Rapid and visual detection of white spot syndrome virus by loop-mediated isothermal amplification

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Abstract

White spot syndrome virus (WSSV) is the most threatening virus that causes mass mortality in shrimp aquaculture worldwide. In this present study, we developed a rapid and visual detection of WSSV based on the principle of loop-mediated isothermal amplification (LAMP) using a set of specifically designed six primers that recognize eight distinct target sequences. The amplification of all reagents simultaneously added with fluorescence reagent (calcein and $MnCl_2$) is performed within a single closed tube on a regular water bath under a constant temperature of 65ºC for 60 min. Upon the completion of reaction, the emission of green fluorescence allows immediate visualization for positive reaction by naked eyes without requirement of additional devices or reagents. This established LAMP protocol is highly specific to WSSV, whereas it is not specific to all other tested shrimp pathogens, including IHHNV, HPV, MBV, BP, TSV, YHV, and IMNV as well as shrimp genomic DNA. In addition, the detection sensitivity of this LAMP assay (10 viral copies) is as comparable as that of nested polymerase chain reaction (PCR), yet it possesses greater advantages than the nested PCR due to its inherent specificity, cost effectiveness, and obviation of handling with the mutagen ethidium bromide for electrophoresis.

Keywords : White spot syndrome virus; Loop-mediated isothermal amplification; WSSV; LAMP; Shrimp

1. Introduction

White spot syndrome virus (WSSV) is the most devastating pathogen causing mass mortality in penaeid shrimp population worldwide. Since the emergence in 1992, this virus has continued over the last decade to affect crustacean industries with significant economic losses (Escobedo-Bonilla et al., 2008). However, it is widely viewed that there is no treatment available at present to interfere with the unrestrained occurrence and the spread of the disease (Sanchez-Paz, 2010). In Americas, shrimp virus diseases, including WSSV infection, are increasingly being managed using a combination of biosecurity and the practice of culturing domesticated specific pathogen-free (SPF) or specific pathogen-resistant (SPR) stocks (Lightner, 2011). Nevertheless, in some developing countries the intervention to prevent pathogens is limited by poor capacity for implementation of effective quarantine and/or biosecurity measures and the illegal or poorly regulated international trans-boundary movement of live aquatic animals (Walker and Winton, 2010).

With regard to detection of WSSV infection at both the lifelong carrier stage and severe acute stage, many methods have been developed and validated in order to help prevent and control the disease transmission or outbreak. These methods include, for instance, indirect fluorescent antibody test, immunohistochemistry, enzyme-linked immunosorbent assay using monoclonal and polyclonal antibodies (Poulos et al.,2001;Sithigorngulet al., 2006; Yoganandhan et al., 2004), in situ hybridization (OIE, 2011), and polymerase chain reaction (PCR) assay (Lo et al., 1997; Flegel, 2011). All of these methods are considered sensitive and accurate to detect the presence of the virus in laboratory. However, these methods are time consuming and require expensive equipments and reagents to perform the analysis, thereby limiting their wide-scale use on a routine basis particularly for inexperienced technicians. A detection method that is not only fast and accurate, but also easy-to-use and economical has been long desired for field application.

Loop-mediated isothermal amplification (LAMP) is a novel alternative nucleic acid amplification method based on the principle of strand displacement DNA synthesis and production of stem-loop DNA structures under a constant temperature (Notomi et al., 2000). This method has drawn a lot of attentions as it offers a rapid, accurate, and cost-effective genetic diagnosis for infectious microorganisms (Mori and Notomi, 2009). For the detection of

WSSV, the LAMP assay was primarily devised using only four primers that recognized six regions of the target DNA sequence (Kono et al., 2004). An improved technique was subsequently reported by this research group who designed a set of six specific primers with two more loop primers to be added into the same tube to shorten the reaction time and employed an inexpensive turbidimeter in order to detect and quantify the amplicons in a real-time fashion (Mekata et al., 2009). Independently, another real-time and quantitative method called fluorescent resonance energy transfer (FRET) LAMP, which requires a LightCycler (Roche) machine and two additional FRET probes, was developed for WSSV allowing the specific LAMP amplicons to be measured in less than an hour (Chou et al., 2011). Moreover, a LAMP-based method which applies a well-developed chromatographic lateral flow dipstick in attempt to detect the WSSV agent in field conditions was almost consequently established (Jaroenram et al., 2009). However, all these methods require sophisticated instrument and/or additional gear, i.e. dipstick, while in fact a rapid and visual detection of specific LAMP amplicons can be achieved by various means. Simply adding a fluorescent metal indicator calcein and $MnCl_2$ to the reaction tube prior to the amplification is one of the most suitable detection systems as clearly recommended and demonstrated by Tomita et al. (2008). This colorimetric method should be helpful since it requires no more equipment other than a water bath or heat block; furthermore, it is inarguably less expensive than the previously mentioned protocols. This method should by itself permit a broader application in most basic laboratories in developing countries.In this present paper, we report the development and validation of LAMP-based assay for WSSV in penaeid shrimp coupled with the simple visual detection of products; meanwhile, its applicability was assessed with field samples.

