Development and Evaluation of a Rapid, Specific, and Sensitive Loop-mediated Isothermal Amplification Assay to Detect Tenacibaculum sp. strain Pbs-1 Associated with Black-spot Shell Disease of Akoya Pearl Oysters

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Research Article

Keywords: black-spot shell disease, Tenacibaculum sp. Pbs-1, Pinctada fucata, Loop-mediated isothermal amplification, detection, assay

Posted Date: August 2nd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1891727/v1

Abstract

Black-spot shell disease decreases pearl quality and threatens pearl oyster survival. Establishment of a rapid, specific, and sensitive assay to detect *Tenacibaculum* sp. strain Pbs-1 associated with black-spot shell disease is of commercial importance. We develop a rapid, specific, and highly sensitive loop-mediated isothermal amplification (LAMP) assay to detect *Tenacibaculum* sp. Pbs-1. A set of five specific primers (two inner, two outer, and a loop) are designed based on the 16S–23S internal spacer region of strain Pbs-1. The optimum reaction temperature, and concentration of inner and loop primers are 63°C, and 1.4 μM and 1.0 μM, respectively. The LAMP product can be detected using agarose gel electrophoresis, and the color change in the reaction tube can be detected visually (by naked eye) following addition of malachite green. Our assay is specific for Pbs-1, with no cross-reactivity with other species in the genus *Tenacibaculum*. The detection limit of the LAMP assay at 35 min is 50 pg, and at 60 min it is 5 fg. We evaluate the LAMP assay using diseased and healthy pearl oysters. Results indicate the suitability and simplicity of this test for rapid field diagnosis of Pbs-1.

Introduction

Aquaculture production in Japan from 1993–2017 has hovered near 1×10^6 t per year (Matsuura et al. 2019). However, according to the Ministry of Agriculture, Forestry and Fisheries statistics in Japan, the domestic production of pearl oysters has decreased year after year, peaking at approximately 125×10^3 t in 1967 and decreasing to approximately 16×10^3 t in 2020. This decrease is mainly attributed to mass mortality events of pearl oysters caused by viral or bacterial infection, such as Akoya oyster disease caused by *Candidatus* Maribrachyspira akoyae (Matsuyama et al. 2017), an unidentified virus in 2019 and 2020 (Matsuyama et al. 2021), and black-spot shell disease caused by *Tenacibaculum* sp. strain Pbs-1 (Sakatoku et al. 2018). Of these, black-spot shell disease—characterized by having localized blackish shell discoloration—causes serious problems in cultured pearl production (Sakatoku et al. 2018), and both decreases pearl quality and threatens pearl oyster survival. Although this disease was first reported in the 1950s, no treatment is available. A rapid, specific, and sensitive pathogen detection method is necessary to more fully understand this disease, and to develop a treatment for it.

Immunological techniques such as ELISA (Swain and Nayak 2003; Hu et al. 2020), immunofluorescence (McCarthy 1975; Liu et al. 2020), immunohistochemistry (Ekman and Norrgren 2003; Khimmakthong et al. 2013), and lateral flow immunoassay (Seo et al. 2020; Su et al. 2015); molecular techniques such as PCR (López et al. 2011), real time PCR (Halaihel et al. 2009; Adamek et al. 2016), and loop-mediated isothermal amplification (LAMP) (Gahlawat et al. 2009; Xu et al. 2010); and nanotechnology-based methods such as MALDI-TOF-MS (Piamsomboon et al. 2020) and flow cytometry (Ryumae et al. 2010), can detect various pathogens in aquaculture. Of these, the LAMP method was developed to amplify specific genes using a designated primer set and a DNA polymerase with strand displacement activity (Notomi et al. 2000). Advantages of this LAMP method are that DNA can be specifically amplified under isothermal conditions, and that gene amplification can be detected visually (by the naked eye) following addition of a dye such as malachite green to the reaction mixture (Lucchi et al. 2016). Therefore, it is

possible to detect the pathogen in the field using minimal equipment (e.g., a water bath or heat block) to maintain constant temperature.

Although 16S rRNA genes are widely used in bacterial taxonomy because they contain variable regions, the internal spacer region (ISR) between the 16S and 23S rRNA genes is known to be more variable among bacterial species than ribosomal genes themselves (Barry et al. 1991; Hassan et al. 2003; Osorio et al. 2005). We develop a rapid, specific, and sensitive LAMP method targeting the ISR of *Tenacibaculum* sp. strain Pbs-1, and provide the first report on the diagnosis of *Tenacibaculum* sp. Pbs-1 associated with black-spot shell disease.

