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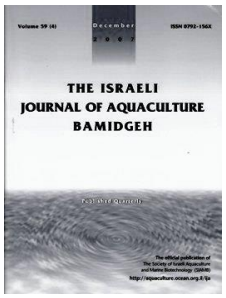

Rapid and Sensitive Detection of *Vibrio natriegens* in *Portunus trituberculatus* with the Loop-Mediated Isothermal Amplification Test

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Abstract

In order to detect *Vibrio natriegens* in *Portunus trituberculatus*, a loop-mediated isothermal amplification (LAMP) method was developed and evaluated. In this study LAMP primers were designed to correspond to the *gyrB* gene sequences. With *Bst* DNA polymerase, the LAMP assay was completed within 100 min at 65˚C in a water bath. Amplification products were observed directly with agar gel or with the naked eye after the addition of SYBR Green I. The sensitivity of the LAMP assay for the detection of *V. natriegens* is about 1.32×10^{-2} fg/mL DNA template, whereas using duplex PCR the detection of *V. natriegens* was possible up to 13.2 pg/mL DNA template. There were no cross-reactions with other *Vibrio* strains indicating a high specificity of the LAMP. The novel LAMP assay in this study can be used as a valuable, rapid, and sensitive detection tool for the detection of *V. natriegens* both in the laboratory and for use in commercial aquaculture.

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Introduction

The swimming crab, *Portunus trituberculatus* (Crustacea: Decapoda: Brachyura), has become an important marine fishery and aquaculture species. It comprises a large proportion of crab aquaculture in China, with the total yield exceeding 100,000 tons in 2011 (Lv et al., 2014). However, with the rapid development of intensive crab culture, the sustainability of the crab aquaculture industry has been threatened by outbreaks of diseases, particularly those caused by bacteria in the genus *Vibrio* (Liu et al., 2007; Yan et al., 2010; Wan et al., 2011; Zhang et al., 2014; Bi et al., 2016).

V. natriegens is a Gram-negative, assumed to be non-pathogenic halophile marine bacterium (Payne et al., 1961). However, recent studies have showed that *V. natriegens* is pathogenic to aquatic animals and mortality rate was greater than 90% (Bi et al., 2016; Deng et al., 2004; Li et al., 2009). Disease has been difficult to control because *V. natriegens* has the fastest growth rate of any known organism, with a reported doubling time of <10 min (Weinstock et al., 2016). Therefore, there is an urgent need for in situ detection of *V. natriegens*, and the method should be fast, sensitive, reliable, easy to carry out, and results easily seen.

Loop-mediated isothermal amplification (LAMP) was first reported in 2000 (Notomi et al., 2000) and has since been used in various aspects of nucleic acid research, and in clinical application as an ideal tool for diagnosis (Dhama et al., 2014). The most innovative features of LAMP, is the simplicity of its protocol, and overall low cost of application when compared to other pre-existing molecular techniques, such as Nucleic Acid Sequence-Based Amplification (NASBA), Self-Sustained Sequence Replication (3SR), Standard Displacement Amplification (SDA), Rolling Circle Amplification (RCA), and most prominently Polymerase Chain Reaction (PCR) (Umar et al., 2015). In aquaculture, LAMP has been used widely for *Vibrio* detection in aquatic animal disease. It shows high specificity, sensitivity, and rapidity under isothermal conditions with the presence of *Bst* DNA polymerase (Wang et al., 2017; Zhou et al., 2016).

In this study, we developed an assay based on the LAMP technique for the detection of *V. natriegens* in *P. trituberculatus*, and investigated its sensitivity, specificity, and application potential in swimming crabs. Our results indicated that by using the LAMP method we identified *V. natriegens* rapidly and accurately. This method will assist in the detection of *V. natriegens* in swimming crab farms.

Materials and Methods

Several hundred swimming crabs were collected from farms located in Lianyungang City of Jiangsu Province, China. *V. natriegens, V. parahaemolyticus, V. harveyi, V. alginolyticus, and V. vulnificus* were previously isolated from swimming crabs and shrimp in our laboratory.

