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Rapid, specific and sensitive detection of *Vibrio vulnificus* by loop-mediated isothermal amplification targeted to *vvhA* gene

ZHANG Lina[1], WANG Mingyi[1, 2], CONG Dianxia[2], DING Shuyan[2], CONG Rinan[2], YUE Jinyong[2], GENG Jianli[2], HU Chengjin[1]*

[1] Department of Laboratory Medicine, General Hospital of Jinan Military Region of PLA, Jinan 250031, China

[2] Department of Central Laboratory, Weihai Municipal Hospital affiliated to Dalian Medical University, Weihai 264200, China

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Abstract

Vibrio vulnificus is an estuarine bacterial pathogen for human. The rapid, specific and sensitive detection of *V. vulnificus* is urgently needed for early disease diagnosis and timely treatment of *V. vulnificus* infection. In the study, a loop-mediated isothermal amplification (LAMP) technique was developed for *V. vulnificus* detection with a set of primers, composed of two out primers and two inner primers targeted to *vvhA* gene. The optimal amplification temperature was 63°C and the reaction only took 35 min. The amplification products could not only be detected by agarose gel electrophoresis with ladder-like pattern bands, but also could be visualized using calcein with naked eye directly. Forty-five strains were tested for the specificity of LAMP assay, and all the *V. vulnificus* strains were identified correctly while other strains were negative results. The sensitive of the new LAMP assay was 100-fold more sensitive than the conventional PCR. Meanwhile, all the *V. vulnificus* strains were detected correctly in spiked, clinical and environmental samples by the new LAMP assay. Compared with other well-known techniques, the new LAMP assay targeted to *vvhA* gene was extremely rapid, simple, sensitive and specific for *V. vulnificus* identification.

Key words: *Vibrio vulnificus*, loop-mediated isothermal amplification, *vvhA* gene

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1 Introduction

Vibrio vulnificus is a halophilic estuarine bacterium, which causes gastroenteritis, necrotizing wound infections, or even leads to severe sepsis with high fatality (Richards and Nuñez, 2006). In the last decade, the infection rate of *V. vulnificus* has increased annually worldwide, probably due to the global warming, which affects ocean temperatures and currents (Böer et al., 2013; Jones et al., 2013).

Pathogenic *V. vulnificus* cycles between two distinct environments, i.e., the estuarine environment and the human body, and is virulent not only for humans but also for aquatic animals, such as marine fish (especially eels) and shell fish. Traditional identification for *V. vulnificus* is based on biochemical tests, which are laborious and time consuming. Furthermore, several *Vibrio* species display similar biochemical characteristics, which limit the application of biochemical tests (O'Hara et al., 2003).

The conventional PCR methods have shown to be highly specificity and relatively time saving than traditional methods for detecting pathogenic bacteria. Noticeably, the novel method, termed loop-mediated isothermal amplification (LAMP), was developed for simple, rapid and sensitive nucleic acid amplification (Notomi et al., 2000). This method can be carried out in a simple heat block or hot water bath, since it only requires a higher isothermal temperature and themostable Bst DNA polymerase. Meanwhile, LAMP was based on a set of two inner and two outer primers that can recognize a total of six distinct sequences on the target gene sequence. The final products are stem-loop structures with several inverted repeats of the target DNA and exhibit cauliflower-like structures with multiple loops. The analysis of the LAMP products is generally accomplished through agarose gel electrophoresis, followed by ethidium bromide staining. Alternatively, the results can be judged with eyes to observe the color change of a mixture with SYBR Green I or calcein (Iwamoto et al., 2003; Ma et al., 2016). LAMP has been successfully used to detect multiple bacterial agents, including those related to major food safety issues, such as *Escherichia coli*, *Vibrio parahaemolyticus*, and *Vibrio cholerae* (Yano et al., 2007; Yamazaki et al., 2008a; Srisuk et al., 2010), especially *V. vulnificus* targeting *vcgC* gene. While only ninety percent *V. vulnificus* clinical strains have the *vcgC* gene in the genome (Li et al., 2010), the LAMP targeting other gene should be established for the detection of *V. vulnificus*.