Materials And Methods

Preparation of bacteria and pearl oysters

The strain Pbs-1 was isolated from diseased Akoya pearl oysters as reported by Sakatoku et al. (2018). Five type strains of the genus *Tenacibaculum* (*T. mesophilum*, *T. amylolyticum*, *T. sediminilitoris*, *T. maritimum*, *T. ascidiaceicola*) were purchased from the National Institute of Technology and Evaluation. Diseased and healthy Akoya pearl oysters, *Pinctada fucata*, were collected from a pearl oyster farm in Ago Bay, Shima City, Mie Prefecture, Japan. After excision from the shell, the shell pieces from Akoya pearl oysters were transferred into 1.5-ml sterile centrifuge tubes and stored at − 30°C prior to DNA extraction.

DNA extraction

Bacterial strains were cultured in marine broth medium (Difco marine broth 2216; Becton, Dickinson and Company, MD, USA) at 25°C for 24 h. After collecting bacteria by centrifugation, bacterial DNA extraction was performed using a Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA). Total DNA was extracted from the shell pieces using a vigorous bead beating DNA extraction kit (FastDNA SPIN Kit for Soil; MP Biomedicals, CA, USA), following the manufacturer's instructions. Genomic DNA was then quantified by spectrophotometer (Nanodrop 1000 Spectrophotometer; Thermo Fisher Scientific K.K., Kanagawa, Japan) and stored at − 30°C for later use.

LAMP assay primer design

The 16S–23S ISR of all bacteria (strains Pbs-1 and 5 type strains of *Tenacibaculum*) was sequenced. The ISR was amplified by PCR using primers 5'-ATACGGAGGGTGCAAGCGTT-3' (forward) and 5'-ACCAGCGGATTTGCCTACCA-3' (reverse), designed based on the 16S rDNA and 23S rDNA sequences of strain Pbs-1, respectively. PCR amplification of the ISR gene of bacteria was performed in a 20-μL volume containing approximately 5 ng of template DNA, 1 × Ex Taq buffer, 200 μM dNTP mix, 0.25 μM of each primer, and 0.5 U Ex Taq HS (TaqDNA polymerase; Takara Bio Inc., Shiga, Japan). PCR reactions involved

an initial denaturation step at 94°C for 3 min, followed by 35 amplification cycles consisting of denaturation at 94°C for 60 s, annealing at 50°C for 60 s, and elongation at 72°C for 60 s. The reaction was terminated with a terminal elongation step at 72°C for 3 min, followed by cooling at 4°C. Amplified products were purified using a QIAquick PCR purification kit (Qiagen K. K., Tokyo, Japan). Purified PCR products were independently ligated into the pGEM-T easy vector system (Promega), following the manufacturer's protocols. Recombinant plasmids were transformed into *Escherichia coli* DH5α competent cells using 10 μL of the ligation mixture, and spread on LB plates containing ampicillin. Insert-containing plasmids were purified using the PureYield Plasmid Miniprep System (Promega), amplified using M13 f and M13 r universal primers, then sequenced using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 3130xl genetic analyzer (Applied Biosystems). Multiple sequence alignment was performed using Genetyx v14 software (Genetyx, Tokyo, Japan). A set of five primers for LAMP was designed using Primer Explorer v5 (https://primerexplorer.jp/lampv5/index.html) to target the ISR of strain Pbs-1. Primer sets comprised two outer primers (F3 and B3), two inner primers (the FIP primer consisted of the complementary sequences of F1c and F2, and the BIP primer consisted of B1c and B2), and a loop primer (LF) (Table 1).

