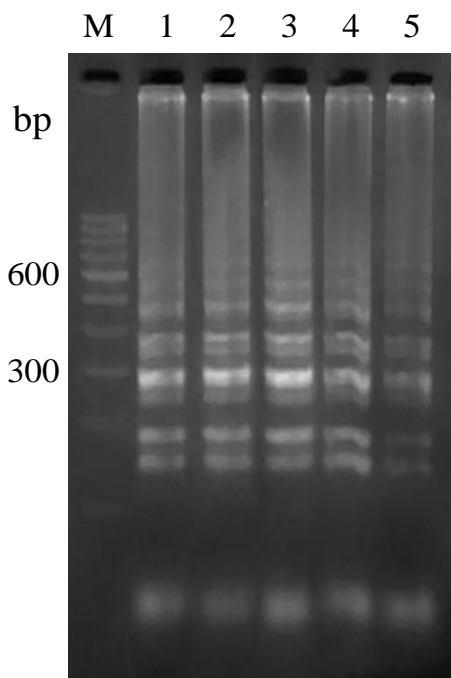


Figure 2. Determination of LAMP conditions: effect of reaction temperature on amount of LAMP product. Lane M: 100 bp DNA ladder; lanes 1–5, LAMP carried out at 58, 60, 62, 64 and 66°C, respectively. All the products were electrophoresed on 2% agarose gels and stained with ethidium bromide.

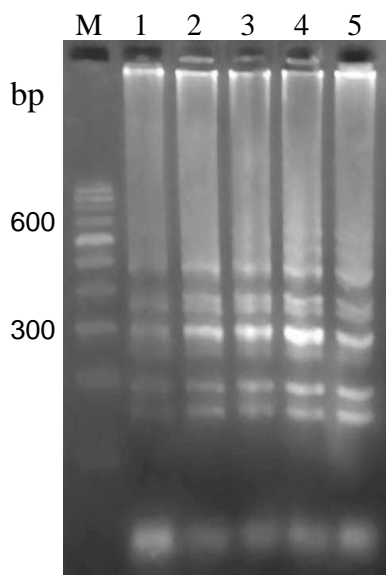


Figure 3. Determination of LAMP conditions: effect of reaction time on amount of LAMP product. Lane M: 100 bp DNA ladder; lanes 1–5, LAMP carried out for 30, 40, 50, 60 and 70 min, respectively. All the products were electrophoresed on 2% agarose gels and stained with ethidium bromide.

and their respective DNA was used for the subsequent LAMP reaction and standard PCR. The LAMP reaction was able to detect *V. alginolyticus* up to 1.8×10^3 cfu/ml (Figure 4). However, standard PCR could detect *V. alginolyticus* up to 1.8×10^5 cfu/ml, indicating a 100-fold higher sensitivity of LAMP than that of the standard PCR method.

Specificity for *Vibrio* spp. detection by LAMP

The LAMP was evaluated for its specificity using 30 *Vibrio* spp. strains and 21 non-*vibrio* micro-organism strains as controls (shown in Table 1). Only the targeted 30 strains of nine *vibrio* species were amplified. No cross-reaction was found with all the related non-*Vibrio* micro-organism strains. These results indicate that the LAMP technique developed in this study is highly specific for pathogenic *Vibrio* species.

Detection of *Vibrio* spp. in infected aquatic organisms by LAMP

As results shown in Figure 5, all the tissue samples from artificially infected fish, shrimps and clams under laboratory conditions were positive by LAMP amplification, whereas all the control samples failed to amplify. The classical microbiological examinations gave identical results as LAMP amplifications.

DISCUSSION

The *Vibrio* spp. is a diverse group of Gram-negative bacteria found exclusively in the aquatic environment, which are associated with infections to commercially important species of mariculture organisms, such as shrimps, bivalves and fish. Some of these species are pathogenic to human and constitute a potential health threat as a result of raw or undercooked seafood. Since pathogenic *Vibrio* spp. has become a major obstacle in aquaculture, as well as a public health problem and the safety of the human food supply (Tantillo et al., 2004; Frans et al., 2011), a rapid and effective detection method would be needed in order to monitor its presence in aquaculture and aquatic products.

LAMP is a convenient diagnostic method, requiring only a conventional water bath or heat block for incubation under isothermal conditions (Notomi et al., 2000). Another useful feature of LAMP is that its products can be observed directly, by naked eye, because a white precipitate of magnesium pyrophosphate forms in the reaction tube (Mori et al., 2001). Adding SYBR Green I to LAMP reactions can increase the ease and sensitivity of detection by the naked eye (Iwamoto et al., 2003). Based on this factor alone, the LAMP assay can be performed without any specialized equipment.

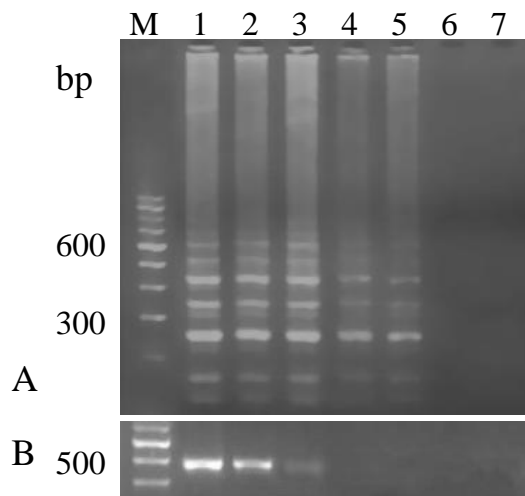


Figure 4. Comparison of the detection limits of the LAMP (A) and PCR (B). Lanes M: 100 bp DNA ladder; lane 1 to 7, The results of LAMP and PCR for different bacterial concentrations (cfu/ml): 1.8×10^7 , 1.8×10^6 , 1.8×10^5 , 1.8×10^4 , 1.8×10^3 , 1.8×10^2 and 1.8×10^1 , respectively. All the products were electrophoresed on 2% agarose gels and stained with ethidium bromide.