Many virulence factors of *V. vulnificus* have been identified to induce pathogenic action both *in vitro* and *in vivo* experiments. The water-soluble pore-forming VvhA toxin with cytolytic and hemolytic activity has been shown to be responsible for the death of the host during the bacterial infection (Sugiyama et al., 2011), which can attach to the host cell membrane to induce hemolysis

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*Corresponding author, E-mail: hcj6289@163.com

by forming small ion permeable pores via colloidal osmotic shock (Kim et al., 1993; Lee et al., 2004). It is vital and specific to identify *V. vulnificus* detection with *vvhA* gene using the molecular techniques. In other preliminary studies, some *V. vulnificus* isolates did not amplified with primers according to *vvhA* gene (Surasilp et al., 2011). Therefore, an alternative primer based on *vvhA* gene was used as a target for identifying *V. vulnificus* by LAMP assay in this study.

2 Materials and methods

2.1 *Bacteria strains, growth conditions and DNA extraction*

A total of 45 bacterial strains (Table 1) were used for the optimization and validation of LAMP assay. *Vibrio vulnificus* M06-24/O strain was kindly provided by Professor Sang Ho Choi, Seoul National University. Other strains were sourced from the Marine Culture Collection of China (Xiamen, China), Center of Marine Microbial Resources Shandong University (Weihai, China) and Weihai Municipal Hospital affiliated to Dalian Medical University (Weihai, China). These strains were identified in our laboratory using VITEK 2 system (bioMérieux Vitek, Durham, NC, USA) and 16S rDNA sequencing.

After selected on thiosulfate citrate bile salt sucrose (TCBS) agar, the strains of *Vibrio* species were grown in alkaline peptone water (APW) supplemented with 2% (w/v) NaCl with incubator shaker at 37°C for 16 h. Other strains were cultured on Campylobacter agar with 8% sheep blood, and subsequently grown in Luria–Bertani broth (LB) at 37°C.

Then, all strains grown in liquid medium were used DNA extraction kit (DV810A, Takara, Japan) to extract DNA according to the manufacturers' protocol. DNA extraction was stored at –20°C before use.

2.2 *Primers design for LAMP assay*

The *vvhA* gene sequence of *V. vulnificus* was based on the available sequence deposited in GenBank (Accession Nos KU680790.1, KC821520.1, AB124803.1, AB124802.1, BA000038.2, M34670.1, CP009262.1, CP014049.1, CP009985.1, CP012740.1, CP011776.1, NZ_CP014049.1, NZ_CP012739.1, NZ_CP009262.1, NZ_CP009985.1, NZ_CP012740.1, and NZ_CP011776.1). The set of four primers composed of two outer primers (F3 and B3) and two inner primers (FIP and BIP) were designed by LAMP primer designing software (http://primerexplorer.jp/e/), which can initiate strand displacement and form the "loop" structure through the reaction respectively. The primers were synthesized by Life Technologies Corporation (Shanghai, China) (Table 2).

2.3 *LAMP assay reaction*

LAMP assay reaction was carried out in a total volume of 25 μL, containing 10×PCR buffer 2.5 μL, 150 mmol/L $MgSO_4$ 1 μL, 10 mmol/L dNTP 3.5 μL, 16 kU/mL Bst DNA polymerase 0.5 μL, 20 μmol/L FIP and BIP 2 μL each, 5 μmol/L F3 and B3 1 μL each, 4 mmol/L calcein 1 μL, 5 mmol/L betaine 4 μL, and DNA template 2 μL. In a heat block, the reaction mixture was heated to 63°C for 30 min and then heated up to 80°C for 5 min to terminate the reaction. After amplification, the solution would turn green in the presence of LAMP amplification products, while it would remain orange in the absence of amplicon. Meanwhile, the PCR products were electrophoresed in 2.0% agarose gels stained with EB and examined under UV illumination for the analysis of ladder-like pattern bands.

