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# Loop-mediated isothermal amplification of the *toxR* gene coupled with lateral flow dipstick (LAMP-LFD) for the novel, rapid and specific visual detection of *Vibrio harveyi*

**Ahmad Mukhlis Abdul Rahman[1,2], Julian Ransangan[3] and Vijay Kumar Subbiah[1]***

[1]Biotechnology Research Institute, Universiti Malaysia Sabah, Kota Kinabalu 88400, Sabah, Malaysia.
[2]Faculty of Chemical Engineering & Technology, Uniciti Alam Campus, Universiti Malaysia Perlis, Sg. Chuchuh, Padang Besar 02100, Perlis, Malaysia.
[3]Borneo Marine Research Institute, Universiti Malaysia Sabah, Kota Kinabalu 88400, Sabah, Malaysia.
Email: vijay@ums.edu.my

## ABSTRACT

**Aims:** *Vibrio harveyi* is a serious pathogen for marine organisms particularly in hatcheries and grow-out ponds that attack their immune system. The rapid detection of *V. harveyi* is urgently needed to prevent bacterial spread. Here we described a rapid and specific visual detection method based on the Loop-Mediated Isothermal Amplification in combination with Lateral Flow Dipstick (LAMP-LFD).
**Methodology and results:** A set of six novel primers were designed to target the *toxR* gene. These include the biotin-labelled inner primer that complements specifically to the target sequences. The resulting biotinylated LAMP amplicons were hybridised to the FAM-labelled probe resulting in lateral flow detection on the dipstick. The addition of loop primers improved the reaction time of LAMP by more than half and rapid detection was observed within 10-15 min. In comparison, the sensitivity of PCR-UV analysis was only at $10^4$ copies while LAMP-LFD was able to detect lower amounts at $10^3$ copies. The LFD provided higher specificity and selectivity since hybridization with specific probes to the LAMP amplicons was employed. In addition, detection of *V. harveyi* infected grouper was successful using the LAMP-LFD method described here.
**Conclusion, significance and impact of study:** LAMP-LFD is specific to *V. harveyi*. Our method provides a useful tool to rapidly detect and monitor the outbreaks of the pathogen.

*Keywords:* Lateral flow dipstick, loop-mediated isothermal amplification, rapid detection, *toxR* gene, *Vibrio harveyi*

## INTRODUCTION

*Vibrio harveyi* is a pathogenic marine bacterium associated with the mass mortalities and outbreak of the marine diseases including luminous vibriosis in hatcheries and grow-out ponds especially in warm water (Diggles *et al.*, 2000; Austin and Zhang, 2006; Zhang *et al.*, 2020). This bacterium has long been recognized as one of the important aquaculture pathogens responsible for debilitating diseases such as a chronic skin ulcer, gastroenteritis and deep dermal lesions for several marine organisms such as fish, mollusks and crustaceans (Kraxberger-Beatty *et al.*, 1990; Diggles *et al.*, 2000; Austin and Zhang, 2006; Shen *et al*., 2017; Mohamad *et al*., 2019a). The bacterium commonly infects its host via the processes of adhesion, invasion, reproduction and toxin release which disrupt the immune response and ultimately leading to the host's death (Deng *et al.*, 2020). The outbreak of the disease has been recognized as a significant constraint to aquaculture industry includes production and trade, affecting both the economic development and socio-economic revenue and causing severe economic losses in many countries in the world (Walker and Subasinghe, 2000; Ma *et al*., 2020; Mohd Yazid *et al.*, 2021).

Rapid, selective and specific visual detection of *V. harveyi* remains a challenging effort for both scientists and breeders. Common non-PCR based detection methods of *V. harveyi* are usually achieved through the procedures of culturing the bacterium, followed by biochemical or immunological tests (Colwell and Chun, 2008; Montánchez *et al*., 2019; Abdelsalam *et al*., 2023). Although the methods are still useful for specific purposes such as to evaluate the host-pathogen response, it requires skilled manpower, dedicated laboratory space and, more importantly, is usually time-consuming (Pang *et al*., 2006; Cao *et al.*, 2010). With the advances of nucleic acid amplification techniques, polymerase chain reaction (PCR) has been widely used for the detection of *V. harveyi*. The most commonly used target region for conventional PCR-based identification of *V. harveyi* is 16S rRNA that requires additional procedure such as

***Corresponding author**


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DNA sequencing and *in silico* analysis (Wei *et al.*, 2019; Xie *et al*., 2020; Yuan *et al*., 2021). However, the discriminatory power of 16S rRNA-based identification for *Vibrio* species is poor due to high genome similarity (Gomez-Gil *et al*., 2004; Hernandez and Olmos, 2004).

Several species-specific taxonomic markers based on the unique target DNA region have been developed to detect *V. harveyi* by conventional PCR including *hly* (hemolysin) (Conejero and Hedreyda, 2004; Haldar *et al*., 2010) and *toxR* (virulence-associated) genes (Conejero and Hedreyda, 2003; Cao *et al.*, 2010). The use of species-specific primers coupled with conventional PCR will reduce the detection time since the DNA sequencing and the complex *in silico* analysis could be eliminated. Detection of *V. harveyi* also has been reported to be performed using a more rapid and sensitive approach compared to conventional PCR known as real-time PCR by manipulating specific unique target DNA regions. The examples of the target region that have been used for the detection of this bacterium by real-time PCR are *mreB* (rod shape-determining) (Mougin *et al*., 2020), *groEL* (protein which belongs to the chaperonin family) (Costa *et al.*, 2022) and 16S rRNA (small subunit ribosomal RNA) genes (Fukui and Sawabe, 2008). Although the PCR-based detection method is a very useful approach to detect *V. harveyi*, the method is still considered laborious, time-consuming and required skilled manpower making it not suitable to be used for point of care detection to get reliable information of the pathogen in the fields especially in the early stage for timely control measures.

Due to the significant constraints of the PCR approach especially for the onsite detection, isothermal amplification has become a powerful approach to overcome the problem. The isothermal amplification protocol eliminates temperature cycling requirements thereby simplifying and speeding up the diagnosis process (Oliveira *et al.*, 2021; Boonbanjing *et al.*, 2022). In 2000, a novel nucleic acid amplification method based on loop-mediated isothermal amplification (LAMP) was reported (Notomi *et al*., 2000). LAMP is a rapid, sensitive, specific and cost-effective approach that can be used for *in situ* testing of target organisms based on the specific target DNA region and it is the approach that is selected and used in this research. The reaction occurs under isothermal condition (60-65 °C) and yields large amount of amplicons in a short time (15-60 min) that can be observed as a ladder-like band on gel electrophoresis (Notomi *et al*., 2000; Nagamine *et al*., 2002; Wang *et al*., 2013). This method is performed using the *Bst* DNA Polymerase with strand displacement activity and a set of four specially-designed primers based on six distinct regions of the target DNA (Notomi *et al*., 2000; Wang *et al*., 2013).

Detection of LAMP products is another critical step in order to develop an accurate visualisation method of the isothermal amplicons. A number of detection methods have been developed to visualise the LAMP products including gel electrophoresis (LAMP-UV analysis), SYBR green dye (LAMP-SYBR green) and a lateral flow dipstick (LAMP-LFD) (Das *et al*., 2022). The visual inspection using SYBR green is performed through the observation of a colour change with the addition of SYBR green. For positive amplification, the colour of the reaction will change from orange to green (Soliman and Al-Matbouli, 2005; Parida *et al*., 2008). The use of SYBR green as a colorimetric visualisation approach reduces the detection time by eliminating the use of gel electrophoresis as well as the gel documentation system (Singh *et al*., 2020). However, visualisation of SYBR green dye intercalated LAMP products based on the colour changes can be subjective and misinterpreted especially when the concentration of amplicons is low (Yongkiettrakul *et al.*, 2020). As such, the use of more selective and easier readout techniques such as LFD is highly useful as an onsite rapid detection method.