2. Materials and methods

2.1. Design of primers for LAMP assay

The highly conserved genomic region of WSSV (GenBank accession number AF332093; genome positions 224,261 to 225,707) which has been used in the conventional nested PCR (Lo et al., 1997) was selected as the target for the primer design. Primers for LAMP assay were designed using Primer Explorer V4 software (http://primerexplorer.jp/elamp4.0.0/index.html) and synthesized as high performance liquid chromatography (HPLC)-grade by BioDesign Co. Ltd. (Pathumthani, Thailand). The primer set, stretching from nucleotides (nt) 224,793 to 225,000

(208 nt; Fig. 1) and comprising two outer primers (F3 and B3), two inner primers (FIP and BIP), and two loop primers (LF and LB), recognized eight distinct sites on the target sequence of the WSSV genome. Nucleotide sequences of the LAMP primers are shown in Table 1.

Table 1 The nucleotide sequences of the primers used in the LAMP assay.

Label	Sequence 5'>3'
F3	TCC AAC TCT GGC ATG ACA AC
B3	GCA GAG CAT TCT GGA TCA GT
FIP	CGT GCT GGC GAT GAC ATT CCA ACA GGA GTC AAC CTC GAC GT
BIP	CCG ACG CCA AGG GAA CTG TCC GTT ACC GTT TCC ATC GGT
LF	CCT AGT ACA CTT CTT GCA TTA TTT GGT TTG AC
LB	CTT CAG CCA TGC CAG CCG T

2.2. DNA extraction

Shrimp specimens used in this study were sent to our laboratory from different areas of Thailand without clinical manifestation of WSSV infection, and their appropriate target organs, i.e. pleopods for WSSV, were sampled and submitted for the examination of diseases. DNA extraction was performed using both a commercial kit and a tissue boiling method. For the QIAamp DNA mini kit (Qiagen), the ablated pleopods were placed in a 1.5 mL microcentrifuge tube and ground in 500 µL phosphate buffered saline (pH 7.2) using a pestle, and the tissue homogenate was centrifuged at 14,000 rpm for 5 min. The clear supernatant (160 µL) was placed in a new 1.5 mL microcentrifuge tube, and 180 µL of lysis buffer and 20 µL of proteinase K were added and incubated at 56ºC until the complete lysis occurred (1-3 h). Total DNA was then isolated according to the manufacturer's instructions. The DNA was eluted with 100 µL of elution buffer, quantified using a Nanodrop spectrophotometer model ND-1000 (NanoDrop Technologies), and stored at -20ºC until further analysis.

The second method of DNA extraction was by boiling as previously reported (Soliman and El-Matbouli, 2005; Moa et al., 2008). Total DNA was extracted from shrimp tissue according to the previous protocols with minor modifications. Specifically, the ablated pleopods were placed and ground using a pestle in a 1.5 mL microcentrifuge tube with 200 µL lysis buffer (Qiagen). Subsequently, the capped tube was placed in boiling water for 15 min. The tube was

then added with 800 µL Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), mixed well, and centrifuged at 14,000 rpm for 3 min. The supernatant containing DNA was used immediately in the WSSV assays.

2.3. LAMP assay

The LAMP assay was performed in a 25 µL of reaction mixture containing 1.6 µM each FIP and BIP, 0.08 µM each F3 and B3, 0.64 µM each LF and LB, 800 µM each deoxyribonucleotide triphosphate (dNTP), 1 M betaine (Sigma), 20 mM Tris-HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 4 mM $MgSO_4$ (Sigma), 0.1% Triton X-100, 8 U *Bst* DNA polymerase (New England Biolabs) and 2 µL template DNA except for the negative control for which the template DNA was omitted from the reaction. To visualize the reaction, 25 µM calcein (Sigma) and 0.5 mM $MnCl_2$ (Sigma) had been previously added to the reaction mixture, as previously described (Tomita et al., 2008; Peng et al., 2011). The amplification was carried out in a conventional water bath. Six temperatures (60, 61, 62, 63, 64, and 65ºC) and seven times (30, 40, 50, 60, 70, 80, and 90 min) were screened to find the optimal temperature and time; consequently, varying concentrations of magnesium, betaine, dNTPs, and $MnCl_2$ were also trialed (Fig. 2). Additionally, the effect of time on the amplification, using four primers (FIP, BIP, F3, B3) and six primers (FIP, BIP, F3, B3, LF, LB), carried out at 65ºC for duration of 10-60 min was determined (Fig. 3). The reaction was terminated by heating at 80ºC for 2 min. The samples with color change to green under daylight were considered as the positive reaction, while the samples with the starting orange color were considered as the negative reaction. The amplified product was also confirmed by 1.5% agarose gel electrophoresis, and the gel was thereafter submerged in an ethidium bromide-containing buffer (0.5 µg L^{-1}) for 15 min and recorded on an ultraviolet transilluminator.

