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Rapid, Simple, and Sensitive Detection of *Vibrio alginolyticus* by Loop-Mediated Isothermal Amplification Assay

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***Vibrio alginolyticus* is widely distributed in marine ecosystems. *V. alginolyticus* is also associated with infection in aquatic animals, and has a significant negative impact on aquaculture. Therefore, devising a rapid, sensitive, and effective detection method for *V. alginolyticus* is necessary. In this study, we developed a detection method for *V. alginolyticus* using loop-mediated isothermal amplification (LAMP) assay, in which the *V. alginolyticus gyr*B (DNA gyrase subunit) gene was used as the target gene. A set of primers was designed to amplify specific DNA sequences by LAMP. Moreover, the reaction conditions were optimized. Results show that the optimum conditions for LAMP assay for the rapid detection of *V. alginolyticus* were 63.5 °C for 45 min. The optimum concentrations of Mg^{2+} and dNTP were 5 and 1 mM, respectively. The LAMP assay had a detection limit of 2.08×10^{-5} ng/μl. LAMP detection of *V. alginolyticus* is more simple and rapid compared with traditional detection methods. The results of LAMP detection can be identified without the aid of sophisticated equipment or a complicated protocol. Thus, the LAMP assay is a potential diagnostic tool for *V. alginolyticus*.**

Key words: Loop-mediated isothermal amplification (LAMP), *Vibrio alginolyticus*, *gyr*B gene, diagnostic tool.

Vibrio alginolyticus has gained attention in the recent years as a prominent fish pathogen. It is a halophilic and mesophilic rod-shaped flagellated Gram-negative bacterium and causes high mortality vibriosis in various fish species such as sea bream, grouper, large yellow croaker, kuruma prawn[1]. And recent studies show that although *V. alginolyticus* is most commonly associated with wound infections, otitis media, and otitis externa[2,3], it is increasingly recognized as an important intestinal pathogen in humans[4]. There are many techniques widely used to control the diseases, such as using antibiotics and chemotherapy. Treatment of vibriosis with antibiotics in the early stages is effective, but once the disease has progressed to its chronic phase, treatment times are longer and relapses are common, resulting in high mortality. Therefore, In order to lessen harm to organism, the early rapid diagnosis of diseases is an important task for the further study of disease control.

The use of conventional detection methods is limited because of the lack of well-equipped laboratory facilities in the culture area. Therefore, developing a simple, rapid, and sensitive

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detection method for *V. alginolyticus* is necessary for fast and local diagnosis. The current detection methods for *V. alginolyticus* mainly include microscopic examination and bacterial culture, followed by biochemical identification[5]. The direct smear method is rapid and economical, but has low specificity and detection rates. Thus, discriminating *V. alginolyticus* from other *Vibrio* species is difficult using this method. API 20E identification is currently the standard technique for the detection of *V. alginolyticus*. However, this method is time-consuming and requires laboratory facilities of relatively high standard. This requirement can lead to a delay, which is beyond the optimum time window for treatment. PCR detection is highly sensitive, and a specific PCR assay is recommended for reasonable quantitative analysis. However, PCR is not suitable for point-of-care testing because it requires high-standard testing equipment and operators[6]. Loop-mediated isothermal amplification (LAMP) is a sensitive, specific, and simple nucleic acid amplification method that can generate up to 10^9-fold amplification in less than 1 h under isothermal conditions (60 °C to 65 °C)[7]. In this study, we developed a rapid LAMP assay, a potentially rapid and simple diagnostic tool for *V. alginolyticus* infection, to detect *V. alginolyticus* using the *V. alginolyticus gyr*B (a Type II topoisomerase found in bacteria) gene as the target gene.

MATERIALS AND METHODS

Primer design

The nucleotide sequence of the *gyr*B gene (GenBank Accession No. EJY3 CP003241.1) specifically expressed by *V. alginolyticus* was retrieved from GenBank, and used as a target gene in the assay. The primers were designed using PrimerExplorer 4.0 online software (http://primer-explore.jp/e/v4-manual/index.html). The LAMP primers were designed according to their conserved regions. Primers were synthesized by Sangon Biotech (Shanghai, China). The primer sequences are shown in Table 1.

