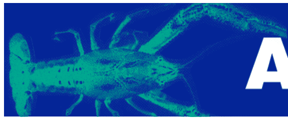
Aquaculture Research

Aquaculture Research, 2013, 1–10 doi: 10.1111/are.12266

Rapid and sensitive detection of *Vibrio harveyi* by loop-mediated isothermal amplification combined with lateral flow dipstick targeted to *vhhP2* gene

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Abstract

Vibrio harveyi is a causative agent of the Vibriosis or luminescent bacterial disease in worldwide aquaculture industry. A reliable assay for identification of *V. harveyi* infection is important to prevent the bacterial spread. In this study, biotinylated loop-mediated isothermal amplification (LAMP) amplicons were produced by a set of four designed primers that recognized specifically the *V. harveyi vhhP2* gene, encoding a putative outer membrane protein with unknown function, followed by hybridization with an fluorescein isothiocyanate (FITC)-labelled probe and lateral flow dipstick (LFD) detection. A novel set of PCR primer was also designed specifically to *vhhP2* gene and appear to be a species-specific tool for *V. harveyi* detection. The optimized time and temperature conditions for the LAMP assay were 90 min at 65°C. The LAMP-LFD and PCR methods accurately identified 22 isolates of *V. harveyi* but did not detect 16 non-*harveyi Vibrio* isolates, and 34 non-*Vibrio* bacterial isolates. The sensitivity of LAMP-LFD for *V. harveyi* detection in pure culture was 1.1×10^2 CFU mL^{-1} or equivalent to 0.6 CFU per reaction, while that of PCR was 6 CFU per reaction. For spiked shrimp sample, the sensitivity of LAMP was 1.8×10^3 CFU g^{-1} or equivalent to 5 CFU per reaction, while that of PCR was 50 CFU per reaction. In conclusion, the established LAMP-LFD methods provided a valuable tool for rapid identification of *V. harveyi* and can be used to distinguish *V. harveyi* from *V. campbellii*.

Keywords: *vhhP2* gene, *Vibrio harveyi*, loop-mediated isothermal amplification (LAMP), PCR

Introduction

Vibrio harveyi is a Gram-negative halophilic bacteria widely distributed in marine environments throughout the world. This bacteria is a major cause of Vibriosis, a fatal disease in penaeid shrimp. There are several reports about massive levels of mortality among penaeid shrimp in both hatchery and grow-out pond caused by Vibriosis which leads to severe economic losses in aquaculture industries worldwide (Liu, Lee & Chen 1996; Alvarez, Austin, Alvarez & Reyez 1998; Lavilla-Pitogo, Leaño & Paner 1998). Therefore, the rapid method for identification and tracking of *V. harveyi* is required.

Traditional identification of *V. harveyi* is achieved through biochemical tests which are laborious, time consuming and several *Vibrio* species display similar biochemical characteristics that limit the identification of species (Davis, Faning, Madden, Steigerwalt, Bradford & Smith 1981; O'Hara, Sowers, Bopp, Duda & Strockbine 2003) especially *V. harveyi* and *Vibrio campbellii* which are closely related species (Vandenberghe, Thompson, Gomez-Gil & Swings 2003; Musa, Seong & Wee 2008).

There are many attempts to develop a species specific marker to identify *V. harveyi* and differentiate *V. harveyi* from *V. campbellii*. Several molecular methods such as polymerase chain reaction (PCR) targeting to *toxR* (Pang, Zhang, Zhong, Chen, Li &

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Austin 2005), *vhh* (Conejero & Hedreyda 2004), *luxN* (Hernandez & Olmos 2004) and *gyrb* (Thaithongnum, Ratanama, Weeradechapol, Sukhoom & Vuddhakul 2006) genes have been used for identification of *V. harveyi*. However, both *toxR* and *vhh* genes could not be used to detect all *V. harveyi* isolates (Conejero & Hedreyda 2004; Pang *et al.* 2005). Similarly, PCR reaction specific to *luxN* and *gyrB* genes could not discriminate *V. harveyi* from *V. campbellii* (Defoirdt, Verstraete & Bossier 2007; Thompson, Gomez-Gil, Vasconcelos & Sawabe 2007).