After the sequence of *vvhA* gene was amplified using the primers of F3 and B3, the amplified specific DNA fragment was cloned into the pMD18-T vector (Takara, Japan) as the positive control in LAMP assay. Sterile water was used as a negative control.

2.4 *Specificity and sensitivity of conventional PCR and LAMP assay*

The specificity of LAMP assay was evaluated to detect *V. vulnificus* under the conditions described above using the strains in Table 1.

Meanwhile, the specificity of conventional PCR was carried out using F3 and B3 primers. After PCR, the amplified products were electrophoresed in 2.0% agarose gels and examined under UV illumination. The PCR products were further validated by DNA sequencing and compared with deposited sequences in NCBI.

The sensitivity of LAMP assay was evaluated using 10-fold serial dilutions of *V. vulnificus* suspension in Table 1, compared to the conventional PCR.

2.5 *Application of LAMP assay in the detection for clinical and environmental samples*

Samples of LAMP assay were separated into Groups A and B. Group A contained 33 spiked clinical samples and environmental samples, which were mixed with reference strains artificially. These samples consisted of 12 wound swab samples, eight sea water samples, nine sediment samples and four oyster samples. Group B were unidentified clinical and environmental samples. Among Group B, nine unidentified clinical samples (seven wound swab samples and two blood samples) were obtained from the Weihai Municipal Hospital and 59 unidentified environmental samples (20 sea water samples, 19 sediment samples and 30 oyster samples) were obtained from different environmental sources.

After pretreated according to *Bacteriological Analytical Manual* (Thompson et al., 2004), all clinical and environmental samples were enriched in APW with 2% NaCl for 16 h, and 0.5 mL APW were transferred into the tube to centrifuge at 12 000 *g* for 5 min. After the supernatant was discarded, the deposit was performed the DNA extraction as described above for LAMP assay. Meanwhile, the enriched samples were plated onto TCBS for regular culture and some single colonies were selected to identify by the standard microbiological procedures using VITEK® 2 Compact.

3 Results

3.1 *The products of LAMP assay*

In the study, the amplification products can turn to green in the reaction tube for naked-eye examination (Fig 1a). Meanwhile, the final LAMP products are mixtures of DNA with various lengths, showing many ladder-like pattern bands on agarose gel due to its characteristic structure (Fig 1b).

3.2 *Specificity and sensitivity of conventional PCR and LAMP assay*

A total of 45 strains were tested for the specificity by LAMP assay. All the *V. vulnificus* strains were detected correctly while all other strains were negative reaction.

A single fragment of approximately 216 bp was amplified by F3 and B3 primers when using *V. vulnificus* genomic DNA (Fig. 2). No PCR product was observed in other *Vibrio* species. After DNA sequencing, all the *V. vulnificus* strains were amplified correctly and all other strains were no amplicons.