Lateral-flow dipstick (LFD) can target DNA sequences rapidly based on nucleic acid hybridization and chromatography in membrane strips as a result for visualisation detection (Nimitphak *et al*., 2008; Blažková *et al*., 2009). It requires no electric devices except a simple heating apparatus. The result can be observed within 5-10 min after the LAMP reaction was completed. LAMP-LFD is based on the principle that biotin-labelled primers biotinylate an amplification product while fluorescein-5-isothiocyanate (FITC)-labelled probes or carboxyfluorescein (FAM)-labelled probes de-hybridize, to simultaneously double-label the product and allow their capture by anti-FITC or anti-FAM antibodies on the LFD. Usually, LFD with one control line and one test line have been used for diagnostic purposes, especially for the detection amplification products of LAMP (Jaroenram *et al*., 2009; Puthawibool *et al*., 2010; Arunrut *et al.*, 2011). In aquaculture, LAMP-LFD has been developed for the detection of bacteria that related to seafood-borne diseases such as *Vibrio parahaemolyticus* (Prompamorn *et al*., 2011) and *Vibrio vulnificus* (Suraslip *et al*., 2011). It has been applied for diagnosis of infectious disease related to marine animals, such as white spot syndrome virus (WSSV) (Jaroenram *et al*., 2009), Taura syndrome virus (TSV) (Kiatpathomchai *et al*., 2008) and *Macrobrachium rosenbergii* nodavirus (Puthawibool *et al*., 2010).

Detection of *V. harveyi* based on the LAMP method in combination with different visualisation of LAMP products procedures have been reported by several researchers with their specific targeted genes. Cao e*t al*. (2010) is a pioneer group of researchers to use LAMP-UV Analysis and LAMP-SYBR green to detect *V. harveyi* based on the *toxR* gene region using the two inner and outer primers in 2010 (Cao *et al.*, 2010). LAMP-UV Analysis and LAMP-hydroxynaphthol blue (HNB) based detection of *V. harveyi* also have been developed based on the other target regions of *dnaJ* gene to improve the number of *V. harveyi* strains to be detected Caipang *et al*. (2012). Detection of *V. harveyi* also has been developed based on the triplex LAMP in combination with UV analysis and SYBR green with the target region of *VhhP2* gene for multiple *Vibrio* species detection (Yu *et al*., 2013). All of the described LAMP-based detection methods had limitations, as the evaluation of the positive result is either

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time-consuming and laborious (gel electrophoresis) or prone to misinterpretation of results due to colour changes that are difficult to distinguish for low-concentration samples.

Due to this, Thongkao and colleagues reported the development of LAMP-LFD method based on the *VhhP2* to detect *V. harveyi* that utilise a more selective approach to detect biotinylated LAMP products and thus eliminate the use of gel electrophoresis (Thongkao *et al*., 2015). However, all of the reported LAMP-based detection methods of *V. harveyi* are time-consuming (more than 60 min) compared to the method that we described here and are less suitable to be used for the field test purposes. Previously, we have described the improved version of LAMP-SYBR green assay for the detection of *V. harveyi* by the addition of loop primers (Rahman *et al*., 2022). However, the use of SYBR green dye intercalated LAMP products based on the colour changes can be subjective and misinterpreted. As such, we attempted to improve the LAMP assay by incorporating biotin-labelled forward inner primer in the LAMP reaction to synthesise biotinylated LAMP products to make it suitable to be detected by streptavidin-based LFD. In addition, we designed a new FAM-labelled hybridization probe to be used in LFD for more selective and specific detection of *V. harveyi*. The modifications made here significantly improved the detection of *V. harveyi*, especially for point-of-care (POC) tests.

## MATERIALS AND METHODS

### Biological samples and extraction of genomic DNA

The target microorganism used in this study is *Vibrio harveyi* srain VHJR7 that was obtained from the Borneo Marine Research Institute, Universiti Malaysia Sabah (UMS), Malaysia. The bacterial strain was previously isolated during a vibriosis outbreak in February 2008 from an infected seabass, *Lates calcarifer* (Bloch), cultured in open net cages in Sabah (Ransangan and Mustafa, 2009). The strain had been previously identified by polymerase chain reaction (PCR), DNA sequencing and *in silico* analysis of 16S rRNA and the hemolysin gene for species confirmation. Genomic DNA of the *V. harveyi* strain VHJR7 was extracted using the I-Genomic DNA Extraction Kit (Intron Biotechnology, Gyeonggi-do, Republic of Korea), according to the manufacturer's instructions. The extracted genomic DNA was then stored at -20 °C until use.

### Synthesis of primers and probe for PCR, LAMP and LFD Assays

A previously validated primer set (Table 1) consisting of two inner primers (*Tox*R2-FIP and *Tox*R2-BIP), two outer primers (*Tox*R2-F3 and *Tox*R2-B3) and two loop primers (*Tox*R2-LF and *Tox*R-LB) that have been developed and reported by us in 2022 was used as a basis to develop and validate a new approach to detect *V. harveyi* by LAMP-LFD assay (Rahman *et al*., 2022). All the primers and probes used in this study were designed using the default parameters of Primer Explorer Version 4 at http://primerexplorer.jp/elamp4.0.0/index.html (accessed on 1 February 2020) (Figure 1). The modification was conducted to the forward inner primer (*Tox*R2-FIP) by labelling it with biotin at 5' end to synthesise biotinylated LAMP amplicons.

A newly synthesised hybridization probe labelled with FAM at the 5' end was introduced in this assay to improve the detection and visualisation of *V. harveyi* biotinylated LAMP amplicons. The DNA probe was designed between *Tox*R2-FIP and *Tox*R2-BIP regions (Figure 1). We ensured that the primers for the unique target sequence that complement to the FAM-labelled hybridization probe were conserved to target all 39 *V. harveyi* strains found in Genbank (Rahman *et al.*, 2022). We also conducted *in silico* verification to ensure that the primers including biotin-labelled primer were specific to *V. harveyi* and not to any other species under the same genera. The outer primers of *Tox*R2-F3 and *Tox*R2-B3 were used to synthesise the PCR amplicon, which was sequenced for validation and subsequently used to produce the template plasmid DNA as well as to compare the sensitivity with LAMP-SYBR green and LAMP-LFD assays.