VhhP2 gene encoding a putative outer membrane protein was originally identified from a pathogenic *V. harveyi* strain T4 from diseased fish by Zhang, Sun and Sun (2008); Zhang, Sun, Cheng and Sun (2008). The experiment conducted by Sun, Hu, Zhang, Bai and Sun (2009) reported that this gene appeared to be a more appropriate species marker for *V. harveyi* as *VhhP2* gene is widely distributed in *V. harveyi* strains in different geographical locations and sources.

A novel nucleic acid amplification method called loop-mediated isothermal amplification (LAMP) was developed for rapid and sensitive nucleic acid amplification by Notomi, Okayama, Masubuchi, Yonekawa, Watanabe, Amino and Hase (2000). This technique is highly specific due to the use of a set of at least four primers that recognize a total of six distinct sequences on the target DNA. The method relies on auto-cycling strand displacement of DNA synthesis performed by the *Bst* DNA polymerase large fragment which can be carried out in a short time under isothermal conditions.

LAMP combined with chromatographic lateral flow dipstick (LFD) has been conducted to simplify and speed up the LAMP-based assay (Jaroenram, Kiatpathomchai & Flegel 2009). This generic LFD dipstick (Milenia Biotec, GieBen, Germany) detects biotin-labelled DNA amplicons that has been hybridized to a fluorescein isothiocyanate (FITC)-labelled DNA probe complex with gold-labelled anti-FITC antibody. The resulting DNA duplex is trapped at the test line by streptavidin forming a reddish-purple colour at the test line. Nonhybridized biotin-labelled primer is also bound here but without showing any colour. Then the solution moves up to the control line where the gold-labelled anti-FITC antibody with or without an attached FITC probe will be trapped forming a reddish colour. The reaction remain of biotin-labelled primer is also bound to streptavidin at the test line but it has no colour or undetected.

This technique has been successfully reported to detect multiple viral and bacterial agents related to shrimp diseases including Taura syndrome virus (TSV) (Kiatpathomchai, Jaroenram, Arunrut, Jitrapakdee & Flegel 2008), white spot syndrome virus (WSSV) (Jaroenram *et al.* 2009), *V. parahaemolyticus* (Prompamorn, Sithigorngul, Rukpratanporn, Longyant, Sridulyakul & Chaivisuthangkura 2011) and *V. vulnificus* (Surasilp, Longyant, Rukpratanporn, Sridulyakul, Sithigorngul & Chaivisuthangkura 2011).

In our preliminary study, some of the verified *V. harveyi* did not yield the PCR positive reactions specific to *toxR* (Pang *et al.* 2005), *vhh* (Conejero & Hedreyda 2004) and *gyrB* (Thaithongnum *et al.* 2006) genes. According to a report by Sun *et al.* (2009) a PCR primer set targeting to the *vhhP2* gene, misidentified one of the *V. campbellii* isolate as *V. harveyi*. Therefore, in this study, a new set of PCR primer and LAMP-LFD assay targeting to *vhhP2* gene of *V. harveyi* were developed. The specificity and sensitivity in both pure cultures and spiked shrimp samples were compared between PCR and LAMP-LFD methods.

Materials and methods

Bacterial strains and DNA extraction

A total of 72 bacterial isolates including 22 *V. harveyi* isolates, 16 non-*V. harveyi* (Table 1) and 34 non-*Vibrio* bacteria were used in this study. All *Vibrio* isolates were cultured using thiosulfate citrate bile salt (TCBS) agar while non-*Vibrio* isolate were cultured using tryptic soy agar (TSA; Difco, Sparks, MD, USA) at 37°C overnight. The origins and sources of all 38 *Vibrio* isolated were shown in Table 1. The other 34 non-*Vibrio* bacteria were obtained from clinical samples, food or environmental sources, as follows: three isolates of *Aeromonas hydrophilla*, three isolates of *Aeromonas caviae*, three isolates of *Aeromonas sobria*, four isolates of *Pseudomonas aeruginosa*, two isolates of *Plesiomonas shigelloides*, one isolate each of *Aeromonas veronii*, *Aeromonas jandei*, *Escherichia coli*, *Edwardsiella tarda*, *Photobacterium damselae* ssp. *damselae*, *P. damselae* ssp. *piscicida*, *Proteus vulgaris*, *Pseudomonas stutzeri*, *Pseudomonas chlororaphis*, *Pseudomonas putida*, *Pseudomonas boreopolis*, *Pseudomonas oleovorans*,

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Table 1 List of bacterial isolates and sources used in this study

