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Research Article

Screening of vibriosis in Asian seabass, *Lates calcarifer* using loop-mediated isothermal amplification (LAMP) assay

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Abstract. The aim of this study was to standardize a loop-mediated isothermal amplification (LAMP) assay for the detection of *Vibrio harveyi*, the causative agent of vibriosis in Asian seabass, *Lates calcarifer*. The dnaJ gene of the bacterial pathogen was used as the target gene for the LAMP assay. It was optimized at an incubation time of 1 h at 63^{0}C. The assay was highly specific for *V. harveyi* and did not cross-react with other bacterial pathogens of fish. However, the assay was able to detect *V. harveyi* that was isolated from infected shrimps. The limit of detection of the LAMP assay was 40 pg of DNA mL^{-1} or 40 fg of the genomic DNA per LAMP reaction and was 10 times more sensitive than conventional PCR in detecting the bacterial pathogen from infected samples. The LAMP products can be quantified spectrophotometrically using hydroxynaphthol blue (HNB) dye and showed positive correlation with the amount of the pathogen. These results demonstrated that LAMP is a simple and sensitive detection technique that has potential application for routine diagnosis of vibrosis caused by *V. harveyi* in Asian seabass and other aquatic species.

Key Words: *Vibrio harveyi*, vibriosis, *Lates calcarifer*, LAMP.

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Introduction

Vibriosis is one of the major bacterial diseases in mariculture systems (Bondad-Reantaso *et al* 2005; Zhang *et al* 2007). This disease is caused by several *Vibrios*, and one of them is *Vibrio harveyi*, a halophilic Gram-negative bacterium that is ubiquitous in the aquatic environments. This pathogen is known to infect fish, shrimp and shellfish either in the culture systems or in the wild (Austin & Zhang 2006). The early diagnosis of infections due to *V. harveyi* could facilitate effective disease surveillance and prevention of the disease in the aquaculture site.

Conventional microbiological, biochemical and immunological methods of detecting *V. harveyi* are costly in labor, materials and time. There are several PCR assays (Oakey *et al* 2003; Hernandez & Olmos 2004; Pang *et al* 2006) that shorten the time required for detection of the pathogen. While PCR assays are considerably faster than conventional methods of pathogen detection, these require post-PCR electrophoresis, which could be labor-intensive and time-consuming (Goto *et al* 2007). Real-time PCR is another technique that has been recently developed for the rapid identification of *V. harveyi* (Fukui & Sawabe 2008). However, this assay is not routinely used because it requires expensive reagents and a thermal cycler with flourescence detector. Hence, a rapid yet cost-effective method of pathogen detection is needed as an alternative to existing assays.

The loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method that has been developed recently (Notomi *et al* 2000). It is an auto-cycling strand displacement DNA synthesis which is performed by an exonuclease-negative *Bst* DNA polymerase under isothermal conditions. The assay can be perfomed in one hour, thus shortening the time for detecting the pathogen. It uses four primers that recognize six distinct sequences of the target gene, ensuring high specificity. LAMP assays have been developed to detect various species of *Vibrio* in the aquatic environnment, including *V. alginolyticus* (Cai *et al* 2010), *V. nigripulchritudo* (Fall *et al* 2011) and *V. parahaemolyticus* (Sun *et al* 2012). It was also used to detect *V. harveyi* in marine shellfish using the toxR as the target gene (Cao *et al* 2010). In these studies, the use of the LAMP method showed that this was faster, easier to perform and has higher specificity and sensitivity than conventional PCR assays.

The Asian seabass, *Lates calcarifer* is a popular species for marine netcage aquaculture in most tropical countries (Tendencia 2002; Ransangan & Manin 2010). This species of fish is also susceptible to *V. harveyi* infections and the diseased fish shows focal hemorrhages in the skin and exophthalmia (Tendencia

2002). The incidence of vibriosis in fish increases in summer when the water temperature rises and there is abundance in organic load (Kim *et al* 1990; Carli *et al* 1993). Previously, we have developed and optimized a conventional PCR assay for the detection of this bacterial pathogen in seabass using *dnaJ* as the target gene (Caipang *et al* 2011a). However, due to some limitations of conventional PCR in the detection of bacterial pathogens, we developed a LAMP protocol as an alternative technique that offers a rapid and more sensitive approach in diagnosing *V. harveyi* infections in fish.