2.4. Nested PCR

The protocol for detection of WSSV by nested PCR was modified from a previous protocol reported by Lo et al. (1997). For the first-step PCR reaction, each sample was amplified in a 25 µL reaction containing 12.5 µL 2x GoTag Green Master Mix (Promega), 300 nM each of outer primers 146F1 and 146R1 (5'-ACTACTAACTTCAGCCTATCTAG-3' and 5'-TAATGCGGGTGTAATGTTCTTACGA-3', respectively) and 2 µL template DNA. For the negative control, nuclease-free water was filled in lieu of the template DNA. The PCR profile was 5 min at 95ºC, followed by 35 cycles of 30 sec at 95ºC, 30 sec at 55ºC and 1.5 min at 72ºC, and a final

extension for 7 min at 72ºC. The amplified product of 1,447 bp was analyzed by 1.5% agarose gel electrophoresis as described above. The second (nested)-step PCR reaction was nearly identical to that of the first step except that inner primers 146F2 and 146R2 (5'-GTAACTGCCCCTTCCATCTCCA-3' and 5'-TACGGCAGCTGCTGCACCTTGT-3', respectively) were instead utilized. The amplified product of the second step (941 bp) was analyzed by 1.5% agarose gel electrophoresis as described herewith above.

2.5. Construction of positive control plasmid DNA

PCR products obtained from the amplification of viral DNA using the PCR primers 146F1 and 146R1 were inserted into pGEM-T Easy vector (Promega) and used as positive control plasmid DNA. Briefly, the amplified product (1,447 bp) was analyzed by agarose gel electrophoresis. The expected ban was then excised from the illuminating gel and cleaned using NucleoSpin Extract II (Machery-Nagel). Subsequently, the amplified product was ligated into a plasmid pGem-T Easy vector (Promega), and the plasmid was used to transform DH5α *Escherichia coli* cells (RBC Bioscience) according to the manufacturer's instructions. The purified recombinant plasmid retrieved from a clone was confirmed by DNA sequencing on both forward and backward directions (data not shown). The plasmid DNA concentration was measured using a Nanodrop spectrophotometer ND-1000 (NanoDrop Technologies), and then the copy number was calculated using an online DNA copy number calculator with the assumption that the sequence lengthequals 208 bp (http://www.endmemo.com/bio/dnacopynum.php).

2.6. Specificity of the LAMP assay

A restriction analysis with *Hinc*II or *Ssp*I endonuclease was used to evaluate the specificity of the LAMP reaction. Enzymatic reaction system was composed as follows: 1 µL (1 µg) of LAMP product, 16.3 µL of sterile deionized water, 2 µL of RE 10x Buffer, 0.2 µL of acetylated bovine serum albumin (10 µg μL^{-1}), and 0.5 µL of *Hinc*II or *Ssp*I (Promega) for 10 U μL^{-1}. The digestion reactions were incubated at 37ºC for 2 h. Finally, 4 µL of digested LAMP product were analyzed by agarose gel electrophoresis. The specificity of the LAMP reaction was also tested using DNA or cDNA from WSSV, infectious hypodermal and hematopoietic necrosis virus (IHHNV), hepatopancreatic parvovirus (HPV), *Penaeus monodon*-type baculovirus (MBV), *Baculovirus penaei* (BP), Taura syndrome virus (TSV), yellow head virus

(YHV), infectious myonecrosis virus (IMNV), necrotizing hepatopancreatitis bacterium (NHPB), and shrimp genomic DNA.

2.7. Sensitivity of the LAMP assay

In order to test the sensitivity, positive control plasmid DNA of known concentration was prepared as elaborated above. Subsequently, the detection limit for the LAMP reaction was determined under optimal conditions using six primers. The reaction was assessed using 1 µL of 10-fold serial dilutions of the recombinant plasmid DNA and compared with nested PCR.

2.8. Applicability of the LAMP assay

Upon the completion of the validations, the LAMP reaction was applied to test for the presence of WSSV in 102 suspicious samples submitted to our laboratory, and the results were compared with the nested PCR results. These shrimp samples were collected from shrimp grow-out farms, hatcheries, and processing plants in Thailand during 2008 and 2010.