**Table 1:** Information of the inner primers (*ToxR2*-FIP and *ToxR2*-BIP), outer primers (*ToxR2*-F3 and *ToxR2*-B3), loop primers (*ToxR2*-LF and *ToxR2*-LB), biotin-labelled forward inner primer (biotin-*Tox*R2-FIP) and FAM-labelled probe based on the *toxR* gene of *V. harveyi.*

| Name | Sequence (5'-3') | Length (bp) |
|---|---|---|
| *ToxR*2-FIP | ATGGTTGAGCTGTCGGTGCTTGTTTCAGAGCCCACTGCTGAGA | 43 |
| *ToxR*2-BIP | TCATCGTGTTAGTTGCCCTGCTTTTTTGTGATTCTGCAGGGTTGG | 45 |
| *ToxR*2-F3 | GAAGCTCCAGTCGTTGACTT | 20 |
| *ToxR*2-B3 | CATCACTGGCACGTTGTGA | 19 |
| *ToxR*2-LF | TCGACGGCTGTTTCTGCTTTT | 21 |
| *ToxR*2-LB | TCCTGTTGGCGTGTTAATGCT | 21 |
| Biotin-*ToxR*2-FIP | Biotin-ATGGTTGAGCTGTCGGTGCTTGTTTCAGAGCCCACTGCTGAGA | 43 |
| FAM-Probe | FAM_GAACACAAACTGGTTATTCAG | 21 |

The primers were designed using the default parameters of Primer Explorer Version 4 at http://primerexplorer.jp/elamp4.0.0/index.html (Rahman *et al*., 2022).

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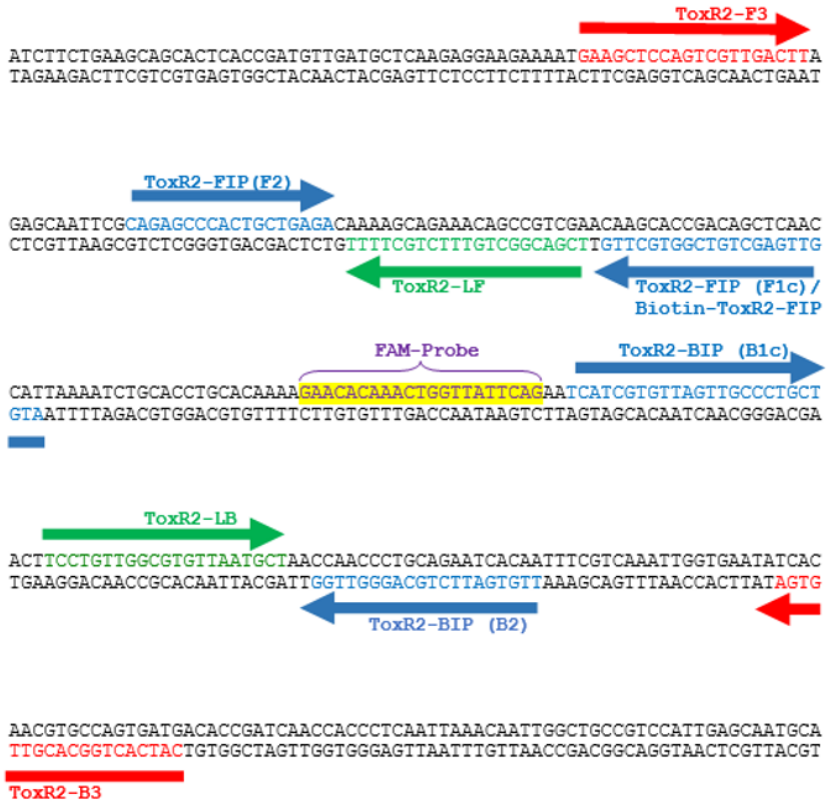


**Figure 1:** The flanking region of the FAM-labelled DNA probes, FIP primer labelled with biotin and other components required for biotin-labelled LAMP reaction, LAMP-UV analysis, LAMP-SYBR green and PCR-UV analysis.

**Preparation of DNA template by conventional PCR**

Conventional PCR was performed with the outer primer pair of *ToxR2*-F3 and *ToxR2*-B3 to amplify the target sites of *V. harveyi* strain VHJR7. The protocol of PCR was performed as described previously, with some modifications with an expected PCR amplicon of 245 bp (Rahman *et al*., 2022). The PCR mixture contained 1× PCR buffer, 0.2 mM of dNTP mix (Promega, USA), 10 pmol each *Tox*R2-F3 and *Tox*R2-B3 primers, 1.5 mM $MgCl_2$ and 1.25 U of *Taq* DNA polymerase (Promega, USA) and sterile deionized water to a final volume of 25 µL. The reaction mixture was subjected to amplification using thermal cycler machine with the following conditions: Initial denaturation time at 95 °C for 3 min, followed by 40 cycles of amplification at 95 °C for 30 sec, 56 °C for 30 sec and 72 °C for 30 sec, with a final extension step at 72 °C for 5 min. The PCR product was then purified using a PCR purification Kit (Qiagen, Germantown, MD, USA). The PCR products were visualised after electrophoresis in 1.5% agarose gels.

**Construction of recombinant plasmid for PCR, LAMP and LFD Assays**

The target sequences for PCR and LAMP reactions were constructed by ligating the synthesised PCR amplicons into the plasmid pGEM-T easy vector (Promega, Madison, WI, USA) according to the manufacturer's protocol. The recombinant plasmid was transformed into 100 µL of *E. coli* strain JM109. The colonies were screened based on the blue-white colonies selection. Positive colonies indicated by a white colour while negative colonies grew with blue colour. The positive colonies were selected and cultured for 16 h in a LB broth medium containing 100 µg/mL of ampicillin. Next, the extraction of plasmid was performed using the PureYield™ Plasmid Miniprep System (Promega, Madison, WI, USA) according to the manufacturer's protocol. The extracted plasmid was then analysed by 1.5% agarose gel electrophoresis and purified for subsequent downstream processes.

**Optimization of reaction time and concentration of FAM-labelled probe for LAMP and LFD**

The LAMP reactions were performed in a total volume of 25 µL reaction mixture containing 5 µL of DNA sample, 1.4 mM deoxyribonucleotide triphosphate (dNTPs), 0.8 M betaine (Sigma-Aldrich, USA), 20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 0.1% Tween-20, 8 U *Bst* DNA Polymerase (NEB), 32 pmol each of the inner primers *Tox*R2-FIP and *Tox*R2-BIP, 8 pmol each of the outer primers *Tox*R2-F3 and *Tox*R2-B3 and 32 pmol of

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the loop primers *Tox*R2-LB and *Tox*R2-LF. The LAMP reaction was performed on a heating block at 65 °C. The reaction was performed at 30, 40, 50 and 60 min for the reaction mixture without loop primers and at 5, 10, 15 and 20 min for the reaction mixture with loop primers. LAMP reactions with and without loop primers were conducted to validate the optimal reaction time. The LAMP amplification products were firstly analysed by 2% agarose gel electrophoresis.

Biotin-labelled LAMP reaction was carried along with normal LAMP. Biotin-labelled LAMP was performed by adding FIP inner primer labelled with biotin at 5' end (Table 1). Other primers and components were added according to the optimised protocol of the LAMP reactions (Rahman *et al*., 2022). After the biotin-labelled LAMP reaction was completed, approximately 5 µL of FAM-labelled DNA probe was added to the biotin-labelled LAMP products with three different concentrations (2 pmol, 20 pmol and 200 pmol) as described by Nimitphak *et al*. (2008) and Kiatpathomchai *et al.* (2008) with slight modification. Then, the mixture of FAM-labelled probes with biotin-labelled LAMP products was hybridised at 63 °C for 5 min. Subsequently, approximately 10 µL of the hybridization product was added to 120 µL of assay buffer in a new tube. Next, the LFD strip was dipped into the tube with the reaction mixture and the result usually appears within 3-5 min. The best concentration of FAM-labelled DNA probes was then determined based on the strongest red colour signal on the test line of LFD.