Materials and Methods

Bacterial strains

Samples of *V. harveyi* grown on trypticase soy agar (TSA, Fluka, Germany) supplemented with 1.5% NaCl were provided by the Fish Health Section of the Aquaculture Department, Southeast Asian Fisheries Development. The bacterium was isolated from diseased juveniles of Asian seabass that were cultured in marine cages in Central Philippines (Caipang *et al* 2011a). The other bacterial strains used in the study were obtained from various sources and routinely cultured on Marine agar.

Extraction of genomic DNA

Genomic DNA of the bacteria was extracted following the procedures described by Kulkarni *et al* (2010) with minor modifications. A loopful of the bacteria was scraped from the TSA plate and placed in a 1-mL microfuge tube containing DNA extraction buffer [10 mM Tris, 125 mM NaCl, 10 mM EDTA, 0.5% SDS and 4M Urea at pH 7.5 (Caipang *et al* 2004)]. The solution was added with 10 µL of Proteinase K (20 µg mL^{-1}) and incubated for 1 hour at 37°C. Genomic DNA was extracted by the addition of phenol:chloroform:isoamyl alcohol (PCI) solution followed by precipitation with ethanol. The DNA pellet was resuspended in 100 µL of 1X TE buffer (pH 7.5) and stored at -20°C until use for the assays.

LAMP primers

The LAMP primers were constructed using the *dnaJ* (Genbank Accesssion No. JF832351.1) as the target gene following Notomi *et al* (2000). The partial fragment of this gene from *V. harveyi* that was isolated from diseased seabass was sequenced in our previous study (Caipang *et al* 2011a). The primers were generated using the Primer Explorer software ver 3.0 (http://primerexplorer.jp/e) and consisted of the sequences, F3, B3, FIP and BIP. The sequences of the primers are the following (in the 5'-3' direction): F3, GCG ATA TCT TTG GCG GTG G; B3, TTG GCG CAT TTG AAC TTG AC; FIP, ACG AAC GGC TTC TTC TAG CGA CTT TTT CAG CAA CGT GCA CAA CG; BIP, GTG ATA CCT GTG ACG GTA GCG GTT TTG TGA CCA TGA CAG GTT CCA C.

The outer primers consisted of F3 and the complementary sequence of B3. The forward inner primer or the FIP is composed of the complementary sequence of F1 and F2. The backward inner primer, BIP consisted of B1 and the complementary sequence of B2. The FIP and BIP primers structure "the loop" during the LAMP reaction, whereas the F3 and B3 are necessary for the strand displacement during DNA synthesis (Notomi *et al* 2000). A TTTT-linker was added within the sequence of both the FIP and BIP primers.

Optimization of the LAMP protocol

The LAMP assay was optimized using a 25 µL reaction volume. It consisted of 12.5 µL reaction mix containing 2x Thermopol buffer (New England Biolabs), 8 mMol L^{-1} $MgSO_4$, 0.8 mMol L^{-1} betaine (Sigma), 2 mMol L^{-1} dNTP (Invitrogen), 2 µL of the FIP and BIP primers (20 pMol), 1 µL of F3 and B3 primers (5 pMol), 1 µL of the Bst DNA polymerase (8 U), 1 µL of the DNA template and 4.5 µL of distilled water. Direct visualization was also done following the protocol of an earlier study (Caipang *et al* 2011b).

The specificity of the LAMP primers was evaluated using the optimized conditions as described earlier with other bacterial pathogens including *V. anguillarum, Vibrio parahaemolyticus, V. harveyi* that was isolated from shrimp, A*eromonas salmonicida* and *Yersinia ruckeri*. To determine the sensitivity of the LAMP assay, ten-fold serial dilutions of the bacterial genomic DNA were prepared at an initial concentration of 0.2 µg genomic DNA mL^{-1}. Two microliters of the DNA template was used for the assay following the optimized conditions.