3. Results

3.1. Optimization of the LAMP assay

The optimal temperature for the LAMP reaction was determined to be between 60 and 65ºC, which could yield the color change to green as observed by naked eyes (Fig. 2A). For ease of later analyses, we opted to set the reaction temperature at 65ºC because at this temperature, the reaction provided the darkest green color as compared to others. The optimal time and concentrations of magnesium, betaine, dNTPs, and $MnCl_2$ were identified as 60 min, 4 mM, 1 M, 0.8 mM, and 0.5 mM, respectively (Figs. 2B–F). The amplification occurred using either 4 or 6 primers. With 4 primers, a LAMP product was detected after 45 min at 65ºC (Fig. 3A), while with 6 primers the amplification product was detected as early as 35 min (Fig. 3B). However, only the reactions with 6 primers provided the color change to green, which was visualized after 50 min. The optimal primer concentration is indicated in Materials and Methods. WSSV DNA extracted either by commercial kit or by boiling resulted in a typical ladder-like pattern of multiple bands of different size up to the loading well as shown in Fig. 2, 3, and 4. With a simultaneous addition of diluted calcein (1.25 µL) plus diluted $MnCl_2$ (0.625 µL) to the reaction tube, positive reactions (generated pyrophosphate) turned green, while negative reactions remained faint orange as illustrated in Fig. 2, 3, and 4.

3.2. Specificity of the LAMP assay

The specificity of LAMP was confirmed by the digestion using *Hinc*II and *Ssp*I restriction enzymes. The amplified products with WSSV resulted in a series of bands by agarose gel electrophoresis. The resultant digested products of 167, 171, and 253 bp with *Hinc*II, and 121, 153, and 267 bp with *Ssp*I were in accordance with the predicted sizes (Fig. 5). On the contrary, specific products in the LAMP assay could not be detected with IHHNV, HPV, MBV, BP, TSV, YHV, IMNV, NHPB, and shrimp genomic DNA (Fig. 6).

3.3. Sensitivity of the LAMP assay

The LAMP reaction was tested using 10-fold serial dilutions of WSSV DNA from purified recombinant plasmid and compared against results from the nested PCR assay. The detection limit of both the LAMP and nested PCR assay was 10 copies (Fig. 7).

3.4. Applicability of the LAMP assay

A total of 102 field samples were tested for WSSV by visualized LAMP and nested PCR. The results revealed that 73 out of 102 were tested positive with both the LAMP and nested PCR, while the other 29 samples were negative (Fig. 8). None of these samples that were negative with the LAMP was tested positive with the nested PCR, and vice versa.

4. Discussion

WSSV is the only member of the DNA virus family *Nimaviridae*. The WSSV disease is characterized by the development of white spots embedded in the exoskeleton associated with very high mortality (OIE, 2011). Due to its worldwide distribution in shrimp aquaculture industry as well as its persistent establishment within wild species populations, it is necessary to develop a quick, sensitive, and accurate diagnostic method for WSSV infection for the routine screening of domesticated shrimp for the presence and absence of this virus. Although PCR is generally accepted as the most extensively used diagnostic method for WSSV, this technique may not be accessible in resource-limited areas especially for most diagnosticians and field practitioners because PCR requires high-precision equipment and expertise to carry out the analysis. Furthermore, inhibitors that may be present in the original sample, such as tissue and soil, usually affect PCR through the interaction with DNA or interference with *Taq* DNA polymerase.

To avoid contamination of unnecessary inhibitors, it is imperative for PCR that the sample is neatly collected using specialized costly purification methods.

LAMP is a new approach to nucleic acid amplification that depends on strand displacement DNA synthesis and production of stem-loop DNA structures completed by *Bst* DNA polymerase (Notomi et al., 2000). An isothermal condition (usually between 60 to 65ºC) required during the LAMP assay can easily be accomplished by setting the single closed tube on a common water bath or heat block thereby eradicating the need for expensive thermal cycling machine. Application of LAMP to detect pathogens, including WSSV, has key advantages owing to its simplicity, efficiency, and low cost. Amplification results can be analyzed by gel electrophoresis, which shows the typical ladder-like pattern. Visible detection of LAMP products is also practical by simple inspection of turbidity caused by the releasing accumulation of magnesium pyrophosphate, or with subsequent addition of SYBR Green, a color change can also be observed without equipment. However, when the sample contains a low amount of virus, it is difficult to check for the turbidity by the unaided eyes unless a more intelligent apparatus namely photometer is used. SYBR Green, although generally thought to be safer than the carcinogen ethidium bromide, is a possible mutagen due to capability of DNA intercalation with high affinity. Besides, SYBR Green cannot be added directly and concomitantly to the reaction mixture, otherwise the amplification is inhibited (Tao et al., 2011). Alternatively, colorimetric visualization of DNA amplification is feasible using manganese bound-calcein that begins to give out green light upon its transformation resulted from magnesium pyrophosphate generated during DNA synthesis.