Bacterial isolates	Origin	LAMP	PCR				Source
			toxR	*vhh*	*gyrB*	*vhhP2*	
Vibrio harveyi (*n* = 22)							
1526	*Penaeus monodon*	+	–	–	+	+	Centex
1114	*P. monodon*	+	–	–	–	+	Centex
VG	*P. monodon*	+	–	–	+	+	Centex
22.30	*P. monodon*	+	–	–	+	+	Centex
14126	*P. monodon*	+	+	+	+	+	Centex
25919	*P. monodon*	+	–	–	+	+	Centex
1039III	*P. monodon*	+	–	–	+	+	Centex
47666-1	Unknown	+	–	+	–	+	DABU
LMG 22888	Unknown	+	–	–	+	+	GB (Belgium)
LMG 22889	Unknown	+	–	–	+	+	GB (Belgium)
LMG 22891	Unknown	+	+	–	+	+	GB (Belgium)
LMG 22893	Unknown	+	+	+	–	+	GB (Belgium)
LMG 22894	Unknown	+	+	+	–	+	GB (Belgium)
LMG 22895	Unknown	+	+	+	–	+	GB (Belgium)
H1	Shrimp ponds	+	–	+	+	+	CPF
H5	Shrimp ponds	+	–	–	+	+	CPF
1P	Green mussel	+	–	–	–	+	DBSWU
4P	Green mussel	+	–	–	–	+	DBSWU
5P	Green mussel	+	–	–	–	+	DBSWU
7P	Green mussel	+	–	–	–	+	DBSWU
1C	Bloody clam	+	–	–	–	+	DBSWU
4C	Bloody clam	+	–	–	–	+	DBSWU
V. campbellii (*n* = 3)							
LMG 21361	Unknown	–	–	–	+	–	GB (Belgium)
LMG 21362	Unknown	–	–	–	+	–	GB (Belgium)
LMG 21363	Unknown	–	–	–	+	–	GB (Belgium)
V. alginolyticus (*n* = 1)							
DMST 22082	Stool	–	–	–	–	–	DMST
V. parahaemolyticus (*n* = 3)							
DMST 15285	Food	–	–	–	–	–	DMST
DMST 21308	Cuttlefish	–	–	–	–	–	DMST
DMST 23797	Stool	–	–	–	–	–	DMST
V. cholerae (*n* = 3)							
DMST 22116	Stool	–	–	–	–	–	DMST
DMST 22126	Rectal swab	–	–	–	–	–	DMST
DMST 22136	Stool	–	–	–	–	–	DMST
V. vulnificus (*n* = 3)							
MT 1506	Unknown	–	–	–	–	–	DABU
4907011	*Penaeus vannamei*	–	–	–	–	–	DBSWU
DMST 27426	Hemoculture	–	–	–	–	–	DMST
V. mimicus (*n* = 1)							
DMST 22090	Food	–	–	–	–	–	DMST
V. fluvialis (*n* = 1)							
DMST 22086	Stool	–	–	–	–	–	DMST
V. shilonii (*n* = 1)							
VS 4907012	*P. vannamei*	–	–	–	–	–	DBSWU
Other non-vibrio bacteria (*n* = 34)		–	–	–	–	–	

+, positive reaction; –, negative reaction; LAMP, loop-mediated isothermal amplification; PCR, polymerase chain reaction; Centex: CENTEX Shrimp, Faculty of Science, Mahidol University, Thailand; CPF: Charoen Pokphand Foods Public; DABU: Department of Aquatic Science, Burapha University, Thailand; DMST: Department of Medical Science, Ministry of Public Health, Thailand; DBSWU: Department of Biology, Faculty of Science, Srinakharinwirot University, Thailand; GB (Belgium): Laboratory of Microbiology, Ghent University, Belgium.

Pseudomonas syringae, *Pseudomonas japonica*, *Pseudomonas fluorescens*, *Salmonella enterica* serotype Enteritidis, *S. enterica* serotype Typhimurium, *Staphylococcus aureus* and *Yersinia ruckeri*. Conventional biochemical tests were performed to identify the bacterial isolates. The 16S rRNA gene analysis was also employed to verify the bacterial identification as previously described (Weisburg, Barns, Pelletier & Lane 1991).