The sensitivity of LAMP assay and conventional PCR with F3

Table 1. Bacterial strains used in this study

Species	Strains or plasmids	Characteristics
Vibrio vulnificus	ATCC 27562	reference strain
	M06-24/O	clinical strain
	VVY066	environmental strain
	201403065529	clinical strain
Vibrio parahaemolyticus	ATCC 17802	reference strain
	I_1-C_1	environmental strain
	I_1-F_4	environmental strain
	I_1-F_{10}	environmental strain
	I_1-G_3	environmental strain
	VPgu	clinical strain
	VPwang	clinical strain
	VPzhao	clinical strain
	VPli	clinical strain
Vibrio alginolyticus	ATCC 17749	reference strain
	VA604	environmental strain
	VA028	environmental strain
	20548934	clinical strain
Vibrio minicus	MCCO000514	clinical strain
	MCCO000515	clinical strain
	MCCO000516	clinical strain
Vibrio fluvialis	MCCO000012	clinical strain
Vibrio furnissii	MCCO000013	environmental strain
Vibrio hollisae	MCCO000027	clinical strain
Vibrio metschnikovii	MCCO000080	clinical strain
Vibrio cincinnatinesis	MCCO000007	clinical strain
Vibrio carchariae	MCCO000006	environmental strain
	MCCO000029	environmental strain
Vibrio fischeri	vf081301	environmental strain
	MCCO000011	environmental strain
Vibrio harveyi	vh020201	environmental strain
	MCCO000015	environmental strain
	MCCO000031	environmental strain
	MCCO000052	environmental strain
Escherichia coli	ATCC25922	reference strain
	coli17802	clinical strain
Klebsiella pneumonia	ATCC700603	reference strain
	kpn101201	clinical strain
Acinetobacter baumannii	aba4488	clinical strain
	aba4489	clinical strain
Pseudomonas aeruginosa	ATCC27853	reference strain
	pa04589	clinical strain
Staphylococcus aureus	ATCC25923	reference strain
	sa040603	clinical strain
Enterococcus faecalis	ATCC 43300	reference strain
	ef052403	clinical strain

Table 2. Target genes and primer sequences in LAMP

Name of primers	Sequence (5′-3′)	Reference
F3	CGGCAACCTCAGATGGTT	this study
B3	CCCTACGTATGCCCTTGTCCC	this study
FIP	ATGAATACCCGTGTCAGGCTTTCCTGACGCCAAAATTGTCC	this study
BIP	CACAGCTGGTTCCATAGTTGGGCGGGTTTCACCCAAAGGT	this study

and B3 primers were analyzed and detected simultaneously. The minimum detection limit of LAMP assay was 4 CFU/tube for *V. vulnificus*, while the conventional PCR was 4×10^2 CFU/tube (Figs 3 and 4).

3.3 *Application of LAMP assay in the detection of spiked samples, clinical and environmental samples*

All the *V. vulnificus* strains were detected correctly in Group A by LAMP assay. Two *V. vulnificus* strains were detected from the

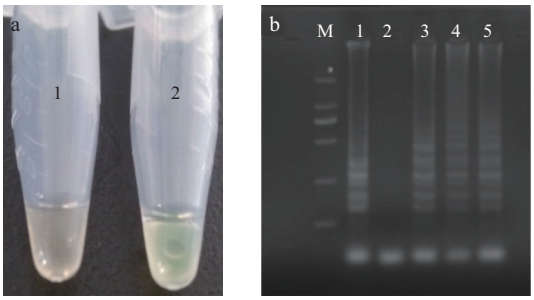

Fig. 1. Confirmation of *vvhA* gene detection by LAMP assay. a. Color change of LAMP assay. Tube 1 is negative reaction and Tube 2 positive reaction. b. 2.0% agarose gel electrophoresis applied to *vvhA* gene detection. M represents DNA marker; Lanes 1, 3, 4 and 5 positive reaction; and Lane 2 negative reaction.

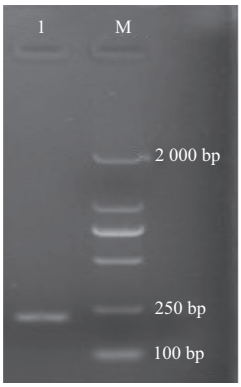

Fig. 2. Electropherogram of PCR products revealed a specific band of 216 bp using F3 and B3 for *V. vulnificus vvhA* gene. M represents DNA marker and Lane 1 positive reaction.

clinical samples of Group B by LAMP assay, including one wound swab sample and one blood sample (Fig. 5). Nine *V. vulnificus* strains were detected from the environmental samples of Group B by LAMP assay, including two sea water samples, four sediment samples, and three oyster samples. All the strains above were also isolated and identified in 48 h by standard microbiological procedures. The results of LAMP assay were consistent with standard microbiological procedures.