LAMP reaction condition optimization

The optimal temperature for the LAMP assay was determined at 60°C, 63°C, and 65°C for 35 min. The LAMP was performed in a 25-μL reaction volume containing 1.4 μM each of FIP and BIP, 0.2 μM each of F3 and B3, 1×Reaction mixture (Loopamp DNA amplification reagent kit; Eiken Chemical Co., Ltd. Tokyo, Japan), 8 U *Bst* DNA polymerase (Eiken Chemical Co., Ltd.), and 50 ng of DNA template. To evaluate the optimal concentration of the two inner primers (FIP and BIP), their concentrations were reacted from 1.0–1.6 μM. The composition of the reaction mixture other than FIP and BIP is the same as above. The LAMP reaction was performed at 63°C for 35 min. To evaluate the optimal concentration of the loop primer (LF), its concentrations were reacted from 0.6–1.2 μM. The composition of the above reaction mixture was used, except that 50 pg of DNA template and LF were used. The LAMP reaction was performed at 63°C for 35 min. The LAMP products were separated by 2% (w/v) agarose gel electrophoresis, stained with ethidium bromide, then photographed using a FAS-Digi PRO (NIPPON Genetics Co., Ltd. Tokyo, Japan).

LAMP assay sensitivity

Ten-fold serial dilutions (50 ng to 50 ag) of DNA extracted from *Tenacibaculum* sp. strain Pbs-1 were used as templates for LAMP. The LAMP was performed in a 25-μL reaction volume containing 1.4 μM each of FIP and BIP, 0.2 μM each of F3 and B3, 1.0 μM LF, 1×Reaction mixture, 8 U *Bst* DNA polymerase, and 2 μL of DNA template. The LAMP reaction was performed at 63°C for 35 min or 60 min. LAMP products were detected by 2% (w/v) agarose gel electrophoresis as described above, and visually following addition of 0.008% (w/v) malachite green (Wako Pure Chemical Industries, Ltd., Japan) to the reaction mixture to observe a color change.

Evaluation of the LAMP assay using clinical samples

Three diseased and three apparently healthy pearl oysters were collected from Ago Bay and tested by LAMP assay in a 25-μL reaction volume containing 1.4 μM each of FIP and BIP, 0.2 μM each of F3 and B3, 1.0 μM LF, 1×Reaction mixture, 8 U *Bst* DNA polymerase, and 2 μL of DNA template. The reaction was performed at 63°C for 60 min. LAMP products were detected by 2% (w/v) agarose gel electrophoresis. The rate for positive detection of strain Pbs-1 in the samples, and assay sensitivity were calculated.

Results

Optimization of Pbs-1 LAMP reaction conditions

The LAMP reaction was performed using DNA from the genus *Tenacibaculum* (strain Pbs-1 and strains from five related species) as a template to determine optimum reaction temperature, and concentrations of inner and loop primers. Reaction temperatures were examined at 60°C, 63°C, and 65°C. Electrophoresis produced clear ladder bands at 60°C and 63°C, but not at 65°C (Fig. 1). We regard 63°C to be the optimal reaction temperature for the Pbs-1 LAMP assay. To evaluate the optimal concentration of the two inner primers, their concentrations were reacted from 1.0–1.6 μM (Fig. 2). Only DNA from strain Pbs-1 was detected at all tested primer concentrations. Amplified products at 1.0 μM or 1.2 μM were visualized as weak bands, while at 1.4 μM and 1.6 μM, clear ladder bands were produced. Therefore, we regard the optimal concentration of inner primers to be 1.4 μM for this LAMP assay. To evaluate the loop primer optimal concentration, its concentrations were reacted from 0.6–1.2 μM (Fig. 3). Electrophoresis produced nonspecific ladder bands at 1.2 μM, but only DNAs from strain Pbs-1 were amplified from 0.6–1.0 μM. Therefore, we regard the optimal loop primer concentration to be 1.0 μM for this LAMP assay. Thus, the optimum conditions for this LAMP reaction are: reaction temperature 63°C, and concentrations of inner and loop primers, 1.4 μM and 1.0 μM, respectively.

Detection limits of LAMP

LAMP reactions were performed at 63°C for 35 min or 60 min, and their respective detection limits were compared using 10-fold serial dilutions (50 ng to 50 ag) using DNA from strain Pbs-1 as the template. Detection limits of Pbs-1 DNA by LAMP assay for 35 min or 60 min were 50 pg (Fig. 4a) and 5 fg (Fig. 4c), respectively. Additionally, by adding malachite green to the reaction mixture, the positive LAMP reaction (dark blue) could be detected visually (Fig. 4b, 4d) (the negative reaction corresponded to light blue). These visual detection results correlated with those of gel electrophoresis.