An isolate of VH 14126 was utilized for the assay of optimization and sensitivity testing. To extract bacterial DNA, a single loopful of culture on TCBS agar or TSA agar was used with QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacture's specification. The extracted DNA was then stored at −70°C until use.

Polymerase chain reaction

Primers specific to *vhhP2* gene of *V. harveyi* were designed from pairwise alignment generated by blastn analysis between nucleotide sequence of *V. harveyi* T4 *vhhP2* gene (GenBank accession no. FJ025787.1) and hypothetical protein gene of *V. harveyi* BAA-1116 (GenBank accession no. CP000789.1), lately identified as *V. campbellii* (Lin, Malanoski, O'Grady, Wimpee, Vuddhakul, Alves, Thompson, Gomez-Gil & Vora 2010). The primers were designed by choosing the specific regions only to *V. harveyi* as a forward primer (5′-CAG CTC CCC GTT TTT TAA ACC-3′) and a reverse primer (5′-CCA CCA TAT CCA TCG ATA TCT GTT-3′). Amplification with *V. harveyi* specific primers was performed with 35 cycles of 94°C for 1 min, 94°C for 30 s, 59°C for 30 s, 72°C for 30 s and final extension at 72°C for 10 min. The amplicon size is 157 bp. DNA samples from nontarget bacteria and other *Vibrio* species (Table 1) were also used as PCR templates to test for the specificity of the designed primers. PCR targeting to *toxR*, *vhh* and *gyrB* gene of *V. harveyi* were performed according to previous report by Pang *et al.* (2005); Conejero and Hedreyda (2004); Thaithongnum *et al.* (2006).

Primers designed for LAMP

Based on the nucleotide sequence of *vhhP2* gene of *V. harveyi* (GenBank accession no. FJ025787.1), a set of four primers were designed using Primer Explorer V4 software (http://primerexplorer.jp/elamp4.0.0/index.html). A forward inner primer (FIP), a backward inner primer (BIP) and two outer primer (F3 and B3) were utilized for LAMP method. The sequences of primers used for amplification of the *vhhP2* gene region were shown in Table 2.

Optimization of LAMP reaction conditions

The LAMP assay was performed in a total of 25 μL of reaction mixture containing 40 pmol each of FIP and BIP, 5 pmol each of F3 and B3, 1.4 mM dNTP mix (Fermentas, Burlington, ON, Canada), 6 mM $MgSO_4$, 0.8 M Betaine (Sigma-Aldrich, St Louis, MO, USA), 8 U *Bst* DNA polymerase large fragment (New England Biolabs, Ipswich, MA, USA) and 1x of supplied buffer and DNA template. The reaction temperature was optimized at 60, 63, 65°C, and LAMP was carried out at predetermined time (60 min), followed by 80°C for 10 min to terminate the reaction. The LAMP products were analysed by 2% agarose gel electrophoresis.

Design and optimization of FITC-labelled probe for lateral flow dipstick (LFD) assay

To confirm the results obtained from agarose gel electrophoresis, the biotin-labelled LAMP reactions were carried out along with normal LAMP. In this condition, the biotin-labelled LAMP was added at the 5′ end of the FIP but other primers and

Table 2 Primers for loop-mediated isothermal amplification (LAMP) designed from *vhhP2* gene of *V. harveyi*

Primer	Gene position	Sequence (5′–3′)
F3	148–165	CAATTCGAAACAGGCGTG
B3	354–371	AGTAAAGCTTGCCACACG
Forward inner primer (FIP)	225–244/TTTT/185–204	CGCCACCACCATATCCATCGTTTTGGTTAGTCAATGGTGGAACA
Backward inner primer (BIP)	249–273/TTTT/312–333	GGATGTAAATGAGTTTGGCTTTCCGTTTTTTGTCCTATGTTATACGGGTTG
FITC probe	283–302	FITC-AACAAGGGCAACAGAAATGG

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components used were the same as those described above.

A DNA probe was designed from the *vhhP2* gene sequence between the FIP and BIP regions (Table 2). The DNA probe was labelled with FITC at the 5′ end (Bio Basic, Markham, Canada). According to the test protocol, after the biotin-labelled LAMP reaction was finished without heating inactivation, 5 μL of DNA probe solution at three different concentrations (200, 20 and 2 pmol) was added to the biotin-labelled LAMP products before hybridization at 63°C for 5 min. Subsequently, 8 μL of the hybridized product was added to 120 μL of the assay buffer in a new tube (Milenia Genline HybriDetect 2T, Gießen, Germany). After that, the LFD strip was dipped into the mixture and the test result appeared after 5–10 min. The concentration of DNA probe that gave the strongest signal on the test line was determined to be the optimal concentration for LFD assays.