4 Discussion

Vibrio vulnificus is a halophilic bacterium that inhabits in aquatic environment and various marine species such as sea sediments and shellfish, which is of major concern as the pathogenic to animals as well as humans (Thompson et al., 2004). Identification for the pathogen in clinical and environmental samples is an increasingly important issue for public health, while classical identification methods for *V. vulnificus* based on biochemical and microbiological tests are time-consuming and unreliable. Therefore, it is necessary to develop a specific, sensitive and rapid molecular method to replace these classical methods.

Now, it has been known that *V. vulnificus* requires many different gene products to maintain its pathogenicity to human, such as *vvhA* gene. The cytolytic activity of VvhA toxin has been characterized using several host cells (Kim et al., 2008; Shinoda et al., 1985) and molecular mechanisms including pore formation and apoptosis were demonstrated to be responsible for its toxicity (Sun et al., 2012).

LAMP assay is a novel isothermal DNA amplification method with high specificity and efficiency, which can target the conserved sequence of pathogen. The mechanism and expected three reaction steps of LAMP were shown in the illustration finished by Notomi et al. (2000). The LAMP assay targeting *vcgC* gene only detect some *V. vulnificus* strains owing to *vcgC* gene in the genome of ninety percent clinical strains. While *vvhA* gene was found in all *V. fulnificus* strains, *vvhA* may serve as a reliable biomarker to detect *V. vulnificus*. In this report, the LAMP assay targeting the *vvhA* gene of *V. vulnificus* has been successfully established. Since LAMP reaction requires isothermal temperature, the reaction can be carried out using a simple heat block or hot water bath. In the study, the optimal LAMP amplification temperature was 63°C and the reaction were completed within 30 min.

The new LAMP technique, which used of the four specific primers targeting to *vvhA* gene of *V. vulnificus*, ensured a high specificity of nucleic acid amplification. In this study, we determined the specificity of the LAMP assay through screening *V. vulnificus* from other strains of *Vibrio* genus and other species. The results were positive for all the *V. vulnificus* strains and negative for the other species tested in the study. The specificity of LAMP