Evaluation of the LAMP assay using DNA from pearl oysters

Evaluation of the LAMP assay was performed using DNA samples extracted from three diseased and three healthy pearl oysters. All diseased oysters tested positive for the presence of strain Pbs-1 when

using the LAMP assay (Fig. 5). The Pbs-1 strain was detected in one apparently healthy oyster.

Discussion

Tenacibaculum sp. strain Pbs-1, the causative agent of black-spot shell disease in *P. fucata*, is recognized as a major problem for the pearl culture industry. To prevent or monitor disease outbreaks, rapid-, specific-, convenient-, and sensitive-detection of strain Pbs-1 is important. Although many highly optimized, highly sensitive, and specific PCR assays exist, they are time-consuming, and require levels of technical expertise and specific instruments (such as thermal cyclers) to perform, rendering them impractical for field detection of strain Pbs-1. The LAMP reaction can be performed from 60–65°C within a short period of time, and requires only a regular water bath or a heating block to maintain constant temperature.

We designed specific primers for the LAMP assay based on the ISR between the 16S and 23S rRNA genes of *Tenacibaculum* sp. Pbs-1. ISR nucleotide sequences were determined from strain Pbs-1 and from five related bacterial strains. The homologies between Pbs-1 and related species (*T. mesophilum*, *T. amylolyticum*, *T. sediminilitoris*, *T. maritimum*, *T. ascidiaceicola*) were 96.5%, 39.2%, 76.9%, 85.9%, and 73.0%, respectively. From alignment results of these sequences, we designed five specific primers to detect Pbs-1 (Table 1).

Our LAMP method reacted well at 60–63°C. Although we conducted analyses in the laboratory, we suggest that this method could be used in the field using a common water bath and/or an inexpensive heating block, and that it may be suitable for field use where precise temperature control is difficult. Excluding the time for electrophoresis, a conventional PCR assay takes 2–3 h (Hassan et al. 2008; López et al. 2011; Wiklund et al. 2000), whereas the LAMP assay can be performed within 1 h. To our knowledge, the LAMP assay of swine vesicular disease virus in pigs is the most rapid method (25 min) (Blomström et al. 2008). In aquaculture, to our knowledge, the shortest reaction times using the LAMP method to detect viruses or bacteria are: 35 min to detect lymphocystis disease virus in Japanese flounder *Paralichthys olivaceus* (Li et al. 2010), and 30 min to detect *Vibrio parahaemolyticus* in artificially contaminated shellfish (Chang et al. 2011). Because our Pbs-1 LAMP assay requires only 35 min, the time taken to perform it is comparable to these other tests.

The detection limits of Pbs-1 DNA by LAMP assay for 35 min or 60 min were 50 pg (Fig. 4a) and 5 fg (Fig. 4c), respectively. The reaction at 60 min was 10,000 times more sensitive than it was at 35 min. This result indicates that the reaction can occur in 35 min if speed is required, and in 60 min if high sensitivity is required. To our knowledge, the most sensitive level of bacterial LAMP is 0.3 fg (Fall et al. 2008). Although the sensitivity of Pbs-1 LAMP reacted at 60 min was slightly inferior, it was sufficiently sensitive to detect Pbs-1. Additionally, by adding malachite green to the reaction mixture, the positive LAMP reaction changed to a dark blue, which could be discerned visually. The correlation between results judged visually with those observed after gel electrophoresis is consistent with previous reports (Sun et al. 2006; En et al. 2008; Ren et al. 2010; Xu et al. 2010). We regard the visual inspection of LAMP products

with malachite green to be an advantage because it eliminates the need for time-consuming electrophoresis and subsequent staining with the carcinogen ethidium bromide. These results suggest that the LAMP assay could be performed in the field.

Evaluation of the LAMP assay was performed with DNA samples extracted from three diseased and three healthy pearl oysters. When the LAMP assay was used, all three diseased oyster samples (as expected) tested positive for Pbs-1. Interestingly, one healthy oyster sample tested positive for Pbs-1 presence (Fig. 5). LAMP products were also digested using the restriction enzyme MseI, and the size of the digested fragment was consistent with the expected size of 136 bp (data not shown). This result indicates that LAMP amplification was performed by Pbs-1 DNA, not by oyster genomic DNA. Furthermore, no cross-reactions with DNA from other *Tenacibaculum* species were observed using the Pbs-1 LAMP assay, indicating that LAMP is highly specific for target DNA sequences (Fig. 2, Fig. 3). These results strongly suggest that the likelihood of there being false positives is very low, and that Pbs-1 at low densities can also be detected from asymptomatically infected oysters.