In the current study, a one-step assay for WSSV is carried out within 60 min in a closed tube containing the mixture of buffer, 3 pairs of primers, *Bst* DNA polymerase and viral template. The primer set consists of two outer primers (forward primer F3 and backward primer B3), two inner primers (forward inner primer FIP and backward inner primer BIP), and two loop primers (forward loop primer LF and backward loop primer LB) which recognize eight distinct site of the target sequence on the WSSV genome, so its specificity is deemed to be very high. Use of six primers instead of four primers has several advantages, including enhancing the specificity of the reaction, eliminating false positive results, and accelerating the reaction time. The result of the LAMP assay is visualized by adding fluorescent reagent, calcein and $MnCl_2$, prior to the reaction to inspect the change of color from orange to green under daylight. Magnesium ions

from pyrophosphate, generated during the LAMP process, substitute manganese ions once bound to calcein, causing the so-called quenching effect that starts fluorescing green light, which is indicative of the presence of target sequence.

Results from previous studies have implied that DNA extraction and purification using commercial kit can be omitted and that a boiling extraction method can be instead employed to prepare DNA samples for the LAMP assays (Soliman and El-Matbouli, 2005; Tao et al., 2011). The simple extraction technique would not only save time and money but also make it easy for most field laboratories to establish standard operating procedures. In this study, shrimp tissue was boiled in lysis buffer to inactivate DNase and elute DNA from tissue. TE buffer was used for the boiled solution to dilute inhibitors which may be derived from tissues, soil, etc. The LAMP assay was capable of detecting DNA prepared by this boiling extraction method. The detection limit of the LAMP assay was determined through amplification of 10-fold serial dilutions of positive control plasmid DNA. The reaction was performed at 65ºC for 60 min and compared with the results of the nested PCR. There was no difference between the detection limits of the LAMP assay and the nested PCR as both yielded positive reaction down to the dilution of 10 viral copies. The results of the specificity test showed that the assay had no cross reaction with other shrimp pathogens. Furthermore, a total of 102 shrimp samples collected from many locations in Thailand were tested using LAMP and nested PCR. The results revealed that the clinical sensitivity of the LAMP assay was consistent with the nested PCR.

Although the LAMP analysis has more advantages than other nucleic acid amplification methods, potential drawbacks are noteworthy. Firstly, purity of primers especially that of FIP and BIP primers is crucial for rapidity and reproducibility of amplification, and use of HPLC-purified FIP and BIP primers is highly recommended (Tomita et al., 2008). For other primers, desalted grade of purity is sufficient. Secondly, length of target region should not be greater than 300 bp, including F2 and B2 sites, because one rate-limiting step for amplification is strand displacement DNA synthesis (Notomi et al., 2000). Last but not least, minute quantity of DNA that contaminates laboratory, equipments and reagents can result in a false positive because of the high sensitivity of the LAMP assay. Consequently, care should be taken when opening the amplification tube, for aerosol that may contaminate the surroundings is minimized. Adding the fluorescent reagent (calcein and $MnCl_2$) prior to amplification may greatly help prevent the contamination as reported recently (Peng et al., 2011).

In this study, the LAMP assay with high sensitivity was executed in a water bath for 60 min, and the amplification was visualized by adding fluorescent reagent before amplification in order to observe the change of color by naked eyes. In conclusion, the newly established LAMP assay represents a reliable tool to detect WSSV in field conditions without requirement of specialized equipment.

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Fig. 1. Nucleotide sequence of the WSSV genome (GenBank accession number AF332093) used for designing the inner (FIP, BIP), outer (F3, B3), and loop (LF, LB) primers for the LAMP assay. FIP is a hybrid primer consisting of the sequence of F1c plus F2; BIP is a hybrid primer consisting of the sequence of B1c plus B2. The primer sequences (or their complementary (c) sequences) are boxed and underlined. Restriction recognition sequences are colored.