Specificity of LAMP and PCR identification

The 72 bacterial isolates as shown in Table 1 were used to investigate the LAMP specificity. DNA templates isolates from bacterial cultures describe earlier were subjected to both LAMP and PCR amplification.

Determinations of sensitivities of LAMP and PCR with pure culture

The sensitivity of the LAMP assay for the detection *V. harveyi* in pure cultures was determined as previously described (Yamazaki, Ishibashi, Kawahara & Inoue 2008) with some modifications using known amounts of *V. harveyi* 14126. In brief, a single colony on TCBS agar was inoculated in 4 ml of tryptic soy broth (TSB; Difco) supplemented with 2% NaCl and incubated overnight at 37°C. Subsequently, 40 μL of TSB culture was transferred to a new 4 mL of TSB and incubated at 37°C with shaking at 225 rev min^{-1} at 37°C to obtain mid-log phase cells ($OD_{600\ nm}$ = 0.5). Serial tenfold dilutions of the cultures were prepared in phosphate buffered saline (PBS). For preparation of DNAs from pure cultures, 100 μL of each dilution was transferred to a 1.5 mL microcentrifuge tube, and was centrifuged at 18 000 *g* for 5 min, then the pellet was resuspended in 50 μL of 25 mM NaOH, and the mixture was heated at 95°C for 5 min. After neutralization with 4 μL of 1 M Tris-HCl buffer (pH 7.5), the suspension was centrifuged and supernatant was used as a template for LAMP and PCR assay. The sensitivity tests were performed in triplicate, the last dilution with all three samples tested positive was considered as the detection limit. The product from each reaction were analysed by 2% agarose gel electrophoresis and by LFD using the appropriate amount of FITC-labelled DNA probe as described above. In parallel, to enumerate the bacteria, 100 μL of each bacterial dilution was spread on TSA supplemented with 2% NaCl in duplicate and incubated at 37°C for overnight. The colonies were counted at the dilution yielding 30–300 colony forming units (CFUs), and the CFU mL^{-1} of bacterial suspension was calculated.

Determinations of sensitivities of LAMP and PCR with spiked shrimp sample

The shrimp samples were tested to be negative for *V. harveyi* according to the microbiological examination by enrichment in alkaline peptone water (APW) for overnight. The shrimp homogenate was cultured onto TCBS agar. The DNA samples were extracted from bacterial colonies and shrimp homogenate as the template for testing the presence of *V. harveyi* by PCR targeted to *vhhP2* gene. Only shrimp homogenates that were negative for *V. harveyi* were used in the following spiked shrimp experiments.

The detection limit of LAMP assay for *V. harveyi* in spiked shrimp samples was evaluated as previously described (Yamazaki, Ishibashi *et al.* 2008) with some modifications using known amounts of *V. harveyi* 14126. Nine milliliters of APW were added to 1 g of the shrimp sample and homogenized thoroughly. Serial tenfold dilutions of mid-log phase *V. harveyi* were prepared as described in above. One hundred microliters of each dilution of *V. harveyi* with known amounts was spiked into 900 μL of each of the shrimp homogenates and mixed well. The shrimp homogenate was centrifuged at 200 *g* for 5 min to remove shrimp tissues. The supernatant was transferred to a new tube and centrifuged at 18 000 *g* for 5 min. After removal of the supernatant, the pellet was resuspended in 100 μL of 25 mM NaOH, and the mixture was heated at 95°C, for 5 min. After neutralization with 8 μL of 1 M Tris-HCl buffer (pH 7.5), debris was pelleted by centrifugation at

20 000 *g* 4°C, for 5 min. For LAMP and PCR assays, 3 μL of each supernatant was used as a template. The sensitivity tests were conducted in triplicate. The last dilution with all three samples tested positive was considered as the detection limit. The product from each reaction was analysed by 2% agarose gel electrophoresis and by LFD using the appropriate amount of FITC-labelled DNA probe as described above.