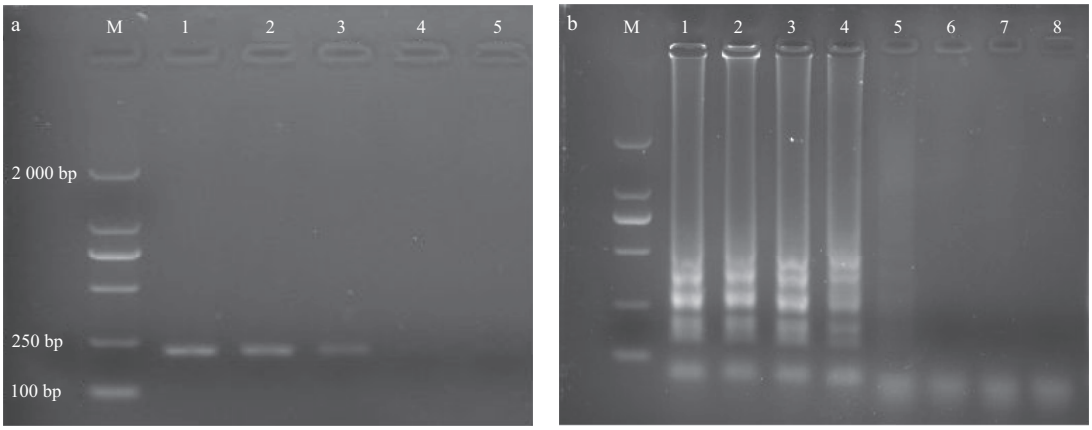

Fig. 3. The detection sensitivity of conventional PCR and LAMP assay. a. Conventional PCR. Lane 1: 4×10^4 CFU/tube, Lane 2: 4×10^3 CFU/tube, Lane 3: 4×10^2 CFU/tube, Lane 4: 4×10^1 CFU/tube, and Lane 5: 4×10^0 CFU/tube. b. LAMP assay. Lane 1: 4×10^4 CFU/tube, Lane 2: 4×10^3 CFU/tube, Lane 3: 4×10^2 CFU/tube, Lane 4: 4×10^1 CFU/tube, Lane 5: 4×10^0 CFU/tube, Lane 6: 4×10^{-1} CFU/tube, Lane 7: 4×10^{-2} CFU/tube, and Lane 8: 4×10^{-3} CFU/tube.

Fig. 4. The sensitivity of LAMP assay by naked-eye examination. Tube 1: 4×10^{3} CFU/tube, Tube 2: 4×10^{2} CFU/tube, Tube 3: 4×10^{1} CFU/tube, Tube 4: 4×10^{0} CFU/tube, Tube 5: 4×10^{-1} CFU/tube, Tube 6: 4×10^{-2} CFU/tube, Tube 7: 4×10^{-3} CFU/tube, Tube 8: 4×10^{-2} CFU/tube, Tube 9: negative control, and Tube 10: positive control.

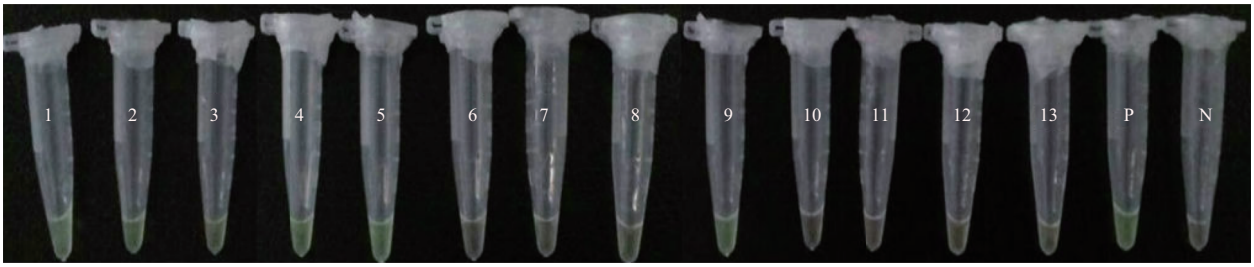

Fig. 5. Partial results of the LAMP assay for clinical and environmental samples (Groups A and B). Tubes 1–5: positive samples (Group A), Tubes 6–8: negative samples (Group B), and Tube 9: positive samples (Group B), and Tubes 10–13: negative samples (Group B). P represents positive control and N negative control.

was in conformity with other studies in the other bacterial detection (Endo et al., 2004; Yeh et al., 2005). Moreover, LAMP assay does not require further processing and the reaction tubes without having to open in the course of the experiment, which can alleviate aerosol contamination effectively.

In this study, the sensitivity of LAMP assay with loop primers in pure culture was 4 CFU per reaction, which was 100 times higher than that of *vvhA* gene using conventional PCR, similar to that of previous LAMP assays (Yamazaki et al., 2008b; Srisuk et al., 2010). These results demonstrate that the LAMP assay has higher sensitivity because loop primers accelerate the reaction by hybridizing to stem-loops formed during LAMP reaction and facilitating strand displacement and amplification (Hara-Kudo et al., 2005; Yano et al., 2007; Jaroenram et al., 2009). Moreover, in the study all clinical and environmental samples were enriched for 16 h before LAMP assay to reduce LAMP inhibitors and raise the sensitivity of LAMP assay.

In conclusion, because of its shorter reaction time, more sensitivity and specificity, and visual discriminatory of positivity, the LAMP method could be appears as valuable alternative tool for detection of *V. vulnificus* in clinical diagnostics, food industry studies, and risk assessment of environment.

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Correction instructions

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