DNA extraction. All *Vibrio* strains were cultured on ZoBell's marine 2216E agar for 24h or 48h at 28°C. Genomic DNA from these strains was extracted using the EasyPure Genomic DNA Kit (Beijing TransGen Biotech Co., Ltd. Beijing, China) according to the manufacturer's protocol. Briefly, the *Vibrio* cells that were cultured in 2 ml ZoBell's marine 2216E medium were harvested by centrifugation at 15,700g for 1 min at 4°C. The supernatant was discarded and lysis buffer was added to the pellets and incubated at 55°C for 15min. The lysate was treated with RNase A (20mg/mL) and the genomic DNA was extracted. DNA quality was assessed by electrophoresis on 1.0% agarose gels and DNA concentration was measured using a NanoDrop 2000 (Thermo Scientific, USA).

Primers designed. Based on the *gyrB* gene sequence (accession numbers: KC912686.1), the primer of LAMP was designed using Primer Explorer V5 software (http://primerexplorer.jp/e/v5_manual/index.html). A forward inner primer (FIP), a reverse inner primer (BIP), and two outer primers (F3 and B3) were chosen and synthesized by Sangon Biotech (Shanghai) Co., Ltd. Information relating to the primers is shown in Table 1 and Figure 1. In addition, 16S rDNA primer (27f/1942r) (Bi et al., 2016) and dulplex PCR primer (data not shown) were used to ascertain the specificity and sensitivity of LAMP assays.

Table 1. Primer names and sequences of LAMP test.

Primer Name	*Sequences (5'-3')*
F3	CAGCACGTGCTCGTGAAC
B3	GGATCTACCTTTCAGCGGC
FIP	AGCAAGTTTGCCCGGAAGGC-TTTT-AAGCTCGTGAAATGACTCGT
BIP	ACATTGTGGAGGGTGACTCTGC-TTTT-GCCTGGTTCTTACGGTTACG

Primer Information | Save

1 ID:1 dimer(minimum)dG=-1.69

label	5'pos	3'pos	len	Tm	5'dG	3'dG	GCrate	Sequence
F3	801	818	18	60.33	-5.90	-4.51	0.61	CAGCACGTGCTCGTGAAG
B3	1000	1019	20	60.23	-4.59	-7.76	0.55	GGATCTTACCTTTCAGCGGC
FIP			40					GTTTGCCTGGAAGGCCTGCT-AAGCTCGTGAAATGACTCGT
BIP			42					ACATTGTGGAGGGTGACTCTGC-GCCTGGTTCTTACGGTTACG
F2	828	847	20	59.10	-5.09	-5.57	0.45	AAGCTCGTGAAATGACTCGT
F1c	868	887	20	65.23	-5.17	-6.08	0.60	GTTTGCCTGGAAGGCCTGCT
B2	974	993	20	60.05	-6.69	-4.67	0.55	GCCTGGTTCTTACGGTTACG
B1c	930	951	22	64.37	-4.21	-5.59	0.55	ACATTGTGGAGGGTGACTCTGC

Fig. 1. The primers information of gyrB-LAMP.

Optimization for LAMP. In order to optimize the amplification system of LAMP assay, PCR experiments were carried out in a 25 μl reaction mixture containing 10 × *Bst*-DNA Polymerase Buffer, 25 mmol/L $MgSO_4$, 10 mmol/L dNTP mixture, 5 mol/L betaine, 10 μmol/L F3, B3, FIP and BIP, template DNA and 8000U Bst DNA polymerase (NEB, Beijing, China). Reaction time was optimized by incubating the mixture for 45, 60, 90, and 120 min at 65°C, while reaction temperature was optimized by incubating the mixture at 60, 63, 65, and 66°C for 60 min. Concentrations of betaine and Mg^{2+} ion in the LAMP reaction solutions were optimized using a series of concentrations from 1-6 mol/L and from 1-5 mol/L, respectively. Reaction was terminated by heating to 80°C for 10 min. The LAMP products were detected by agarose gel electrophoresis and naked-eye observation after the addition of SYBR Green I (Beijing TransGen Biotech Co., Ltd. Beijing, China).