Reaction protocol and system optimization for LAMP

DNA of *V. alginolyticus* was extracted according to the instructions provided in a bacterial DNA extraction kit (TaKaRa). The concentration of bacterial genomic DNA was determined by a nucleic acid and protein analyzer (Biodropsis BD-1000). All LAMP reactions were performed in a 25 µl reaction volume containing 1.6 µM of each of the FIP and BIP primers, 0.2 µM of the F3 and B3 primers, 6.0 mM $MgCl_2$, 1.0 M betaine, 1.0 mM deoxynucleoside triphosphate (dNTP), 2.5 µl of reaction reagent, and 8 U Bst DNA polymerase. The mixture in the reaction tube was incubated isothermally in a thermostat water bath at 57.5 °C, 59.8 °C, 60.8 °C, 63.5 °C, and 65 °C, respectively, for 60 min, and then at 80 °C for 2 min to terminate the reaction. After amplification, the LAMP products were examined by electrophoresis on 2% agarose gels stained with ethidium bromide. After determining the optimum reaction temperature, the LAMP reaction time was set at 15, 30, 45, and 60 min to determine the optimum reaction time. The concentrations of Mg^{2+} (3 mM to 7 mM) and dNTP (0.6 mM to 1.2 mM) were optimized.

Sensitivity of the LAMP assay

To evaluate the sensitivities of the *gyr*B LAMP assay, 20.8 ng/µl genomic DNA of *V. alginolyticus* was diluted into seven serial solutions. From every diluted solution, an aliquot of 1 µl was subjected for LAMP amplification. To determine the detection limit, the reaction products were analyzed by electrophoresis on agarose gels and visually inspected after the addition of 1 µl of 1000× GeneFinder™. All detection assays were performed in triplicate. The bacterial DNA template was replaced by sterile water as a negative control.

Specificity of the LAMP assay

We used the following 13 strains of bacteria in this study: *V. alginolyticus*, *Vibrio splendidus*, *V. parahaemolyticus*, *V. anguillarum*, *V. harveyi*, *Grimontia hollisae*, *Photobacterium damselae subsp. Damselae*, *Salinivibrio costicola subsp. Costicola*, *Edwardsiella ictaluri*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudoalteromonas*, and *Bacillus subtilis*. These 13 strains of bacteria were recovered, cultured, and passaged. The DNA of these strains was extracted by the aforementioned method to investigate the specificity of the LAMP method. The bacterial DNA template was replaced by sterile water as a negative control. The reaction products were analyzed by electrophoresis and visual inspection.

RESULTS

Reaction protocol and system optimization for LAMP

Genomic DNA of *V. alginolyticus* was used as a template to optimize the reaction system. The gradient bands of amplified products were observed after visualization of the electrophoresis results at 57 °C to 65 °C and 30 min to 60 min (Fig. 1 and Fig. 2). The optimum temperature and time were determined as 63.5 °C and 45 min, respectively. The products were amplified when the concentrations of Mg^{2+} and dNTP were 3 mM to 7 mM and 0.6 mM to 1.2 mM, respectively (Fig. 3 and Fig. 4). After repeated testing, the optimum concentrations of Mg^{2+} and dNTP were determined as 5 and 1 mM, respectively.

Sensitivity of the LAMP assay

The following process was conducted to determine the detection limit of the LAMP assay. LAMP amplification of DNA was performed. DNA was extracted from seven serial 10-fold dilutions of *V. alginolyticus*, ranging from 2.08×10^{-6} ng/µl to 2.08 ng/µl. The gradient bands of amplified products were observed in solutions of 2.08×10^{-5} ng/µl to 2.08 ng/µl (Fig. 5A). GeneFinder™ was added into the reaction system to examine the results of naked-eye observations of LAMP. After the addition of GeneFinder™, a pale brown color appeared in the negative reaction tubes, whereas a yellowish-green color was found in the positive reaction tubes (Fig. 5B). With a detection limit of 2.08×10^{-5} ng/µl, the sensitivity of the naked-eye observation test was comparable with that of electrophoresis.

Specificity of the LAMP assay

Thirteen bacterial strains were amplified to determine the specificity of the LAMP assay for *V. alginolyticus*. The results after LAMP reaction are shown in Fig. 6. Among all the tested bacteria, only the amplified *V. alginolyticus* DNA yielded positive results, whereas the amplified DNA from other bacteria rendered negative results. These results indicate that the *gyr*B LAMP assay was specific for *V. alginolyticus*.