For purposes of comparison, the sensitivity and specificity of the LAMP assay in detecting *V. harveyi* in seabass was compared with conventional PCR (Caipang *et al* 2011a).

Quantification using turbidimetric analysis

To determine whether the LAMP products could be used in quantitative analysis, the samples were subjected to spectrophotometric measurement. The individual tubes containing the reaction mixtures (20 µL) were added with 75 µL of distilled water and 5 µL of 3 mM hydroxynaphthol blue dye (HNB, Sigma). Positive samples showed a sky blue coloration, whereas negative samples had violet coloration. The mixture was transferred to the individual wells of a flat bottom 96-well plates and read using a spectrophotometer (Fluostar) at an absorbance of 590 nm. An OD590 absorbance cut-off was set based on the absorbance value of the negative samples plus two standard deviations (Ma *et al* 2010).

Results and Discussion

A LAMP assay was developed and optimized for the detection of *V. harveyi* in seabass using the *dnaJ* gene as the target for analysis. It resulted in the detection of the bacterial pathogen both by gel electrophoresis (Fig. 1a) and visualization under ultra-violet light (Fig. 1b).

During gel electrophoresis, the positive samples showed laddering patterns, while under UV light they showed intense staining in the presence of the dye in contrast to the faint coloration observed in the negative samples.

The LAMP assay was optimized at 63°C for 1 hr. Although the enzyme that was used in the assay, Bst DNA polymerase had an optimum activity at 65°C (Li *et al* 2010), yet LAMP assays showed that this enzyme is also efficient at lower temperatures (Parida *et al* 2004; Kulkarni *et al* 2009; Caipang *et al* 2011b) including this study. The efficient amplification activity of this enzyme at lower temperatures, i.e., 60-64°C ensure that slight temperature variations during the assay will not have adverse consequences on the reaction. This is true when crude apparatus is used such as water bath containers where there is less control of thermal conditions (Li *et al* 2010). The efficient enzyme activity of this enzyme at a wide temperature range makes the

LAMP assay suitable for field use where temperature fluctuations likely happen because of the use of low precision equipment. The LAMP assay is able to detect a wide array of pathogens better than other detection methods because it does not require expensive equipment and the reaction is carried out under isothermal conditions (Notomi *et al* 2000). In the diagnosis of vibriosis in seabass, the LAMP assay can be completed in an hour, and this was faster than either conventional or nested-PCR assays. In addition, the speed and accuracy of detection have implications in providing effective management procedures particularly when making crucial decisions to prevent massive mortalities. The LAMP assay was highly specific for the detection of *V. harveyi* in seabass with no cross-reactions with other bacterial pathogens in fish (Fig. 2). However, the assay was able to detect *V. harveyi* that was isolated from infected shrimps, indicating the potential of this assay in detecting this bacterium in other aquatic species. In terms of the sensitivity of the LAMP assay, ten-fold serial dilutions of the bacterial genomic DNA were prepared at a starting concentration of 0.2 µg mL^{-1} and a template of 2 µL. The limit of detection of the assay was 40 pg of genomic DNA mL^{-1} or 40 fg of genomic DNA for every LAMP reaction (Fig. 3), whereas a conventional PCR assay for this bacterium has a detection limit of 100 fg of genomic DNA per PCR reaction (Caipang *et al* 2011a). Previous LAMP assays developed for the detection of other *Vibrio* species showed that this technique was more sensitive in detecting the pathogen than conventional PCR (Cai *et al* 2010; Cao *et al* 2010; Sun *et al* 2012).