Specificity of LAMP. Four other marine pathogenic *Vibrio*s, *V. parahaemolyticus*, *V. harveyi*, *V. alginolyticu,* and *V. vulnificus*, were used to investigate LAMP specificity. DNA templates of each strain were prepared by the method described in "DNA extraction". The template DNA was subjected to both LAMP and 16S rDNA PCR amplification. Each sample was tested three times with LAMP method.

Sensitivity of LAMP. In order to determine the sensitivity of LAMP assay, the DNA from *V. natriegens* (132μg/mL) was serially diluted ten times and 1μL of each dilution was used as a template for the LAMP and dulplex PCR assays. Dulplex PCR was performed in a 25μL volume of reaction mixture containing 13μL 2×Master Mix, 1μL 10 μmol/L vhh-F/vhh-R, 2μL tox-F/tox-R, 1μL genomic DNA, and 5μL ultrapure water. The program involved the initial denaturation of 5 min at 95^0C, 30 cycles of 30 s at 94^0C, 45 s at 53.3^0C, 1min at 72^0C, and a final 10 min extension at 72^0C. PCR products were electrophoresed to verify the presence of the expected 308 and 526 bp bands.

LAMP application for rapid diagnosis. From hundreds of swimming crabs that were collected from farms located in Lianyungang City of Jiangsu Province, China, 90 swimming crabs were randomly chosen and were used to detect the existence or nonexistence of *V. natriegens* using LAMP assays. In addition, muscle from each crab was homogenized in 2 ml saline. The homogenates were plated onto ZoBell's marine 2216E agar. These plates were incubated at 28^0C for 24 h. Single colony cultures were prepared in ZoBell's marine 2216E broth, and a drop of culture was placed on formvar-coated copper grids and fixed with 2.5% glutaraldehyde for 1min. After removal of excess fluid by blotting on filter paper, the wet residues were immediately covered with the 2% sodium phosphotungstate for 30-40 s, and then withdrawn in the same fashion. The grid was air-dried and was observed and photographed by a Hitachi 600-2A TEM.

Results

Optimized LAMP reaction. After optimization, the reaction mixture (25μL) contained 2.5μL 10 × *Bst*-DNA Polymerase Buffer, 2μL 25 mmol/L $MgSO_4$, 2.5μL 10 mmol/L dNTP mixture, 5μL 5 mol/L betaine, 0.4μL 10 μmol/L each of F3 and B3, 1.6μL each of FIP and BIP, 1μL template DNA, 8U Bst DNA polymerase, and 8μL ultrapure water. 65°C was found to be the optimal temperature as at this temperature LAMP products displayed clearer amplification of DNA than at other temperatures including 60, 63, and 66°C (data not shown). To determine the optimum duration of the LAMP assay, four different reaction times, 45, 60, 90, and 120 min were used in the LAMP reaction. Although we could detect well-formed bands at 60 min, the reaction time was extended to 90 min to ensure positive detection of lower concentration templates in the system.

Specificity of LAMP. When LAMP was carried out using the DNA template from *V. natriegens*, *V. parahaemolyticus*, *V. harveyi*, *V. vulnificus,* and *V. alginolyticus*, color change in the reaction tubes with *V. natriegens* genomic DNA could be seen (Fig. 2A) within a 60 min incubation period, and the specific ladder of multiple bands was produced by 1.5% agarose gel electrophoresis (Fig. 2B). In the reaction of *V. parahaemolyticus*, *V. harveyi*, *V. alginolyticus,* and *V. vulnificus* strains, there was no color change in the reaction tubes after a 60 min incubation period and the specific ladder of multiple bands was not produced by 1.5% agarose gel electrophoresis. This result demonstrated that the LAMP primers were specific to *V. natriegens* identification. At the same time, their DNA was amplified by PCRs primed by 27f/1942r primer in order to indicate the existence of four another *Vibrio* strains genomic DNA. The DNA products were unambiguously observed in PCRs (Fig. 2C).