DISCUSSION

LAMP is a simple technique that rapidly amplifies specific DNA sequences with high

Table 1. Primers of *gyr*B gene of *Vibrio alginolyticus* for LAMP assay

Primers	Sequences(5'→3')	Length/bp
F3	GCGGGTATTAAACTGGCGG	19
B3	TTCGTAGCTGTACTGGCTTG	20
FIP	TTGCTCTGGCGTTAGGCGAGTTTTCGTTACCCAAGTGCTTTGGT	44
BIP	TGCCATGACGCATCAGTTGTGGTTTTACCCACTTCTTTCGCATTCA	46

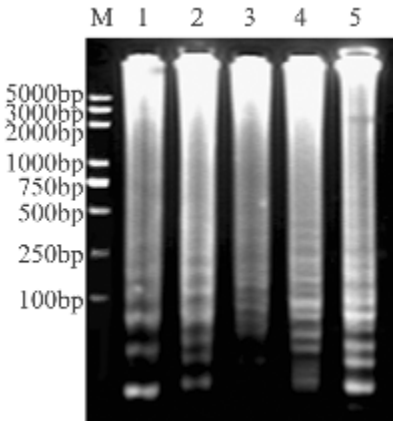


Fig. 1. Optimization of temperature. Lane 1~5. 57.5, 59.8, 60.8, 63.5, 65 ºC; M. 5000 bp marker

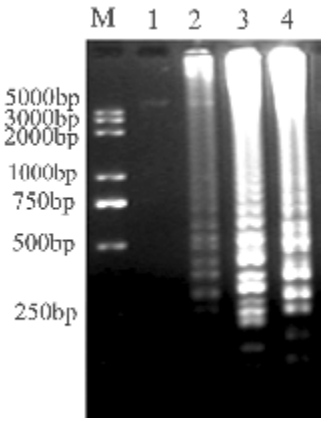


Fig. 2. Optimization of time. Lane 1~4. 15, 30, 45, 60 min; M. 5000 bp marker

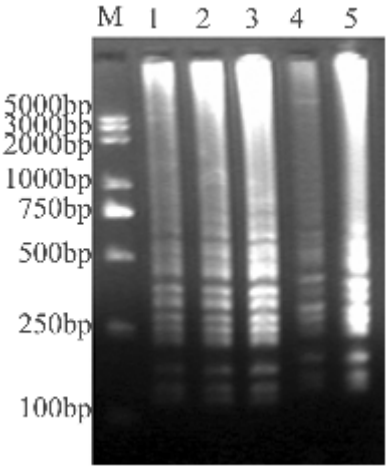


Fig. 3. Optimization of Mg^{2+}. Lane 1~5. 3, 4, 5, 6, 7 mM; M. 5000 bp marker

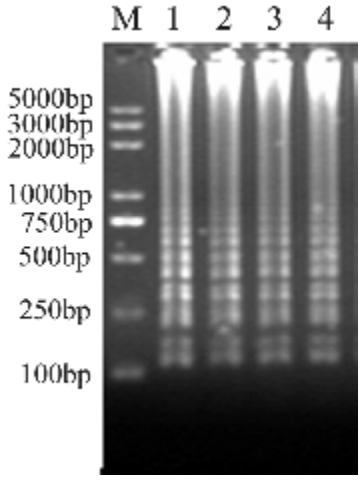
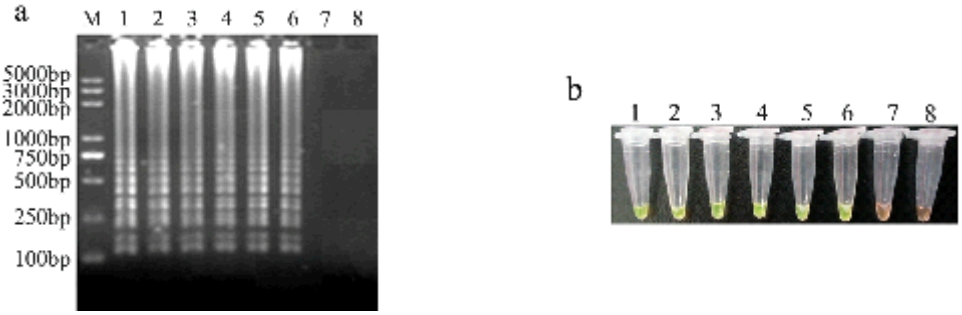


Fig. 4. Optimization of dNTPs. Lane 1~4. 0.6, 0.8, 1.0, 1.2 mM; M. 5000 bp marker



a Electrophoresis analysis of *Vibrio alginolyticus* DNA LAMP reaction. Lane 1~7. *Vibrio alginolyticus* concentration: 2.08, 2.08×10^{-1}, 2.08×10^{-2}, 2.08×10^{-3}, 2.08×10^{-4}, 2.08×10^{-5}, 2.08×10^{-6} ng/µl; lane 8. negative control; M. 5000 bp marker, b Visual inspection of LAMP amplification products. Lane 1~7. *Vibrio alginolyticus* concentration: 2.08, 2.08×10^{-1}, 2.08×10^{-2}, 2.08×10^{-3}, 2.08×10^{-4}, 2.08×10^{-5}, 2.08×10^{-6} ng/µl; lane 8. negative control