For basis of comparison, the genome size of *V. vulnificus* is approximately 5.3 Mbp (Chen *et al* 2003). Assuming that this genome size is used for the calculation of genome equivalents

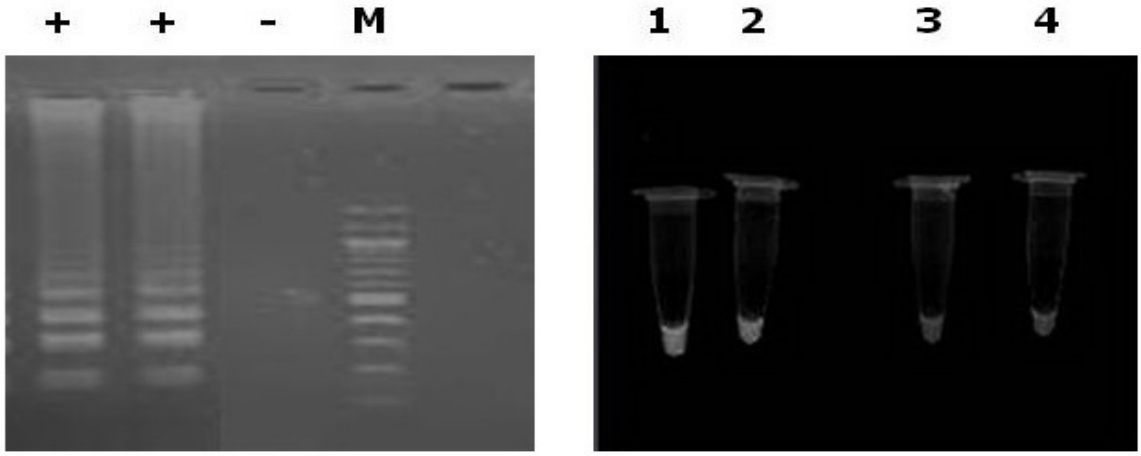

Figure 1. Detection of *V. harveyi* by LAMP reaction using (A) gel-electrophoresis and (B) visualization under UV light with SYBR Safe dye. In Figure 1A, + are positive samples, is the negative control, distilled water and M is the 100-bp DNA marker. In Figure 1b tubes 1-2 are the *V. harveyi*-positive samples, while tubes 3-4 are the negative controls.

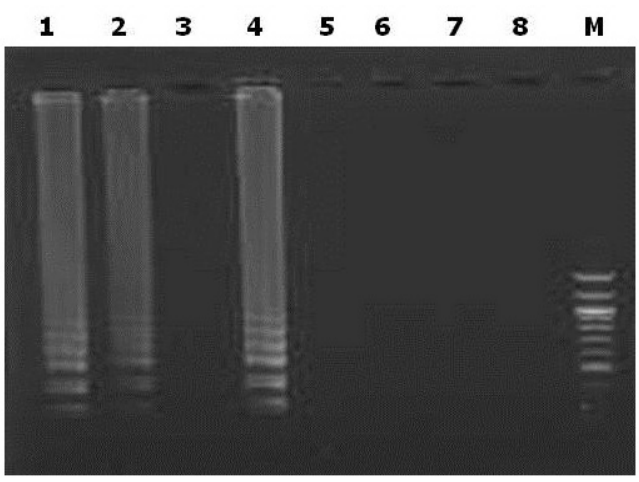

Figure 2. Specificity of the LAMP assay targeting the *dnaJ* gene of *V. harveyi*. Lanes 1-2: *V. harveyi* from Asian seabass, Lane 3: *V. parahaemolyticus*, Lane 4: *V. harveyi* from shrimp, Lane 5: *V. anguillarum*, Lane 6: *Aeromonas salmonicida*, Lane 7: *Yersinia ruckeri*, Lane 8: negative control (distilled water), Lane M: 100-bp DNA marker. This is a representative of three independent LAMP assays.