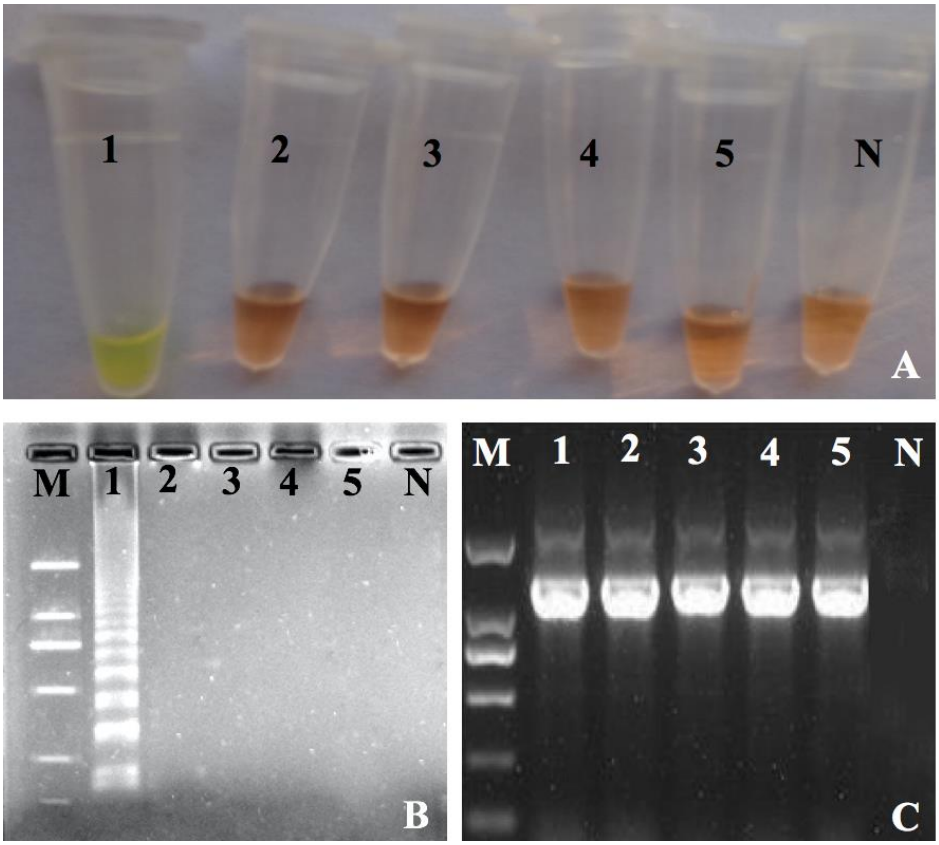

Fig 2. Specificity test of gyrB-LAMP and 16S DNA PCR. (A) Color reaction with SYBR Green I; (B) Electrophoresis of gyrB-LAMP; (C) Electrophoresis of 16S DNA PCR. M: DL DNA marker; 1-5: *V. natriegens*, *V. parahaemolyticus*, *V. harveyi*, *V. alginolyticus* and *V. vulnificus*; N: negative control.

Sensitivity of LAMP. The reaction was tested using 1μL of the 10-fold serial dilutions of *V. natriegens* DNA and compared to results from duplex PCR assay. The detection limit of the *gyrB* of LAMP was 1.32×10^{-2}fg/mL (Fig. 3A and 3B), and LAMP reaction followed by colorimetric analysis could be completed within 100 minutes. However, the detection limit of duplex PCR based on vhh and toxR genes was 13.2 pg/mL, but the following gel electrophoresis required about 3 h for completion (Fig. 3C).