Results and discussion

In this study, the first LAMP-LFD assay and newly developed PCR technique targeted to the *vhhP2* gene were established for specific identification of *V. harveyi*. The PCR assay was specific to *V. harveyi* and could distinguish *V. harveyi* from *V. campbellii* (Table 1). The *V. campbellii* has been reported as a closely related species having nearly indistinguishable phenotypes (Gomez-Gil, Soto-Rodriguez, Garcia-Gasca, Roque, Vazquez-Juarez, Thompson & Swings 2004; Sawabe, Kita-Tsukamoto & Thompson 2007). Recently, the comparative genomic analyses indicated that *V. harveyi* BAA-1116 and HY01 were indeed *V. campbellii* (Lin *et al.* 2010). Therefore, in our study the PCR primers were newly designed specific to *vhhP2* nucleotides of *V. harveyi* but not to that of *V. harveyi* BAA-1116.

To determine the optimal temperature and time for LAMP assay, three different temperatures including 60, 63 and 65°C was used to perform LAMP reaction for 60 min. At all tested temperatures, the LAMP products displayed the ladder-like pattern characteristic on agarose gels. However, at 65°C the LAMP products displayed the clearest amplification of DNA (Fig. 1a, lane 3). Therefore, the temperature at 65°C was selected for the subsequent LAMP assays. To determine the optimum time for LAMP assay, five different reaction times including 30, 45, 60, 75 and 90 min were used in the LAMP reaction. The LAMP amplicons could be observed at 60 min and 75 min; however, at 90 min, the intensity of LAMP amplicons were stronger and clearly detected (Fig. 1b, lane 5). Therefore, the reaction time of 90 min was chosen as an optimal reaction time for LAMP assay.

For LAMP-LFD method, the appropriate amount of FITC-labelled probe at 200, 20 and 2 pmol used in the hybridization reaction were tested. The result showed that the 20 pmol of DNA probe yielded the highest intensity of purple colour at the test line (data not shown). Therefore, the concentration of

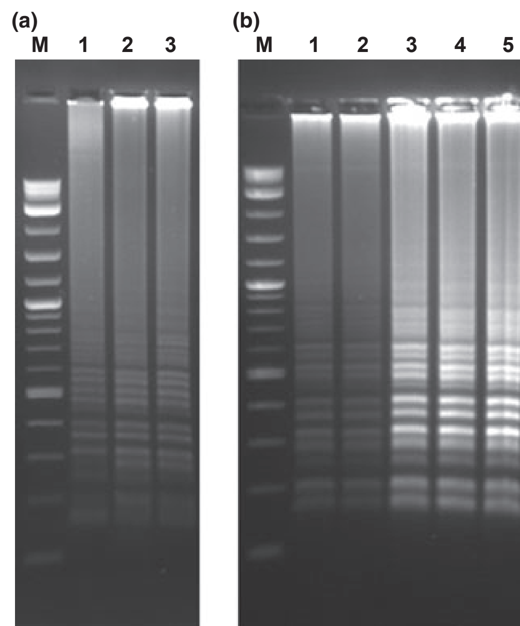

Figure 1 Determination of LAMP conditions at different temperatures (a) and time (b) using DNA extracted from VH 14126 bacterial isolate. (a) Temperatures: lanes 1–3, the reaction was carried out at 60, 63 and 65°C for 60 min, respectively. (b) Time: lanes 1–5, the reaction was carried out at 65°C for 30, 45, 60, 75 and 90 min, respectively; lane M, molecular marker.

20 pmol was used for all subsequent assays. The specificity of LAMP-LFD assay was tested against various *V. harveyi* isolates, non-*harveyi Vibrio* isolates, and various bacterial species as shown in Table 1 and Fig. 2. LAMP products were detected in all 22 *V. harveyi* isolates, whereas no LAMP amplicons were revealed in 16 other *Vibrio* spp. and 34 non-*Vibrio* isolates. All the results of LAMP assay agreed with that of *vhhP2* PCR (Table 1). Therefore, the LAMP-LFD demonstrated high specificity to *V. harveyi*.