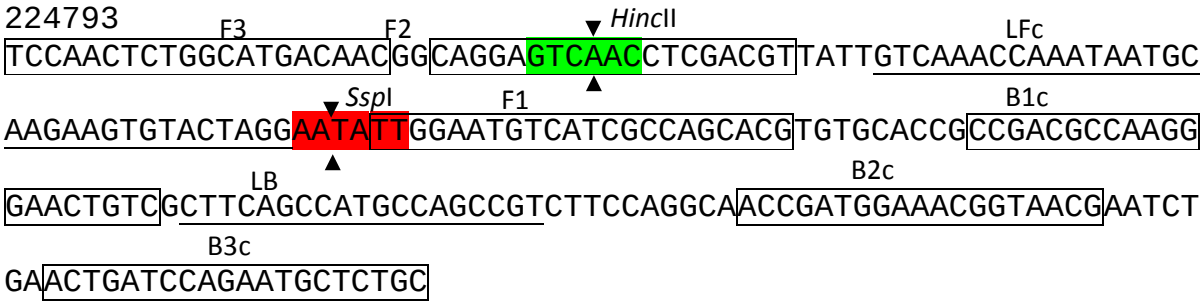


Fig. 2. The optimal condition and concentration of LAMP for the detection of WSSV. The optimal condition and concentration with six primers is monitored by visual detection of color change and agarose gel electrophoresis. A. Optimal temperatures. M: 100-bp DNA molecular weight marker; 1–6: 60, 61, 62, 63, 64, and 65ºC, respectively. B. Reaction times. 1–7: 30, 40, 50, 60, 70, 80, and 90 min, respectively. C. Concentrations of magnesium. 1–5: 2, 3, 4, 6, and 8 mM, respectively. D. Concentrations of betaine. 1–4: 0, 0.5, 1.0, and 1.5 M, respectively. E. Concentrations of dNTPs. 1–5: 0.2, 0.4, 0.8, 1.4, and 1.6 mM, respectively. F. Concentrations of $MnCl_2$. 1–4: 0, 0.5, 1.0, and 1.5 mM, respectively. (+): the color change to green, i.e. positive reaction of the visualized LAMP; (–): the starting orange color remains unchanged.

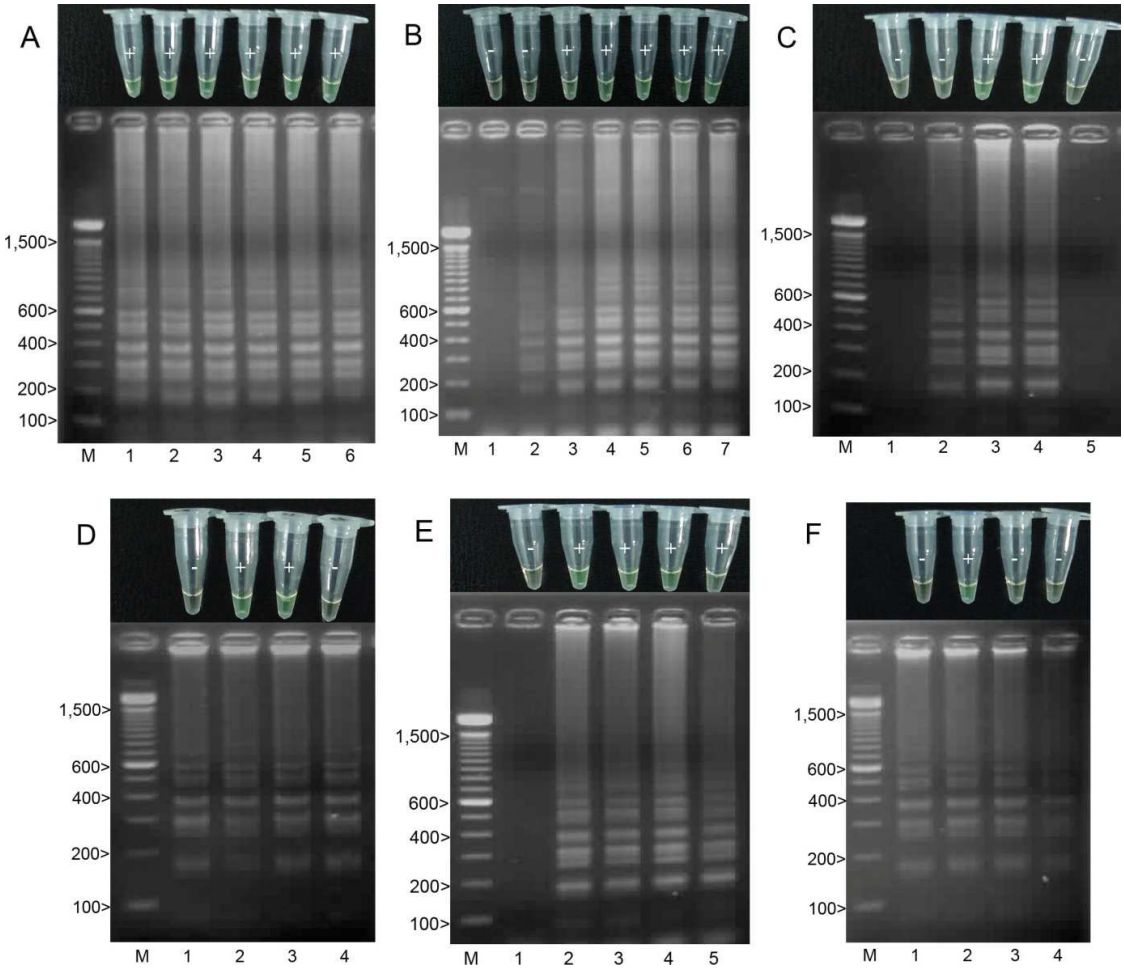


Fig. 3. The effect of time on amplification of WSSV DNA by LAMP assay, using (A) four primers (FIP, BIP, F3, B3) and (B) six primers (FIP, BIP, F3, B3, LF, LB), carried out at 65ºC for duration of 10-60 min. The results are observed by visual detection of color change and agarose gel electrophoresis. M: 100-bp DNA molecular weight marker; –ve: negative control; (+): the color change to green, i.e. positive reaction of the visualized LAMP; (–): the starting orange color remains unchanged.