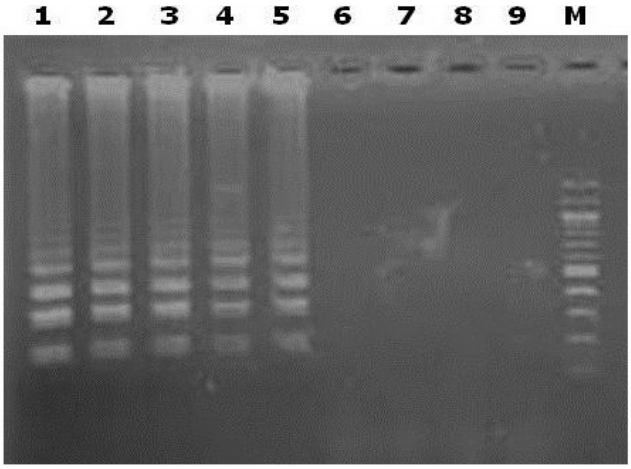

Figure 3. Sensitivity of the LAMP assay to detect *V. harveyi* isolated from Asian sea bass. Lane 1: 0.2 µg DNA mL^{-1}, Lane 2: 20 ng DNA mL^{-1}, Lane 3: 2 ng DNA mL^{-1}, Lane 4: 200 pg mL^{-1}, Lane 5: 20 pg DNA mL^{-1}, Lane 6: 2 pg DNA mL^{-1}, Lane 7: 200 fg DNA mL^{-1}, Lane 8: 20 fg DNA mL^{-1}, Lane 9: negative control (distilled water), Lane M: 100-bp DNA marker. This is a representative of three independent LAMP assays.