**Visualisation of *V. harveyi* LAMP amplification products by LAMP-UV analysis, LAMP-SYBR green and LAMP-LFD**

The reaction mixture for the LAMP-UV analysis was prepared based on the optimum protocol as described earlier. The analysis of LAMP-UV was performed at 65 °C for 15 min. The resulting amplicon of LAMP reaction was electrophoresed using a 2% agarose gel, stained with ethidium bromide (0.5 µg/mL) and visualised under UV light. For LAMP-SYBR green assay, the initial LAMP reaction was conducted using the optimised protocol at 65 °C for 15 min. Approximately, 2 µL of 1:10 diluted SYBR green I nucleic acid gel stain was added directly to all LAMP reaction tubes and any change of colour was visually observed.

The newly developed LAMP-LFD assay for detection of *V. harveyi* was performed by conducting the biotin-labelled LAMP reaction as described earlier above. After the biotin-labelled LAMP reaction completed, 5 µL of FAM-labelled DNA probe (optimum concentration) was added to the biotin-labelled LAMP products. The mixture of FAM-labelled probe with biotin-labelled LAMP products was left to hybridise at 63 °C for 5 min. Next, approximately 10 µL of the hybridization products was added to 120 µL of assay buffer in a new tube. Finally, the LFD strip was dipped into the tube with the reaction mixture and the result appeared approximately within 3-5 min.

**Specificity test for the detection of *V. harveyi* LAMP assays in combination with UV analysis, SYBR green and LFD**

The reference strains of the *Vibrio* and non-*Vibrio* bacterial samples were included in a specificity test of the LAMP assays in combination with UV analysis, SYBR green and LFD to ensure that the developed procedure was specific to detect *V. harveyi*. These strains were *Vibrio parahaemolyticus* ATCC 17802, *Vibrio alginolyticus* ATCC 17749, *Vibrio aguillarum* ATCC 19264 and *Aeromonas salmonicida* subsp. *salmonicida* ATCC strain 33658. DNA templates were isolated from the bacterial cultures as described above for *V. harveyi*, according to the manufacturer's procedures. The LAMP-UV analysis, LAMP-SYBR green and LAMP-LFD assays were carried out at 65 °C for 15 min followed by their respective procedure to visualise the amplification products.

**Sensitivity test for the detection of *V. harveyi* using PCR-UV analysis, LAMP-UV analysis, LAMP-SYBR green and LAMP-LFD**

The sensitivity evaluation of the LAMP-LFD in comparison with LAMP-UV-analysis, LAMP-SYBR green and conventional PCR was performed using the same set of 10-fold serial dilution of the *V. harveyi* recombinant plasmid DNA samples ($10^7$ copies to 5 copies). The amplification products were then visualised in three ways; UV analysis (PCR and LAMP-UV Analysis), SYBR green dye (LAMP-SYBR green) and LFD (LAMP-LFD). The LAMP reaction was performed for all *V. harveyi* recombinant plasmid DNA samples at 65 °C for 15 min.

The resulting amplicons of the LAMP and PCR reactions which were stained with ethidium bromide were analysed on 2% agarose gel electrophoresis with the aid of a gel-documentation system. For visualisation using SYBR green dye, approximately 2 µL of 1:10 diluted SYBR green I nucleic acid gel stain was added directly to all reaction tubes containing LAMP product amplicons, and any changes of colour were visually observed. The optimised protocol of visualisation of biotinylated LAMP products by LFD was performed as described earlier. The result of LAMP-LFD was evaluated based on the formation of a test and control line on the dipstick.

**Evaluation of LAMP-LFD assay with grouper infected with *V. harveyi***

Cell suspensions of *V. harveyi* in PBS were prepared prior to inoculation into healthy tiger groupers (*Epinephelus fuscoguttatus*) for field evaluation of the newly developed detection method by LAMP-LFD. The glycerol stock of *V. harveyi* was cultured in tryptic soy agar (TSA) supplemented with 2% NaCl at 30 °C overnight. Next, approximately 1-2 mL of the cultured bacteria cells was transferred into a 2 mL microcentrifuge tube. Then, the tube was centrifuged for 1 min at 10,000× *g* and the supernatant were discarded. To wash the cell,

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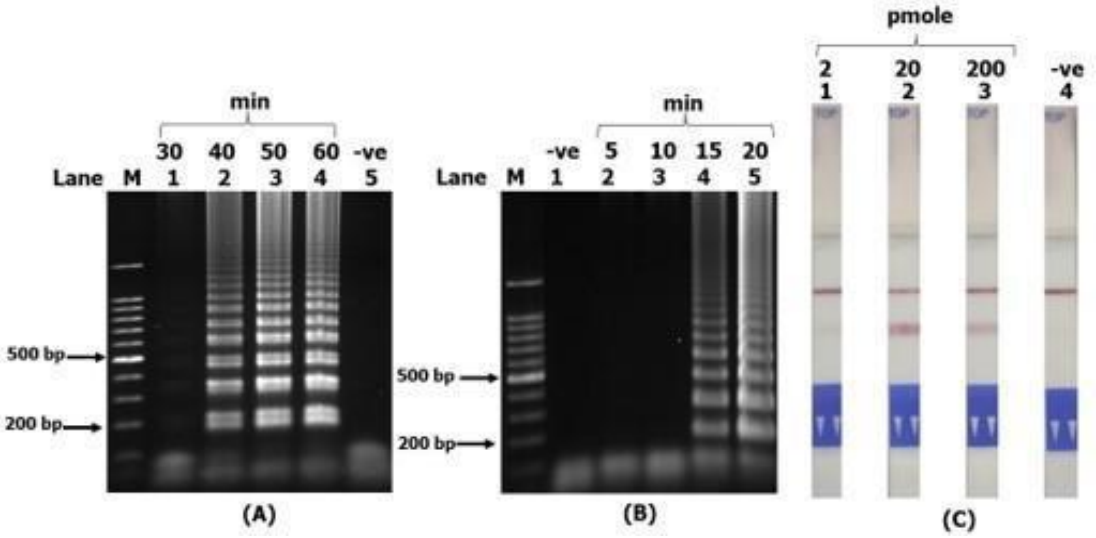


**Figure 2:** LAMP reactions with different reaction temperatures and different concentrations of FAM-labelled hybridization probe for LFD assay. (A) Reaction mixture without loop primers, Lane M: 100 bp DNA ladder, Lane 5: Negative control (distilled water); the reaction time of LAMP is indicated on the top of each lane. (B) Reaction mixture with loop primers, Lane M: 100 bp DNA ladder, Lane 1: Negative control (distilled water); the reaction time of LAMP is indicated on the top of each lane. All products were analysed by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. (C) Visual appearance of LAMP coupled with LFD with different concentration of FAM-labelled hybridization probe, dipstick 4: Negative control (distilled water); the concentration of FAM-labelled hybridization probe is indicated on the top of each dipstick.

the pellet was resuspended in PBS, centrifuged for 1 min at 10,000× *g* and the supernatant were discarded. This step was repeated three times. Finally, the pellet was resuspended with approximately 1 mL of PBS.

Three groupers were injected intraperitoneally with $10^4$ CFU/g of the *V. harveyi* PBS cell suspension. After the mortalities among the test group, we performed rapid detection using LAMP-LFD. A control group was included for comparison purposes. Fish from both the treated and control groups were dissected, and the internal tissues were used for the subsequent preparation of the DNA template using the rapid-boiling method (Arunrut *et al*., 2011). Briefly, 200 µL of 0.025 N sodium hydroxide (NaOH) and 0.0125% sodium dodecyl sulphate (SDS) were added to the tissues and allowed to boil for 5 min. Approximately 200 µL of the supernatant solution was used directly for the subsequent LAMP-LFD assay.