Until now, only PCR-DGGE (denaturing gradient gel electrophoresis) or culture methods were able to detect the pathogen *Tenacibaculum* sp. strain Pbs-1 (Sakatoku et al. 2018). Black-spot shell disease caused by *Tenacibaculum* sp. strain Pbs-1 also occurs in pearl farms in Asia such as Vietnam (Sakatoku et al. 2018). Regarding cost and simplicity, our assay is a rapid, simple, and economically viable alternative to use to perform large-scale shellfish screening both nationally and internationally.

Conclusions

The LAMP-based assay described herein represents a specific, rapid, and simple diagnostic method to detect *Tenacibaculum* sp. strain Pbs-1 associated with black-spot shell disease. Ours is the first report to detail the LAMP method for the molecular diagnosis of strain Pbs-1. Reactions are performed in a single tube, incubated for 35 min or 60 min in a water bath or on a heating block at 63°C. In addition to its high specificity, the detection limit of the LAMP assay is 50 pg at 35 min and 5 fg at 60 min. Although LAMP products can be detected by agarose gel electrophoresis, they can also be detected visually by adding malachite green to the reaction mixture, facilitating field detection of Pbs-1. This technique has considerable potential for routine diagnosis of Pbs-1 in less-well-equipped hatcheries and/or pearl farms.

Declarations

Acknowledgments

This study was supported in part by grants to A.S. (Grant-in-Aid for Scientific Research [C] No. 20K06203 by JSPS and Nos. 20E003 and 21E018 by Kurita Water and Environment Foundation). We also thank Steve O'Shea, PhD, from Edanz (https://jp.edanz.com/ac), for editing a draft of this manuscript.

Funding declaration

The authors declare they have no financial interests.

Conflict of Interest

The authors declare no competing financial interests.

Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contribution

Conceptualization: [Akihiro Sakatoku and Tadashi Isshiki], Methodology: [Akihiro SAKATOKU], Formal analysis and investigation: [Takaya Suzuki, Yuri Tatamiya and Makoto Seki], Writing - original draft preparation: [Akihiro Sakatoku]; Writing - review and editing: [Daisuke Tanaka, Shogo Nakamura and Tadashi Isshiki], Funding acquisition: [Akihiro Sakatoku], Supervision: [Akihiro Sakatoku and Tadashi Isshiki]

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Tables

Table1. Primers used in the study.

Primer	Length (bp)	Sequence (5’-3’)
F3	18	CAAGAGGTCATCGGTTCG
B3	25	CGAGCTCTTTATAATTCTTGTTCTT
FIP (F1c+F2)	45	TGCCTGCAATACTCTCAGAAATAATACTCCGATATTCTCCACTGG
BIP (B1c+B2)	45	ACTCGCCACAACGTTCATTGGATGTTATCTAACTCTCTATCTTGA
LF	22	GCAATGTCCAGAGATAATAAGA

Figures

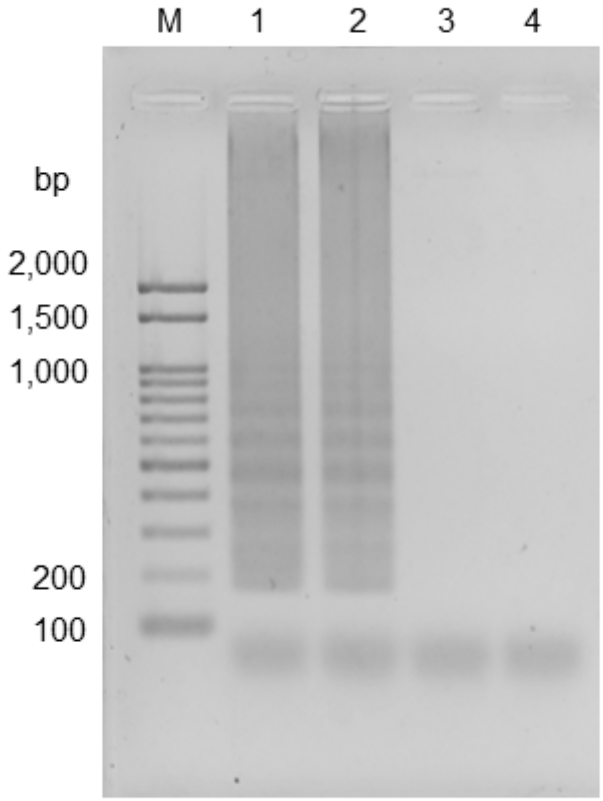

Figure 1

Determination of LAMP reaction conditions at different temperatures by agarose gel electrophoresis. M, marker; 1, 60°C; 2, 63°C; 3, 65°C; 4, negative control