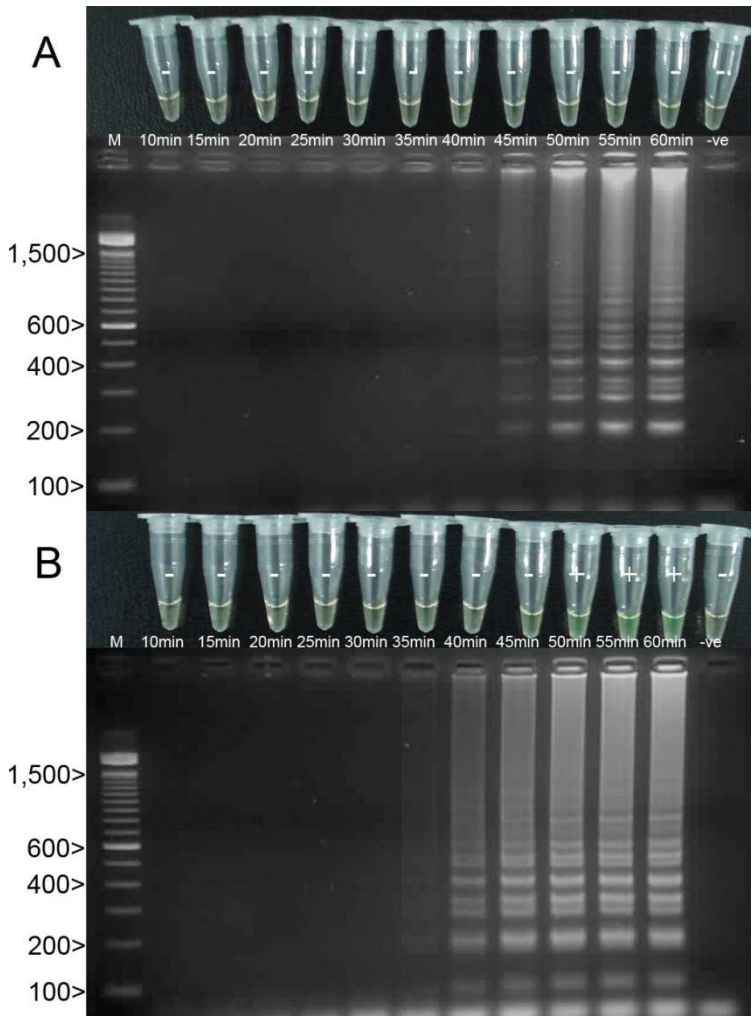


Fig. 4. Agarose gel electrophoresis of LAMP products of WSSV DNA extracted by boiling. The reaction was carried out at 65ºC using the 6 primer set. M: 100-bp DNA molecular weight marker; 1–3: WSSV DNA samples extracted by boiling; 4: negative shrimp tissue; 5: negative control.

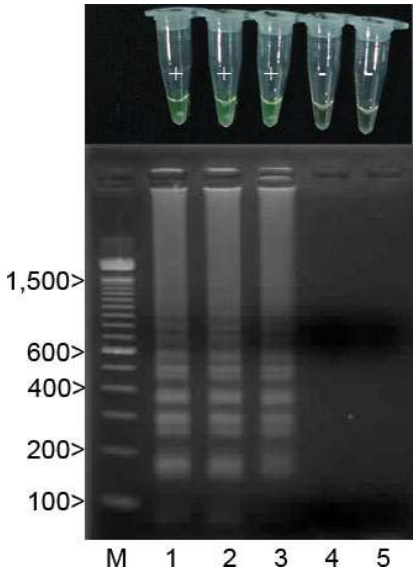


Fig. 5. Restriction analysis of LAMP products. M: 100-bp DNA molecular weight marker; 1 and 3: LAMP products; 2 and 4: LAMP products digested with *Hinc*II and *Ssp*I, respectively.