## RESULTS

### Optimal reaction time and concentration of FAM-labelled probe for LAMP and LFD

The primers and probe synthesised in this study were subjected to the LAMP and LFD reactions in order to evaluate the optimum reaction time and concentration of probe for rapid, sensitive, specific and selective detection of *V. harveyi*. This study confirmed that loop primers including a forward loop primer (*ToxR2*-LF) and a backward loop primer (*ToxR2*-LB) provide the best result with shorter reaction time for LAMP reaction. The results for LAMP reactions at different temperatures were electrophoresed, stained with ethidium bromide and visualised under UV light by gel documentation system (Figure 2A and 2B). The ladder-like band was indicative for positive results of the LAMP-UV analysis.

LAMP reaction with loop primers requires only 15 min to detect *V. harveyi* compared to the reaction without loop primer that requires longer time from 40 to 60 min to complete the reaction (Figure 2A and 2B). The primers bind to the loop region of the stem-loop DNA structure before initiating strand-displacement DNA synthesis. Loop primers were used in all subsequent procedures throughout this study including LAMP-SYBR green and LAMP-LFD due to the fact that the reaction time was reduced drastically compared with the LAMP reaction without loop primers.

The hybridization probe was designed based on the specific target sequence of *V. harveyi* and labelled with FAM. The FAM-labelled probe was directly added to the biotin-labelled LAMP products with three different concentrations (2 pmol, 20 pmol and 200 pmol) and all the mixtures were then successfully hybridised at 63 °C for 5 min. Positive test results consisted of red lines at both the test and control lines. On the other hand, the negative test result consisted of only one red line at the control line. The best concentration of hybridization probes labelled with FAM is at 20 pmol since it produced the strongest red colour signal on the test line of LFD (Figure 2C). Lowest intensity of red colour signal of the test line was produced by the reaction with 2 pmol of FAM-labelled hybridization probe.

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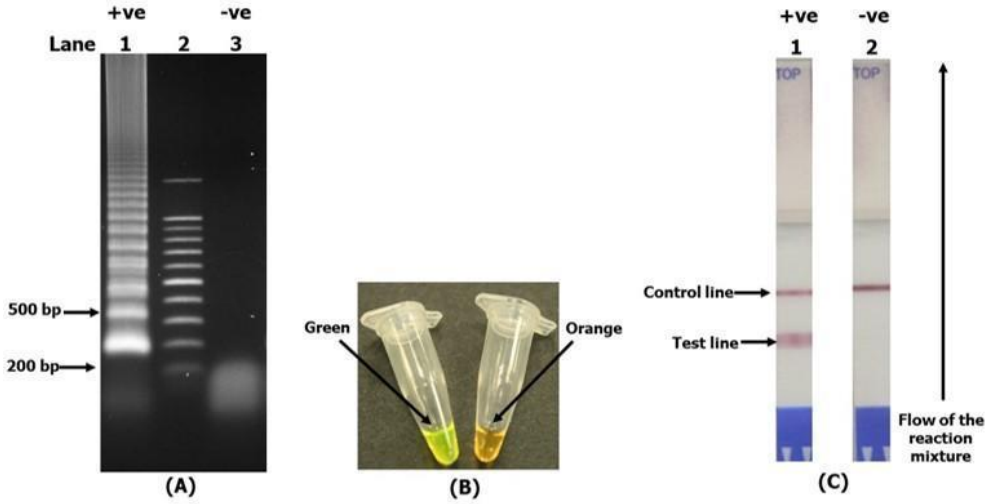


**Figure 3:** Visualisation of LAMP amplicons by UV analysis, SYBR green and LFD. (A) An optimised LAMP-UV analysis protocol was validated for subsequent downstream tests, Lane 1: *V. harveyi* (genomic DNA), Lane 2: 100 bp DNA ladder, Lane 3: Negative control (distilled water). (B) Visual appearance of LAMP coupled with SYBR green. Positive sample (green) and negative sample (orange). (C) Biotin labelled LAMP reaction and LFD. Positive test results consisted of red lines at both the test and control lines. On the other hand, the negative test result consisted of only one red line at the control line (as indicated on top of each dipstick).

**Visualisation of *V. harveyi* LAMP amplification products by LAMP-UV analysis, LAMP-SYBR green and LAMP-LFD**

Visualisation of *V. harveyi* LAMP amplification products based on three different approaches were performed using electrophoresis and UV analysis, intercalating dye of SYBR green and LFD. LAMP reaction was conducted with the optimal protocol at 65 °C for 15 min by heating block. For LAMP-UV analysis assay, a ladder-like band was obtained after the LAMP amplicons were electrophoresed, stained with ethidium bromide and visualised under UV light. The ladder-like band was indicative of a successful LAMP reaction while no ladder-like band was observed for negative control (Figure 3A).

For LAMP-SYBR green assay, approximately 2 µL of 1:10 diluted SYBR green I nucleic acid gel stain was added directly to all LAMP reaction tubes and any change of colour was visually observed. The colour changed from orange to green for positive reaction and remained orange for negative reaction (Figure 3B). LAMP-LFD is a novel newly developed assay that was used to detect the amplification products of LAMP reaction. The *Tox*R2-FIP primer was successfully labelled with biotin at the 5'-end. Other primers and reagent components were conducted using the optimised protocol of the LAMP reactions with the addition of loop primers. The biotin-labelled LAMP products were successfully prepared at 65 °C for 15 min. Hybridization of FAM-labelled probes with biotinylated LAMP amplicons was successfully performed with 20 pmol probe concentration. Positive results appeared with double red colour signals at control and test lines while negative control reported a single red colour signal at the control line (Figure 3C).

**Specificity test of LAMP-UV Analysis, LAMP-SYBR green and LAMP-LFD assays**

Based on the established LAMP-LFD protocol, four reference species were used including *Vibrio parahaemolyticus*, *V. alginolyticus*, *V. aguillarium* and *Aeromonas salmonicida* sub sp. *salmonicida* as negative control to evaluate the specificity of the developed assay. We observed positive test results of ladder-like bands with only the *V. harveyi toxR* gene (Figure 4A). All of the other species showed negative test results. LAMP-SYBR green also showed consistent results with only the *V. harveyi* sample produced green colour at the end of the reaction (Figure 4B). All of the LAMP-LFD assay results agreed with those of the LAMP-UV Analysis test, which were visualised by LFD control and test colour lines (Figure 4C). The result indicated that LAMP-LFD assay is specific to the *V. harveyi tox-R* gene only due to the fact that no false-positive result occurred in the specificity test.