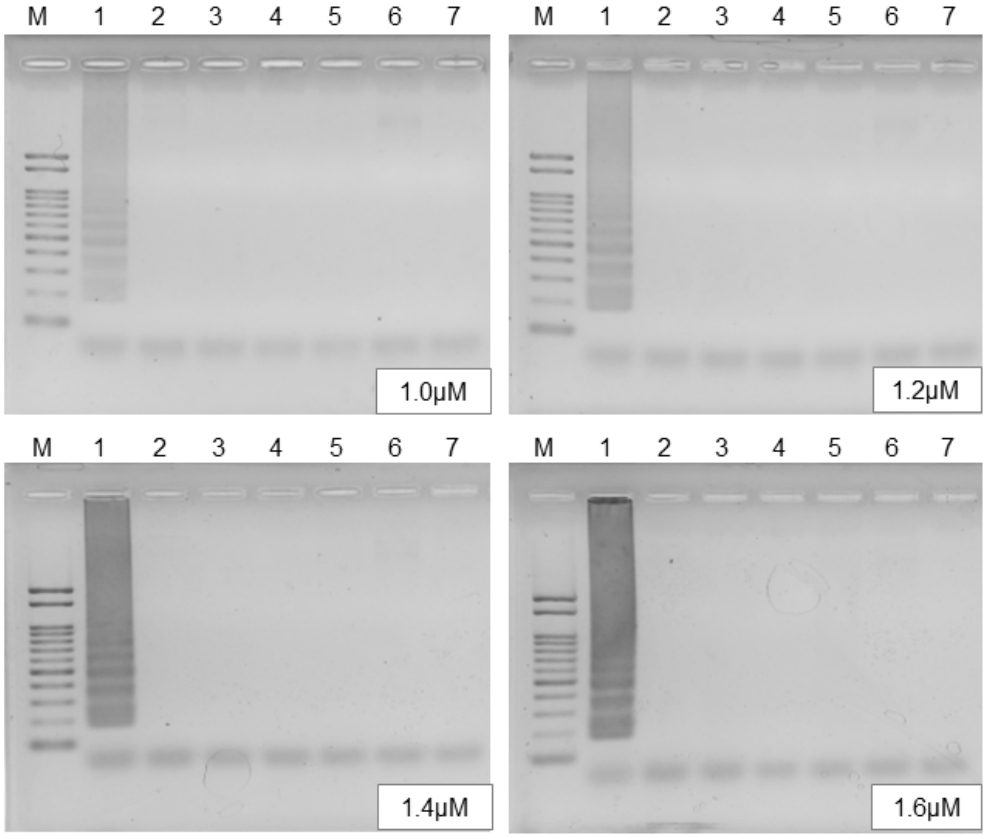

Figure 2

Determination of LAMP reaction conditions at different concentrations of FIP and BIP by agarose gel electrophoresis. M, marker; 1, *Tenacibaculum* sp. Pbs-1; 2, *T. mesophilum*; 3, *T. amylolyticum*; 4, *T. sediminilitoris*; 5, *T. maritimum*; 6, *T. ascidiaceicola*; 7, negative control