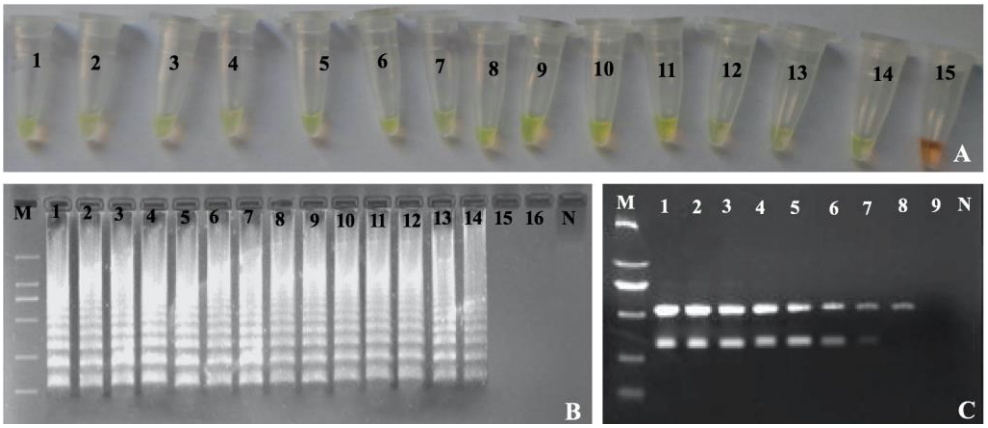

Fig 3. Sensitivity comparison of gyrB-LAMP and duplex PCR methods. (A) Color reaction with SYBR Green I; (B) Electrophoresis of gyrB-LAMP; (C) Electrophoresis of duplex PCR. M: DL DNA marker; 1-14: various dilutions of *V. natriegens* DNA template: 1.32×10^{2}; 1.32×10^{1}; 1.32×10^{0}; 1.32×10^{-1};1.32×10^{-2}; 1.32×10^{-3}; 1.32×10^{-4}; 1.32×10^{-5}; 1.32×10^{-6}; 1.32×10^{-7}; 1.32×10^{-8}; 1.32×10^{-9}; $1.32\times10^{-10;}$ 1.32×10^{-11}; N: negative control.

LAMP application for rapid diagnosis. Of the 90 swimming crabs tested, LAMP detected 34 crabs which were infected by *V. natriegens* (Fig. 4A and 4B). By comparison, in vitro culture of muscle homogenate, isolation and examination by transmission electron microscopy (TEM) by negative staining (Fig 5), *V. natriegens* isolates could be detected in only 18 crabs since the isolation technique could only detect living *V. natriegens.* In the muscle homogenate of the remaining 72 crabs, *V. natriegens* was not observed, even when incubation time was extended to one week. Therefore, the LAMP technique was more efficient, sensitive, and accurate than the in-vitro isolation procedure for the detection of the *V. natriegens* pathogen in swimming crabs.

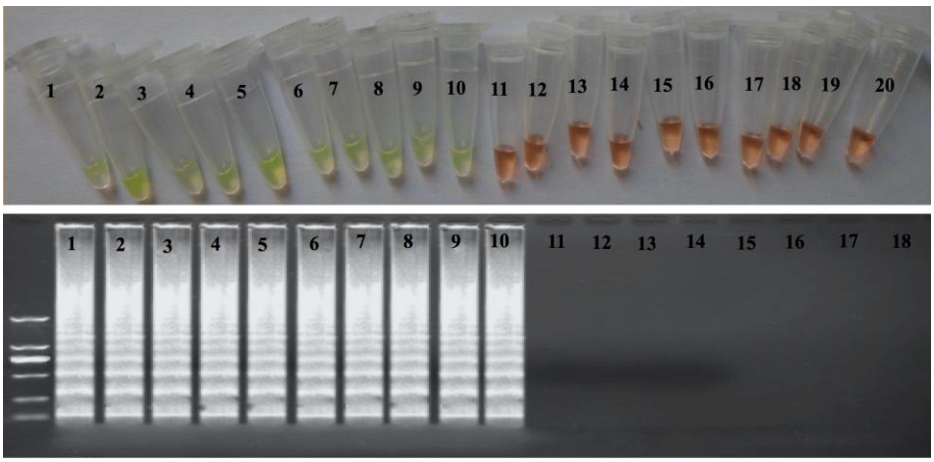

Fig 4 Application of gyrB-LAMP to detect *V. natriegens* from *P. trituberculatus*. (A) Color reaction with SYBR Green I; (B) Electrophoresis of gyrB-LAMP; lane1-10: positive results of gyrB-LAMP; Lane 11-20: negative *results of gyrB-LAMP.*