Fig. 5. Sensitivity of the LAMP Assay

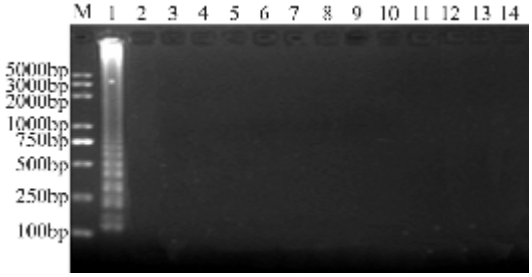


Fig. 6. Specificity of the LAMP Assay. Lane 1~13. *Vibrio alginolyticus*, *Vibrio splendidus*, *Vibrio parahaemolyticus*, *Vibrio anguillarum*, *Vibrio harveyi*, *Grimontia hollisae*, *Photobacterium damselae subsp. Damselae*, *Salinivibrio costicola subsp. Costicola*, *Edwardsiella ictaluri*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudoalteromonas*, *Bacillus subtilis*; lane 14. negative control; M. 5000 bp marker

sensitivity under isothermal conditions[7]. In the LAMP assay, two pairs of primers are specially designed to recognize six regions within the target gene. Strand displacement DNA synthesis is initiated and self-cycled continuously using high-activity *Bacillus stearothermophilus* DNA polymerase[6].

The concentration of Mg^{2+} in the LAMP assay is the main factor affecting the LAMP reaction. Very high or very low Mg^{2+} concentration can affect the amplification efficiency. The reaction temperature is also an important factor, which is mainly determined by the activity of *Bst* DNA polymerase. When the temperature is at the optimum temperature for enzymatic reaction, the amplified product will reach the maximum yield within a short time. dNTPs are raw materials of the

reaction, but adding too many is wasteful and reduces reaction specificity of the reaction; adding too little will reduce reaction sensitivity. Betaine is added to maintain activity and stability of the enzyme.

In this process, the stem-loop amplification product was obtained under isothermal conditions of 57 °C to 65 °C. An extensive and rapid amplification of the target gene was achieved within 30 min to 60 min. When the concentrations of Mg^{2+} and dNTP ranged from 3 mM to 7 mM and 0.6 mM to 1.2 mM, respectively, the target gene could be amplified. The reaction was identified using agarose gel electrophoresis[7,8], and observed by the naked eye by applying appropriate chromogenic reagents such as calcein[9] or hydroxynaphthol blue[10].

In this study, we developed a sensitive and specific LAMP assay based on the *gyr*B gene[5] of *V. alginolyticus*. Several publications have suggested that *gyrB* provides suitable sequence data for bacterial phylogenies, possessing essential attributes such as limited horizontal transmission and presence in all bacterial groups[11,12]. We found that the established LAMP method was specific for *V. alginolyticus*, and had no amplified products for other bacteria, thereby suggesting high specificity of the assay. This result also indicates that the primers designed for the reaction were highly specific to the *V. alginolyticus gyr*B gene. The high specificity of the assay was attributed to the two pairs of primers, which could recognize six independent regions within the target gene. By contrast, only one pair of primer is used in the PCR assay. The *V. alginolyticus gyr*B gene shows high specificity in the detection and identification of *V. alginolyticus*. Conventional PCR assay is also highly specific. However, the current PCR method has limited applications in the field and point-of-care testing because it requires high-level laboratory equipment, such as PCR gene amplifier, water bath, centrifuge, and super-clean bench, and has a complicated reaction protocol. Unlike the thermal cycles essential to PCR assay, the LAMP reaction can be performed under isothermal conditions[6]. The LAMP method developed for the detection of *V. alginolyticus* demonstrated a low detection limit of 2.08×10^{-5} ng/µl. This result was similar to the reported detection limits for the identification of other pathogens, such as *V. parahaemolyticus*[13].

The LAMP method has several limitations. For instance, it places higher standards on primers (i.e., more than one pair of primers are needed for amplification), and the length of amplified target sequences should be lower than 300 bp. The electrophoresis results of LAMP reaction products are illustrated in the form of gradient bands, which cannot be easily discriminated from nonspecific amplification of other DNA. The reaction is highly vulnerable to contamination because of its high sensitivity, and requires close attention[8].

CONCLUSIONS

LAMP is extensively used in the detection of viruses[14,15], bacteria[16,17], and parasites[18] for food safety[19,20] and aquaculture[21,22]. LAMP is characterized as a rapid testing technique with high sensitivity and specificity. However, no literature explains the rapid detection of *V. alginolyticus* using the LAMP assay. To fulfill the requirements of point-of-care testing, we developed a rapid LAMP assay for detecting *V. alginolyticus* using *V. alginolyticus gyr*B as the target gene.

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