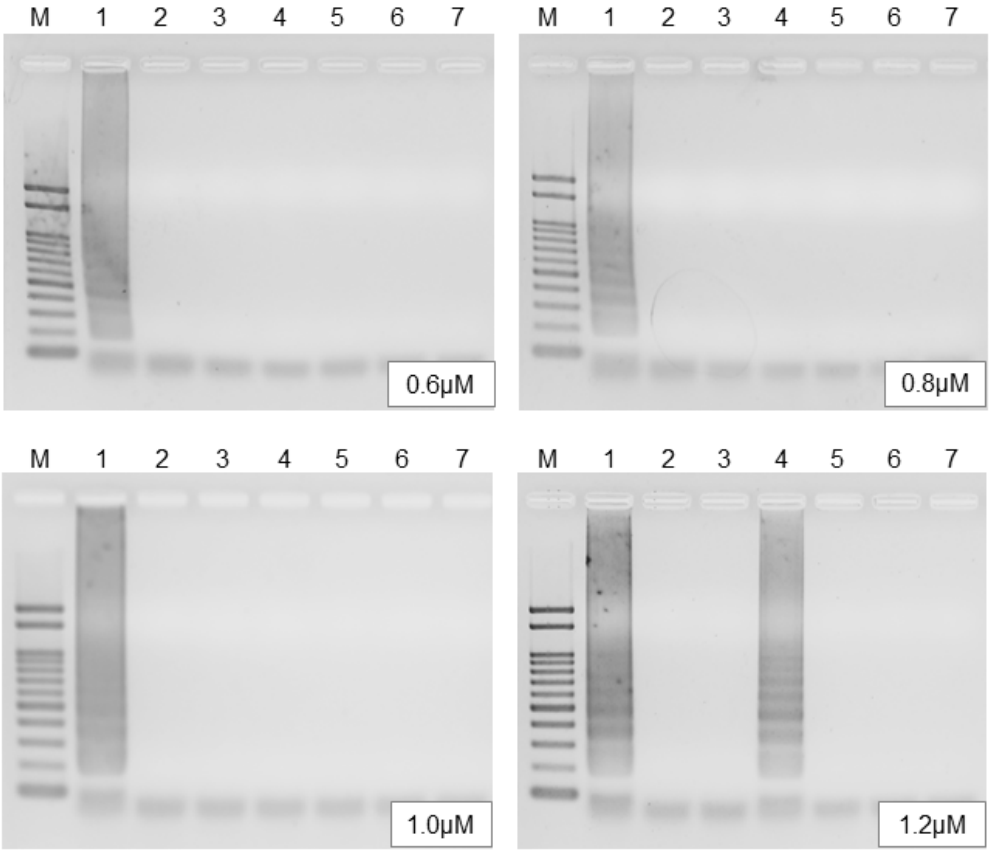

Figure 3

Determination of LAMP reaction conditions at different concentrations of LF by agarose gel electrophoresis. M, marker; 1, *Tenacibaculum* sp. Pbs-1; 2, *T. mesophilum*; 3, *T. amylolyticum*; 4, *T. sediminilitoris*; 5, *T. maritimum*; 6, *T. ascidiaceicola*; 7, negative control