To determine the detection sensitivity of the LAMP-LFD assay for *V. harveyi*, a serial tenfold dilutions of DNA templates extracted from pure culture was used in the LAMP reaction. Based on the initial inoculums of *V. harveyi* (1.1×10^7 CFU mL^{-1}), the sensitivity of LAMP-LFD for the detection of *V. harveyi* in pure culture was 1.1×10^2 CFU mL^{-1} or equivalent to 0.6 CFU per reaction, which was 10 times higher than that of PCR (6 CFU per reaction) (Figs 3a and 4a). The sensitivity of LAMP-LFD in this study was higher than that of previous LAMP assay for *V. harveyi* detection (17.2 CFU per reaction; Cao, Wu, Jian & Lu 2010), *V. corallilyticus*

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(8 CFU per reaction; Liu, Wang, Xu, Ding & Zhou 2010), cholera toxin-producing *V. cholerae* (2.9 CFU per reaction; Yamazaki, Seto, Taguchi, Ishibashi & Inoue 2008), *V. parahaemolyticus* (2.0 CFU per reaction; Yamazaki, Ishibashi *et al.* 2008), *V. vulnificus* (2.8 CFU per reaction; Surasilp *et al.* 2011) and thermostable direct haemolysin (TDH)-producing *V. parahaemolyticus* (1.0 CFU per reaction; Nemoto, Sugawara, Akahane, Hashimoto, Kojima, Ikedo, Konuma & Hara-Kudo 2009). However, the greater sensitivity at 0.6 CFU per reaction may concern with high amplification efficiency which may affect the testing DNA in sample from dead cells or viable but nonculturable cells (Ramaiah, Ravel & Straube 2002; Sun, Chen, Zhong, Zhang, Wang, Guo & Dong 2008).

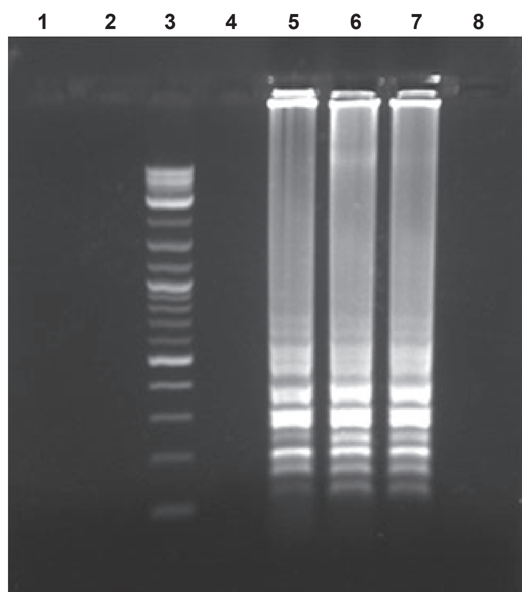

Figure 2 A representative of specificity test of LAMP for *V. harveyi* detection. Lane (1) *V. campbellii* LMG 21361; lane (2) *V. campbellii* LMG 21362; lane (3) molecular marker; lane (4) *V. alginolyticus* DMST 22082; lane (5) *V. harveyi* 14126 (Centex); lane (6) *V. harveyi* LMG 22895 (Belgium); lane (7) *V. harveyi* 1P (DBSWU); lane (8) *V. parahaemolyticus* DMST 23797.

In the case of spiked samples, the sensitivity of LAMP (5 CFU per reaction) was 10 times higher than that of PCR (50 CFU per reaction) (Figs 3b and 4b) and was approximately three times higher than that of previous LAMP assays for *V. harveyi* detection in added shellfish (17.2 CFU per reaction; Cao *et al.* 2010). The sensitivity result of the developed LAMP-LFD assay was comparable to that of LAMP assays for cholera toxin-producing *V. cholerae* in spiked human faeces (1.4 CFU per reaction; Yamazaki, Seto *et al.* 2008), *V. parahaemolyticus* in spiked shrimp sample (2.0 CFU per reaction; Yamazaki, Ishibashi *et al.* 2008), *Vibrio vulnificus* detection in spiked oyster sample (11 CFU per reaction; Surasilp *et al.* 2011) and *V. cholerae* in spiked shrimp sample (20 CFU per reaction; Srisuk, Chaivisuthangkura, Rukpratanporn, Longyant, Sridulyakul & Sithigorngul 2010).