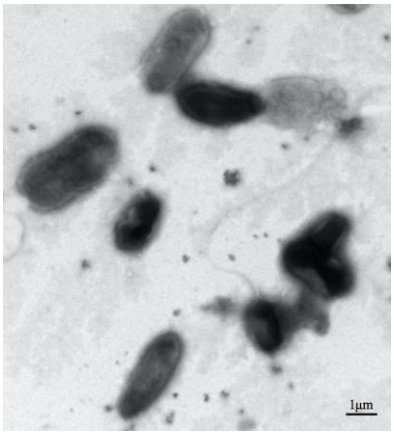

Fig 5. Negatively stained electron micrograph of *V. natriegens* from the tested *P. trituberculatus* showing its morphological characteristics. Scale bar, 1μm.

Discussion

V. natriegens was initially thought to be a non-pathogenic marine bacterium isolated from salt marshes (Payne et al., 1961). However, it has been verified as a local, highly prevalent, pathogen and has been isolated from aquatic animals and the environment. The pathogenicity of *V. natriegens* to aquatic animals seems to have been underestimated (Bi et al., 2016; Deng et al., 2004; Li et al., 2009).

Traditionally, the detection of *Vibrio* species has been performed using a series of culture-based methods which involve enrichment, colony formation on selective agar medium, morphological, and biochemical tests. However, they are time-consuming and labor intensive. As the gold standard for *Vibrio* species detection, polymerase chain reaction (PCR), and real-time PCR are also impractical for in situ detection, due to the long operation time, and expensive, non-portable, instruments (Wang et al., 2017; Zhou et al., 2016; Garrido et al., 2012). In order to simply, rapidly, and accurately identify *V. natriegens*, a *gyrB*-LAMP method was developed. The *gyrB* gene that encodes the B subunit protein of bacterial DNA gyrase (topoisomerase type II) has been used for molecular identification (Yamamoto & Harayama 1995). Both the conserved and the variable regions of this gene are suitable for the development of PCR primers (Kumar et al., 2006; Luo & Hu 2008). The *gyrB*-targeted PCR methods have been developed for the identification of *Vibrio* (Kumar et al., 2006; Luo & Hu 2008; Zhou et al., 2007; Cai et al., 2010). These studies demonstrate the preferred choice of the *gyrB* gene for molecular identification of closely related genomic species.

In this study, the established LAMP method was found to be a rapid, simple, and sensitive detection tool for species *gyrB*-targeted diagnosis of *V. natriegens*. It does not require gel electrophoresis, expensive equipment, and trained personnel. It is only necessary to maintain a constant temperature of 65^0C for 1 h for the reaction. It can detect *V. natriegens* up to 1.32×10^{-11} fg/mL DNA template whereas using duplex PCR the detection of *V. natriegens* is possible up to 1.32×10^{-5} fg/mL DNA template. All characterization mentioned above make LAMP a better choice than PCR and culture-based methods for the diagnosis of *V. natriegens* in cases where lower concentrations of bacteria are expected. Hence, the LAMP assay could be developed into a field test and made available to aquaculturists and producers in an effort to improve the prevention of *V. natriegens*.

In conclusion, the LAMP method described in this study represents a new sensitive, specific and rapid protocol for the detection of *V. natriegens*. It provides an important diagnostic tool for the detection of *V. natriegens* infection both in the laboratory and in the aquatic product market.

Acknowledgements

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