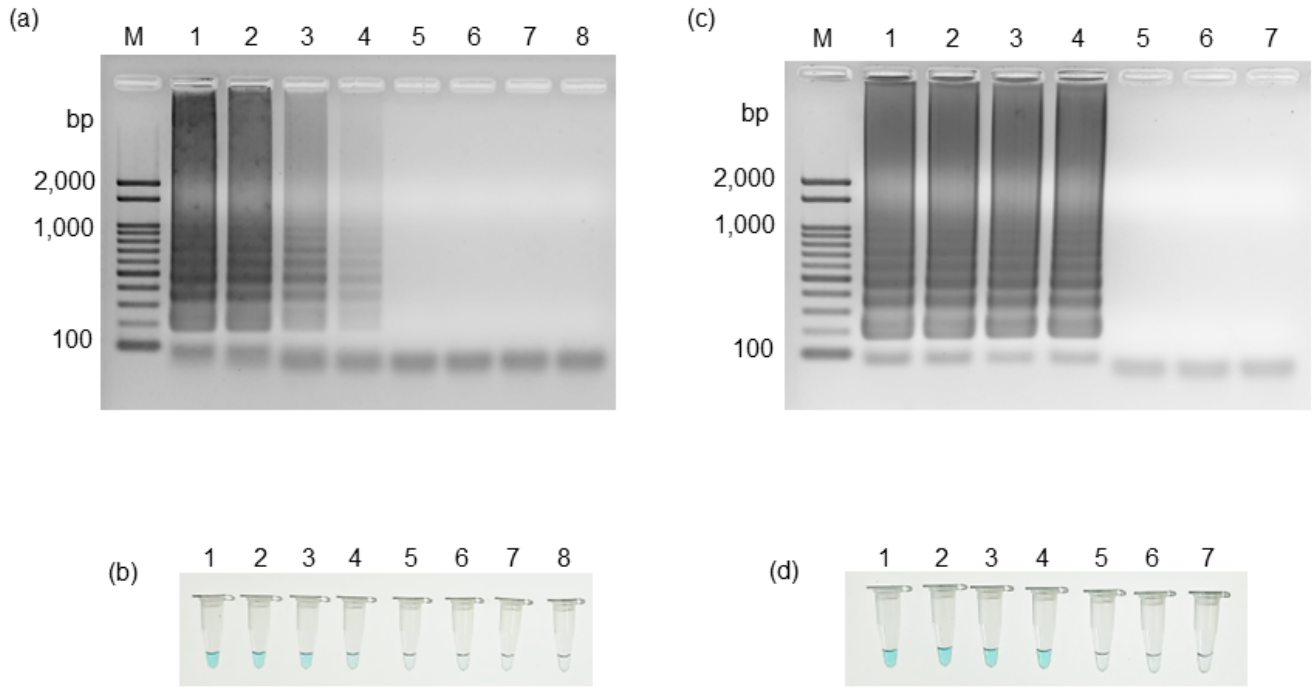

Figure 4

Sensitivities of LAMP assays for detection of Pbs-1. The LAMP reaction was performed at 63°C for 35 min, and then products were detected using (a) agarose gel electrophoresis and (b) visually by adding malachite green. M, marker; 1, 50 ng; 2, 5.0 ng; 3, 0.5 ng; 4, 50 pg; 5, 5.0 pg; 6, 0.5 pg; 7, 50 fg; 8, negative control. The LAMP reaction was performed at 63°C for 60 min, and then products were detected using (c) agarose gel electrophoresis and (d) visually by adding malachite green. M, marker; 1, 50 ng; 2, 50 pg; 3, 50 fg; 4, 5.0 fg; 5, 0.5 fg; 6, 50 ag; 7, negative control

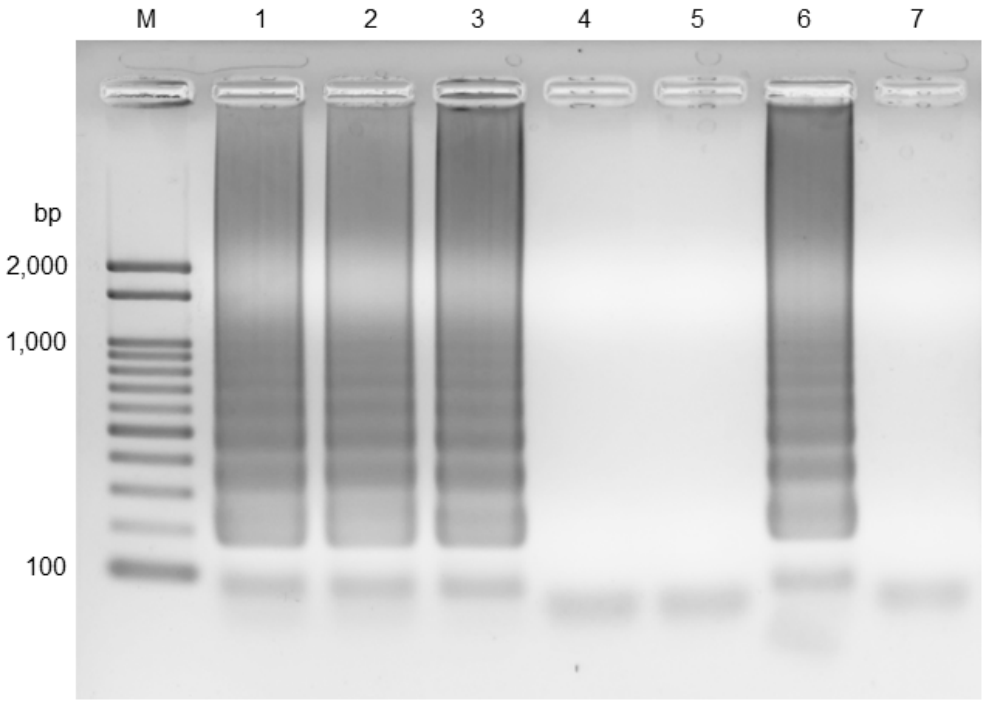

Figure 5

Feasibility of LAMP assay. M, marker; 1–3, DNA from diseased pearl oysters; 4–6, DNA from healthy pearl oysters; 7, negative control