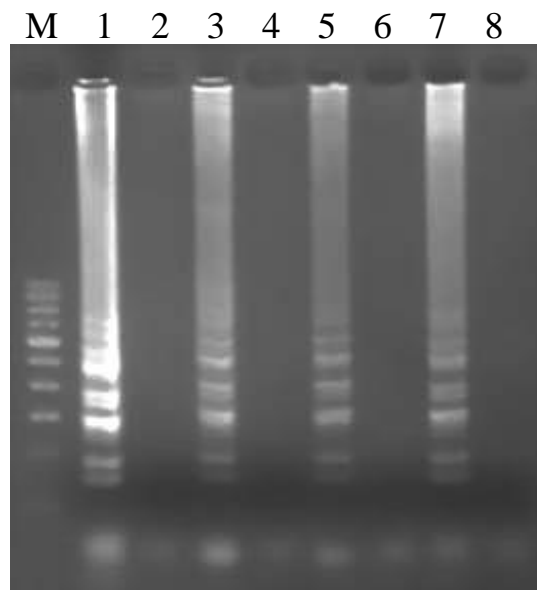


Figure 5. Detection of *Vibrio* spp. in infected aquatic organisms by LAMP. Lanes M, 100 bp DNA ladder; lane 1, infected banded grouper; lane 2, non-infected banded grouper; lane 3, infected greasyback shrimp; lane 4, non-infected greasyback shrimp; lane 5, infected manila clam; lane 6, non-infected manila clam; lane 7, *V. alginolyticus* served as positive control; lane 8, double distilled water served as negative control.

Although several LAMP assays have been developed for rapid detection of *Vibrio* spp., including *V. vulnificus*

(Han and Ge, 2008, 2010b; Ren et al., 2009; Li et al., 2010), *V. cholerae* (Yamazaki et al., 2008b; Srisuk et al., 2010), *V. parahaemolyticus* (Yamazaki et al., 2008a, 2011), *V. nigripulchritudo* (Fall et al., 2008, 2011), *V. corallilyticus* (Liu et al., 2010), *V. harveyi* (Cao et al., 2010), *V. alginolyticus* (Cai et al., 2010) in recent years, but LAMP assay in all these reports was used to detect and discriminate rapidly only one strain of pathogenic *Vibrio* species in every reaction. Because the overwhelming majority of *Vibrio* species are causative agents of vibriosis in aquatic organisms and food-borne illnesses, the universal detection of *Vibrio* spp. with one single assay significantly increase the speed of detection and thereby improve the microbiological safety of aquatic organisms and seafood.

In this study, a universal LAMP assay targeted to the conserved regions of 16S rRNA gene sequences of pathogenic *Vibrio* spp. was successfully developed. The optimal condition for detecting *Vibrio* spp. DNA was determined at 62°C for 60 min. However, detection was possible within 30 min. Further, amplification of the LAMP assay could be judged by visual assessment using the naked eye after adding SYBR Green I, without the need for electrophoresis. This suggests that it is possible to detect the pathogenic *Vibrio* spp. in a very short time (<60 min) and the rapidity of this protocol makes it preferable over other conventional and molecular methods of detection mentioned above.

The LAMP method used for *Vibrio* spp. detection was found to be highly sensitive, as it could detect *V. alginolyticus* up to 1.8×10^3 cfu/ml, whereas by standard PCR the detection it was possible up to 1.8×10^5 cfu/ml. This indicates that LAMP is 100 times more sensitive than the standard PCR. This result is consistent with previous studies and demonstrated higher sensitivity of LAMP compared to that of PCR (Yamazaki et al., 2008a; Srisuk et al., 2010; Han and Ge, 2008, 2011; Ren et al., 2009; Li et al., 2010; Prompamorn et al., 2011). This increased sensitivity makes LAMP a better choice than standard PCR for the diagnosis of pathogenic *Vibrio* spp. Moreover, the LAMP reaction was positive for all *Vibrio* species and negative for all non-*Vibrio* species tested, demonstrating that these primers were specific for identification of pathogenic *Vibrio* spp. All the samples of aquatic organisms infected artificially with *V. alginolyticus* were positive by LAMP amplification, while all the controls were negative, which was identical to the result by classical microbiological examination.

The LAMP reaction does not progress without the hybridization of six distinct sequences in the target DNA by four different highly specific primers, and thus it is highly specific. The efficiency of LAMP does not seem to be affected by the presence of non target genomic DNA in the reaction mixture, which is highly desirable in the development of a diagnostic system (Fall et al., 2008). Although, there is a limitation of carry-over contamination to the method, it can be overcome effectively by using uracil-N-glycosylase (UNG) and 2'-Deoxyuridine

5'-Triphosphate (dUTP) in the LAMP reaction mixture (He and Xu, 2011).

In conclusion, a universal LAMP assay developed in this study was a highly specific, sensitive, rapid method for the detection of pathogenic *Vibrio* species in aquatic organisms. This is the first report to use the universal LAMP technique for the detection of *Vibrio* species. This protocol is very useful for the detection of *Vibrio* infection in aquatic organisms and even if the infection is at a relatively low level, which is of great significance for ensuring the good health of aquatic organisms and reduction of economic loss in aquaculture.

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