**Sensitivity test of PCR-UV analysis, LAMP-UV analysis, LAMP-SYBR green and LAMP-LFD for detection *V. harveyi***

The sensitivity of the LAMP-based assay was compared with conventional PCR using a set of *V. harveyi* recombinant plasmid samples with different concentrations from $10^7$ to 5 copies. The conventional PCR reaction was performed using *ToxR2*-F3 and *ToxR2*-B3 primers. Both primers acted as the outer forward and reverse primers that bound at specific flanking regions, yielding a PCR amplicon of approximately 245 bp. The detection limit of the various methods was determined from the lowest amount of *V.*

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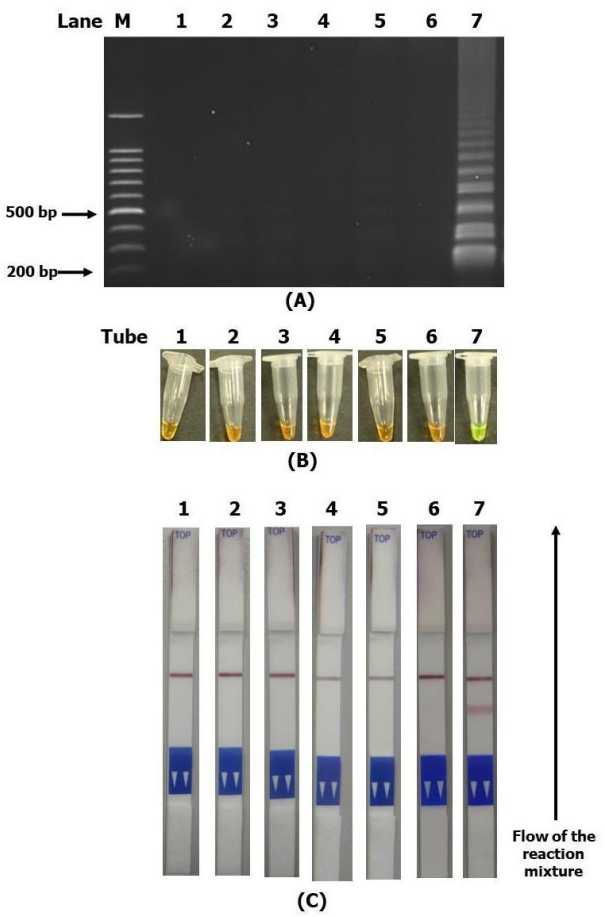


**Figure 4:** Comparative results of molecular specificity for the LAMP-LFD assay for the detection of *V. harveyi* based on the *Tox*R gene. (A) LAMP amplicons visualised with gel electrophoresis in a 1.5% agarose gel and stained with ethidium bromide; (B) The corresponding LAMP-SYBR green assay; (C) LAMP-LFD assay using the same reference species for specificity test. Lane M: 100 bp DNA ladder, Lanes 1-2: Negative controls (distilled water), Lane 3: *Aeromonas salmonicides* subsp. *salmonicida* ATCC strain 33658, Lane 4: *V. aguillarium* ATCC 19264, Lane 5: *V. alginolyticus* ATCC 17749, Lane 6: *V. parahaemolyticus* ATCC 17802, Lane 7: *V. harveyi* strain VHJR 7.

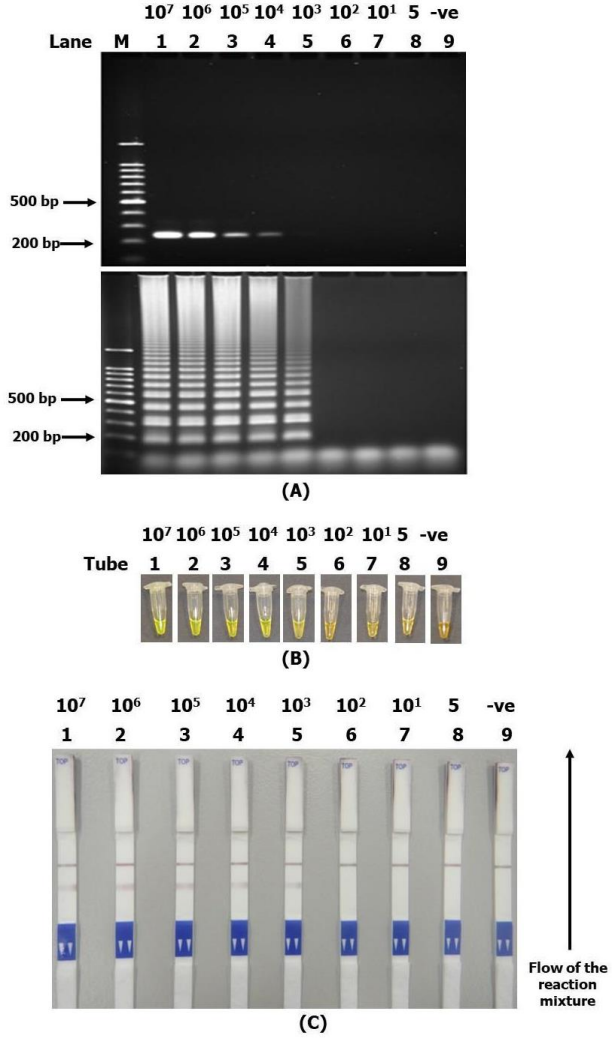


**Figure 5:** Comparative results of molecular sensitivity for PCR, LAMP-UV, LAMP-SYBR green and LAMP-LFD using different concentrations of recombinant plasmid DNA of *V. harveyi* from $10^7$ to 5 copies. PCR (A, top), LAMP-UV analysis (A, bottom), LAMP-SYBR green (B) and LAMP-LFD (C). The specific concentrations of samples are indicated on the top of each lane, tube and dipstick. Lane M: 100 bp DNA ladder; Lane 10: Negative control (distilled water).

*harveyi* recombinant plasmid samples that can be detected. The detection limit for conventional PCR was at $10^4$ copies of *V. harveyi* recombinant plasmid samples (Figure 5A, top).

Based on the sensitivity test, LAMP-UV analysis (Figure 5A, bottom), LAMP-SYBR green (Figure 5B) and LAMP-LFD (Figure 5C) are greater in sensitivity than those obtained using conventional PCR reactions. The sensitivity test of the LAMP-UV analysis, LAMP-SYBR green and LAMP-LFD assays shows that the method is able to detect even as low as $10^3$ of *V. harveyi* recombinant plasmid samples.

### *In vivo* evaluation of infected grouper using LAMP-LFD assay

Groupers were infected with a cell suspension of the *V. harveyi* strain VHJR7 and evaluated whether the newly introduced biotin-labelled *Tox*R2-FIP primer, FAM-labelled hybridization probe for the LAMP-LFD assay

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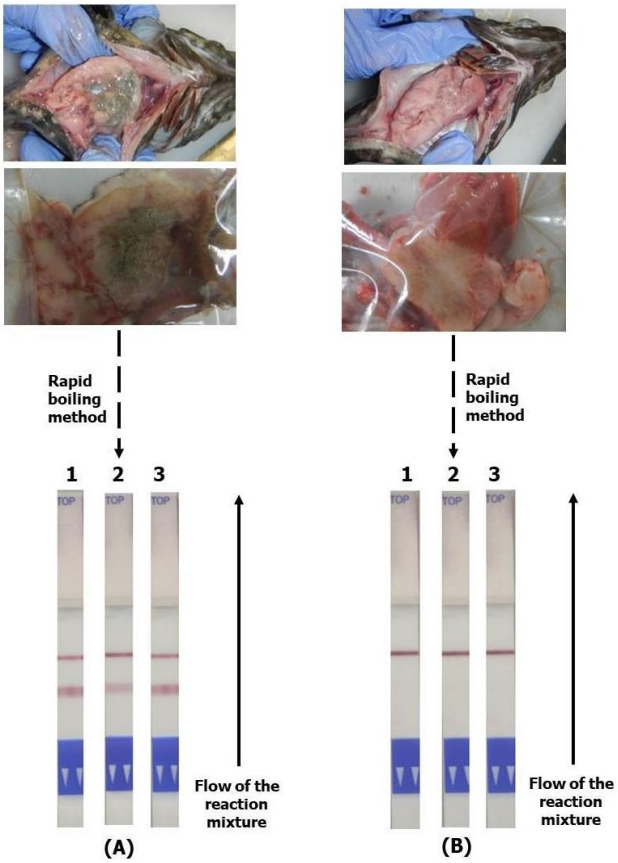