The sensitivity of LAMP-LFD in both pure culture and spiked shrimp sample was 10 times

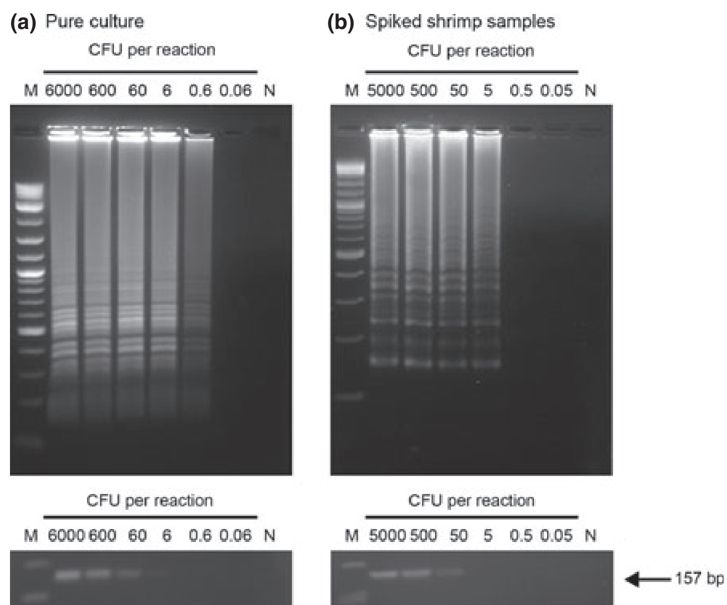

Figure 3 Sensitivity comparison of LAMP-LFD (top) and PCR (bottom panel) assays for the detection of *V. harveyi* in pure cultures (a) and spiked shrimp samples (b). The bacterial CFU per reaction was indicated at the top of each lane. Lane M, molecular marker and lane N, no template control (negative control).

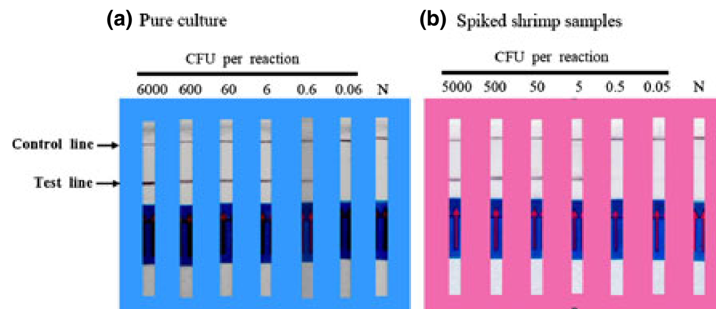

Figure 4 Sensitivity of LAMP-LFD for the detection of *V. harveyi* in pure culture (a) and spiked shrimp samples (b). The bacterial CFU per reaction was indicated at the top of each lane. N, negative control.

higher than that of PCR assay. These results agreed with previous reports stating higher sensitivity of LAMP-LFD compared with that of PCR (Jaroenram *et al.* 2009; Khunthong, Jaroenram, Arunrat, Suebsing, Mungsantisuk & Kiatpathomchai 2012). Previous studies suggested that LAMP assay was more tolerate to certain inhibitors in the food contexts (Han & Ge 2008; Yamazaki, Ishibashi *et al.* 2008; Techathuvanan, Draughon & D'Souza 2010).

In previous study, LAMP amplicons were detected by gel electrophoresis followed by staining with carcinogenic ethidium bromide which is not suitable for field applications.

The LAMP-LFD products can be easily detected by applying the membrane into the assay buffer which can reduce the total time for LAMP assays by 60 min. Furthermore, the specificity of the LAMP-LFD assay can be enhanced since the hybridization with specific probe to LAMP amplicons is utilized. Recently, The LAMP-LFD was also established for the detection *V. parahaemolyticus* and *V. vulnificus* in contaminated food sample (Prompamorn *et al.* 2011; Surasilp *et al.* 2011). Therefore, this technique can be applied to study *V. harveyi* infection which has caused the major economic losses in aquaculture industry (Ruangpan, Tabkaew & Sangrungruang 1995).

In conclusion, the first LAMP-LFD method for *V. harveyi* detection was successfully established. This method could be used for *V. harveyi* differentiation from all other tested *Vibrio* species including a closely related species, *V. campbellii*. It had the sensitivity of approximately 5 CFU per reaction with spiked shrimp samples. The developed LAMP-LFD assay is a sensitive, rapid, simple and valuable tool for the detection of *V. harveyi* for monitoring the disease outbreak in aquaculture farm.

Acknowledgments

The authors are indebted to all institutes in Table 1 for kindly providing us bacterial species. This work was supported by The Office of Commission on Higher Education, Thailand to K.T. and National Research Council of Thailand to P.C. and K.S.

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