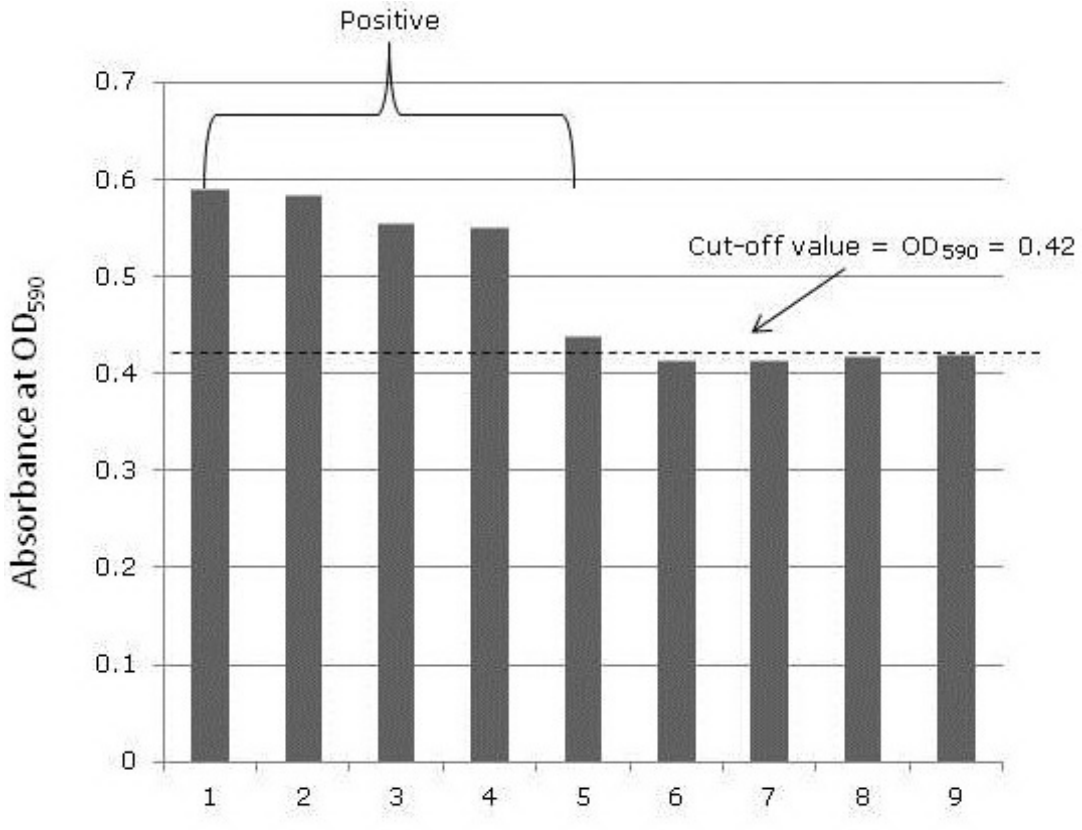

Figure 4. Absorbance of the LAMP products at OD590. 1: 0.2 µg DNA mL^{-1}, 2: 20 ng DNA mL^{-1}, 3: 2 ng DNA mL^{-1}, 4: 200 pg mL^{-1}, 5: 20 pg DNA mL^{-1}, 6: 2 pg DNA mL^{-1}, 7: 200 fg DNA mL^{-1}, 8: 20 fg DNA mL^{-1}, 9: negative control (distilled water). A dashed horizontal line represents the cut-off value that was calculated based on the absorbance reading obtained in the negative control. Values above the cut-off line are considered positive, while the samples are negative if they are below it. Samples were done in triplicate.

that can be detected by the LAMP assay, then the sensitivity of this assay in detecting *V. harveyi* is approximately 7-8 genome equivalents (GE) per LAMP reaction. A conventional PCR that was previously developed for this bacterium showed that it can detect 18-20 GEs (Caipang *et al* 2011a).

Aside from detecting the pathogen visually through the use of dyes such as SYBR Safe, hydroxynaphthol blue (HNB) and ethidium bromide, the LAMP products were quantified spectrophotometrically at an absorbance of OD590. In an earlier study, turbidimetric analysis of LAMP products using a spectrophotometer was done to quantify the viral load of red seabream iridovirus (RSIV) in fish (Caipang *et al* 2004). Results showed that there was a high degree of correlation between the turbidimetric reading and the amount of viral DNA, indicating the potential of this assay to quantify viral load in an infected host. Improvements in the spectrophotometric quantification of LAMP products using dyes also showed good correlation between the reading and the amount of the pathogen (Goto *et al* 2010; Ma *et al* 2010; Nie *et al* 2011). In these studies, HNB was added in the reaction to enhance the reading and to visualize the presence or absence of the pathogen through a change in the color of the reaction (Ma *et al* 2010).

In this study, it was observed that upon the addition of HNB after LAMP reaction, the samples that were negative for the pathogen had a violet coloration, while the samples that were positive for the pathogen turned sky blue. When these samples, were quantified using a spectrophotometer, the threshold of detection was at 10-5 dilution, which was also observed visually (Fig. 4). The cut-off for detection was 0.42. It was also observed that higher OD values corresponded with more amounts of the genomic DNA of the pathogen, thus the present assay can potentially quantify the bacterial load in the host.

The results of our LAMP assay for the detection of *V. harveyi* in Asian seabass reinforces the notion that this assay provides a specific and highly sensitive method for diagnosing the disease during the early stages of infection. A previous study on the LAMP method for the detection of *V. harveyi* in marine shellfish used the *toxR* as the gene candidate (Cao *et al* 2010). In the present work, we used the *dnaJ* gene to generate LAMP primers to detect *V. harveyi* because this gene can differentiate closely related *Vibrio* species (Nhung *et al* 2007), thus a high degree of species identity and specificity is attained. In the Philippines, *V. harveyi* is known to infect shrimps (de la Peña *et al* 2005) as well as it causes vibriosis in seahorse (Tendencia 2004) and seabass (Tendencia 2002). The LAMP assay that we have developed for the detection of *V. harveyi* in seabass could be used to detect this pathogen in other aquatic species. Thus, additional tests are needed to valídate the assay to diagnose vibriosis due to *V. harveyi* among these species of aquatic organisms.

Conclusions

We developed a LAMP assay for the detection of *V. harveyi* in Asian seabass, *L. calcarifer* using *dnaJ* as the target gene. The presence or absence of the pathogen can be detected visually upon the addition of dye or through gel electrophoresis. The assay was highly specific. It was also able to detect the

pathogen in infected shrimp. It was ten times more sensitive than conventional PCR in detecting the pathogen. The LAMP products can be quantified spectrophotometrically and showed correlation with the amount of the pathogen in infected samples.

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