**Figure 6:** Field test evaluation of the newly developed novel LAMP-LFD method on the grouper infected with *V. harveyi*. (A) Sample of fish infected by *V. harveyi*; (B) Sample control of non-infected fish. Tissue samples were collected after 48 hrs of treatment and the DNA template was prepared using the rapid-boiling method. The corresponding LAMP-LFD shows positive results of the *V. harveyi* infection for the infected fish sample (A), but negative for the control group sample (B).

could detect the presence of the pathogen in the fish. The rapid-boiling method was used to reduce the reaction time for DNA-isolation procedure. This took an additional 5 min on top of the 15 min required for the main step of LAMP amplification. After the biotinylated-LAMP amplicons were synthesised, approximately additional 7 min was required for probe hybridization and LFD procedure. The results indicate that the rapid-boiling method coupled with the newly developed LAMP-LFD assay was able to rapidly detect the presence of *V. harveyi* (Figure 6A). Additionally, we could not detect the presence of *V. harveyi* in the control of uninfected fish samples (Figure 6B).

## DISCUSSION

While the aquaculture industry includes tiger shrimp, groupers and Asian seabass is gearing towards expansion, the increase of vibriosis incidences caused by *V. harveyi s*eems to be parallel to aquaculture industry development worldwide (Mohamad *et al*., 2019b; Xie *et al*., 2020; Zhang *et al*., 2020). Developing rapid, selective, sensitive and specific diagnostic methods suitable for field testing is essential for early screening, prevention, control and treatment of *V. harveyi* infection. In 2022, we reported a LAMP-SYBR green protocol for *V. harveyi* detection that consisted of LAMP reaction by six primers followed by the addition of intercalating dye of SYBR green (Rahman *et al*., 2022). In this study, we improved upon more selective and specific visual rapid detection of *V. harveyi* by LAMP-LFD assay. This is the first report on the improved rapid detection of *V. havevi* using LAMP reaction with the addition of biotin-labelled inner primer, loop primers, FAM-labelled hybridization probe coupled with LFD as a single detection method. The detection method of *V. harveyi* bacteria based on the LAMP-LFD that we have developed here only takes less than half an hour which is much faster than that reported in 2015 by Thongkao *et al.* (2015) which required around 90 min.

In developing LAMP-LFD assay, the target region should be given considerable attention in order to introduce suitable biotin-labelled inner primers as well as to design specific and selective FAM-labelled hybridization probes for significant output. We decided to design the FAM-labelled hybridization probe (FAM-probe) and biotin-labelled forward inner primer (biotin-*ToxR*2-FIP) based on the partial sequence of the *toxR* gene that have been reported by our research team in 2022 (Rahman *et al*., 2022). The target region was previously discovered based on the re-examination of the *toxR* gene sequences available at GenBank (Figure 1). The target region has several advantages including being suitable to the loop primers design process and able to capture as high as 39 strains of *V. harveyi* based on the sequences at GenBank compared to other target region such as *dnaJ* and *VhhP2* genes (Rahman *et al.*, 2022).

Four regular primers that was synthesised and used in this study are forward inner primer (*Tox*R2-FIP), backward inner primer (*Tox*R2-BIP), forward outer primer (*Tox*R2-F3) and backward outer primer (*Tox*R2-B3) with length of approximately 246 bp (Figure 1). The regular principle of LAMP reaction depends on the recognition of the six specific sequences on target DNA region by the four sets of primers, in the presence of DNA polymerase having high strand displacement activity (Garg *et al*., 2022; Park, 2022). The regular primers used for LAMP reaction in this study meet all the optimum condition of good LAMP primers based on the four critical key factors that need to be considered involving melting temperature (Tm), GC content, stability at the end of each primer and the formation of secondary structures (Primer Explorer V4, 2009). The regular LAMP primers information file was very important for development of loop primers using the same software (Primer Explorer V4, 2009; Rahman *et al*.,

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2022). Forward loop primer (*Tox*R2-LF) and backward loop primer (*Tox*R2-LB) were then designed based on the information file of the selected regular LAMP primers. If the reaction rate of the amplification increases, the loop primers were selected (Wang and Liu, 2015; Ali *et al*., 2022).

DNA synthesised from the loop primers was displaced by the extension from the 3′ end of the target DNA, thus generating structures which were not present in the conventional LAMP (Nagamine *et al*., 2002). The LAMP reaction without loop primers was able to detect *V. harveyi* around 40 to 60 min (Figure 2A). However, the LAMP reactions with loop primers required approximately 10-15 min only to detect *V. harveyi* (Figure 2B). Addition of loop primers increases the rate of DNA amplification in LAMP (Nagamine *et al*., 2002; Nam *et al.*, 2022). These primers essentially hybridise to the stem-loop structures except for the loops that had been hybridised by the inner primers, FIP or BIP (Nagamine *et al*., 2002). The LAMP reaction in this study is approximately 10-15 min which is more rapid compared to previous LAMP reaction without loop primers for detection of *V. harveyi* that took approximately 60 min (Cao *et al.*, 2010). In previous work, the addition of loop primers also resulting in reduction of the detection time to 20 min for infectious spleen and kidney necrosis virus (ISKNV) (Ding *et al*., 2010), 30 min for hematopoietic necrosis virus (IHHNV), 30 min for citrus bacterial cancer (CBC) (Rigano *et al*., 2010) and 30 min for *Mycobacterium tuberculosis* (Liang *et al*., 2023).

There are two main components for LAMP-LFD which includes biotin-labelled LAMP products and FAM-labelled DNA probes. The production of biotin-labelled LAMP products is achieved through biotin-labelled LAMP reaction conditions, in line with the approach used by others (Allgöwer *et al*., 2020; Sharma *et al*., 2021). The *Tox*R2-FIP primer was labelled with biotin at the 5' end. Other primers and reagents were added with the optimised protocol of the LAMP reactions. The biotin-labelled LAMP products were successfully obtained at 65 °C for 15 min. The biotin-labelled primer used in this study is essential to produce biotin-labelled LAMP products that react with streptavidin in the LFD. Together, both FAM-labelled DNA probe and the produced biotin-labelled LAMP product will migrate towards the test line during the assay and only the biotin-labelled LAMP product will be trapped by streptavidin at the test line, thus signalling the positive test result. Immobilised streptavidin can be used to retrieve and characterise moieties of interest. Biotin-streptavidin interaction as one of the strongest non-covalent interactions has some unique characteristics that make it a very good 'bridge system' in many applications including nucleic acid hybridization assays (Chivers *et al.*, 2011; Mukama *et al*., 2020). The same principle of biotin and streptavidin molecules interaction concept was also applied in other LAMP-LFD assays such as for the detection of classical swine fever virus (Chowdry *et al.*, 2014), human enterovirus coxsackievirus (Yan *et al*., 2015) and *Staphylococcus aureus* (Nawattanapaiboon *et al*., 2016).

Different concentrations of FAM-labelled DNA probes were tested to evaluate the optimal concentration for LFD assay. The best concentration of hybridization probes labelled with FAM is at 20 pmol (Figure 2C). Most of the previous LAMP-LFD works for detection of specific organisms such *Vibrio parahaemolyticus* (Prompamorn *et al*., 2011), *Vibrio vulnificus* (Suraslip *et al*., 2011), shrimp yellow head virus (Khunthong *et al.*, 2013), shrimp hepatopancreatic parvovirus (Nimitphak *et al*., 2008) and HPV (Kumvongpin *et al*., 2017) also used approximately 20 pmol of the hybridization probe as an optimum concentration. During the assay, the gold-labelled 1° Ab binds with both FAM-labelled DNA probes duplexed with biotin-labelled LAMP product and free FAM-labelled DNA probes and migrates to control and test lines. As they reach the control line, there are 2° Ab that will trap the gold-labelled 1° Ab attached to a free FAM-labelled DNA probe and the reaction produces the reddish colour of control line (Chowdry *et al*., 2014; Wong *et al*., 2018).

Three different visualisations of LAMP amplicons were established and compared in this study included LAMP-UV analysis, LAMP-SYBR green and LAMP-LFD (Figure 3). The assays were successfully conducted using the optimum protocol as described in the methodology section. The ladder-like bands were generated for positive LAMP-UV reaction due to the final products that contain complex dumb-bell like DNA structures, stem-loop DNA structures with various lengths and cauliflower-like DNA structures (Gill *et al*., 2011) (Figure 3A). For LAMP-SYBR green, the colour changed from orange to green for positive reaction and remained orange for negative reaction resulting in easier detection by visual inspection, eliminating the need for gel electrophoresis and greatly reducing the time taken for result analysis (Figure 3B). LAMP-LFD assay provides easier and straightforward result interpretation based on the control and test lines (Figure 3C). Although both LAMP-SYBR green and LAMP-LFD provide visual results, the specificity and selectivity of the LAMP-LFD method can be increased compared to LAMP-SYBR green and LAMP-UV analysis since the hybridization with specific probe to LAMP amplicons is employed (Mamba *et al*., 2018).

LAMP is theoretically a method that barely produces non-specific products. When the appropriate starting material for LAMP cycling is produced, DNA synthesis ensued quickly because the products included sequences to be recognized by the loop primers. If there is even one amplicon that is not recognized, positive amplification cannot be achieved in the specific period of time. Therefore, it is particularly difficult to produce non-specific products when using loop primers. Based on the molecular specificity test, all the LAMP-based assays correctly managed to detect only the *V. harveyi toxR* gene and none of the controls, i.e., three *Vibrio* species (*V. aguillarium, V. alginolyticus* and *V. parahaemolyticus*) and one non-*Vibrio* (*Aeromonas salmonicides* subsp. *salmonicida*) strain (Figure 4). The number of controls could be increased in future for further confirmation since we only tested against limited numbers of species

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available during the development process. LAMP-LFD is very specific as the probe targets a specific complementary sequence within LAMP products, in contrast to nonspecific binding of double stranded DNA by intercalating dye of the LAMP-SYBR green.

Njiru *et al.* (2011) reported that the use of nonspecific DNA intercalating dye may produce false positives when partially processed templates are used (Njiru *et al*., 2011). SYBR green dye could not differentiate some false positive products and thus limiting its use as detection format as the intercalating dyes usually bind to any double stranded DNA including the primer dimers leading to erroneous results interpretation (Liu *et al*., 2008). Here, LAMP-LFD assay eradicated the toxicity of fluorescent dye and the possibility of false positives. The use of LAMP-LFD also offers a remarkable advantage in terms of the molecular sensitivity of the assay. The detection limit of PCR was only at $10^4$ copies while LAMP-LFD, LAMP-SYBR green and LAMP-UV analysis were able to detect low amounts even at $10^3$ copies (Figure 5). Although the sensitivity of LAMP-LFD, LAMP-SYBR green and LAMP-UV analysis are the same, there are several advantages of using LFD compared to SYBR green and UV analysis (Figure 5).

The combination of LFD and SYBR green eliminates the need for gel electrophoresis. When detecting low concentrations of DNA with SYBR green, distinguishing between positive and negative samples could be confusing, as the results may appear as either light green or orange. However, this problem can be prevented by utilising LFD, which relies on the specific probe hybridization concept. With LFD, a positive test result shows red lines at both the test and control lines, while a negative test result displays only one red line at the control line. In this study, LAMP-LFD, LAMP-SYBR green and LAMP-UV analysis were 10 times greater in sensitivity than that obtained using conventional PCR reaction. Others have reported that the sensitivity of both PCR and LAMP methods were the same for the detection of Koi Herpesvirus and pathogenic *Leptospira* (Soliman and El-Matbouli, 2005; Lin *et al*., 2009). Arunrut *et al.* also compared the application of LAMP-LFD with PCR reaction in studying a rapid detection of IHHNV and concluded that LAMP reaction is 10 times greater sensitivity than one-step PCR (Arunrut *et al*., 2011).

The newly developed LAMP-LFD assay was then subjected to *in vivo* test to detect the presence of *V. harveyi* in groupers infected with the pathogen. The rapid boiling method was successfully conducted to isolate DNA samples for the LAMP-LFD assay. The possibility of heating the samples (tissue) directly to obtain a template shortens the overall assay time. The application of the rapid boiling method also has been reported for preparation of the sample to perform LAMP-LFD for rapid detection of IHHNV (Arunrut *et al.*, 2011). A simple and rapid extraction method with high sensitivity and specificity detection method and the use of inexpensive equipment are among the key advantages of the LAMP-LFD method. Other studies have shown superior tolerance of LAMP tests for biological substances (Kaneko *et al*., 2007) and heat processed blood (Yamada *et al*., 2006) has been used successfully in detection of malaria (Poon *et al*., 2006). The LAMP-LFD in combination with the rapid boiling method showed positive results of the *Vibrio harveyi* infection for samples in the test group (Figure 6A), but negative for samples in the control group (Figure 6B).

Although the results for this *in vivo* test are very encouraging, there are limitations that can be improved in the future. We do take note of the limited types of different tissue and inoculation doses used in *in vivo* tests. A more-thorough screening will be needed especially for the efficacy of the protocol and its constraints in field trials for further confirmation. In this study, the novel LAMP-LFD assay based on the partial *toxR* gene is a simplest, more convenient, rapid, specific, selective, sensitive and particularly cost-effective alternative as the results can be visualised by naked eye that is more suitable and feasible in the field than previously published methods, i.e., conventional PCR, LAMP-UV analysis, LAMP-SYBR green as well as the LAMP-LFD based on the *vhhP2* gene assays. This method will provide a very useful tool to help aquaculturists and fish and prawn breeders to rapidly monitor the outbreaks of this pathogen in order to prevent mass mortality of fishes and prawns.

## CONCLUSION

The innovative use of loop primers in the LAMP-LFD method developed in this study significantly enhances the speed and accuracy of *V. harveyi* detection. This method is highly convenient, requiring less than 30 min with a simple heating device, and it does not necessitate expensive equipment or sophisticated technical personnel. These qualities make it particularly well-suited for low-equipment setting laboratories and on-site detection in the field. It can be readily adapted for the rapid surveillance and detection of *V. harveyi* in fish and other aquatic animals, particularly in developing countries where limited resources and the prevalence of this pathogen make such a detection method highly relevant. Since there are some limitations in this study, especially in the field test aspect, we thought that it would be best to put out the LAMP-LFD protocols for researchers to start testing them, as it would be impossible to obtain all strains of *V. harveyi* for screening. The assay will provide a useful tool for the rapid, selective and specific detection of the *V. harveyi* infection.

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