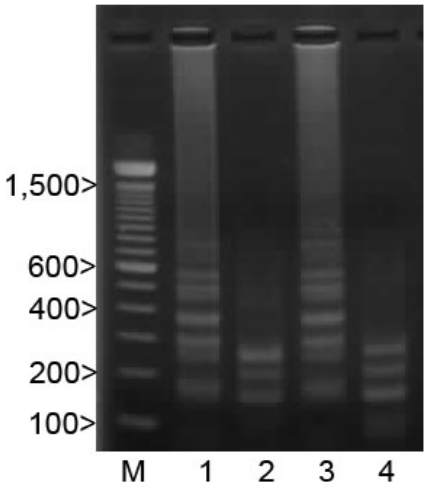


Fig. 6. Specificity of the WSSV LAMP assay. The reaction was carried out at 65ºC for 60 min using six primers. M: 100-bp DNA molecular weight marker; 1: WSSV DNA; 2: IHHNV DNA; 3 HPV DNA; 4: MBV DNA; 5: BP DNA; 6: TSV cDNA; 7: YHV cDNA; 8: IMNV cDNA; 9: NHPB DNA; 10: shrimp genomic DNA; 11: negative control.

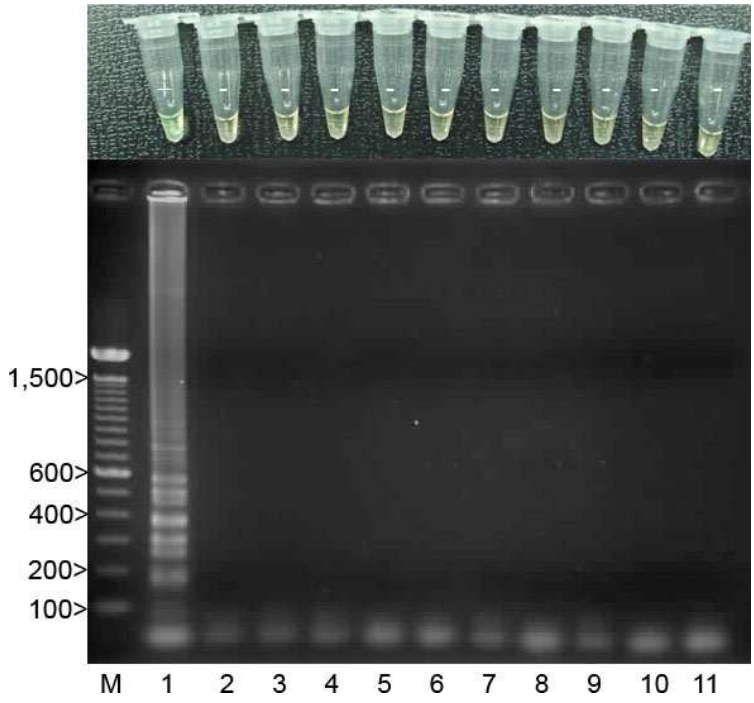


Fig. 7. Comparison of the sensitivity of (A) the LAMP assay and (B) nested PCR using 10-fold serial dilutions of WSSV DNA from a recombinant plasmid. The amplification shows a ladder-like pattern and detected purified WSSV DNA at a dilution of 10 copies. Nested PCR product for WSSV DNA is 941 bp. M: 100-bp DNA molecular weight marker; 1–6: dilutions of 10^5, 10^4, 10^3, 10^2, 10 and 1 viral copy, respectively; 7: negative control; (+): the color change to green, i.e. positive reaction of the visualized LAMP; (–): the starting orange color remains unchanged.

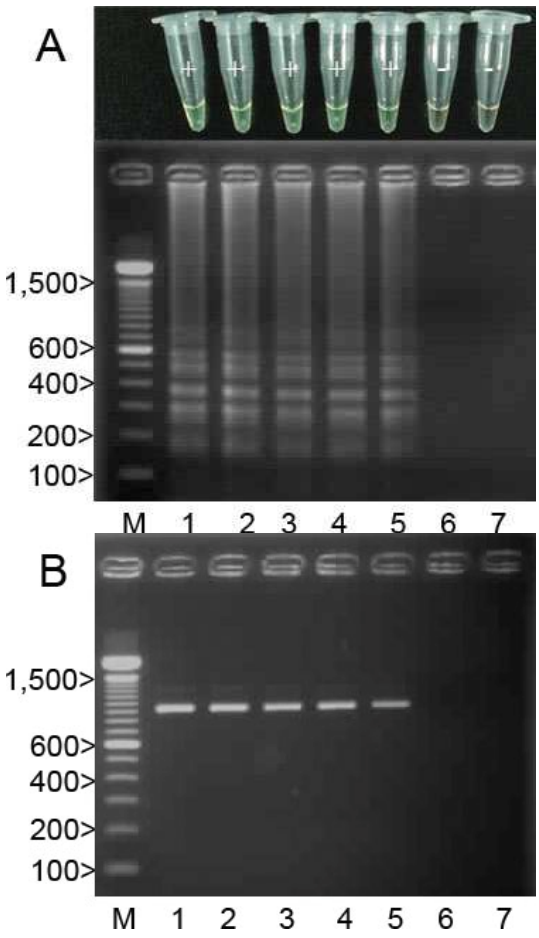
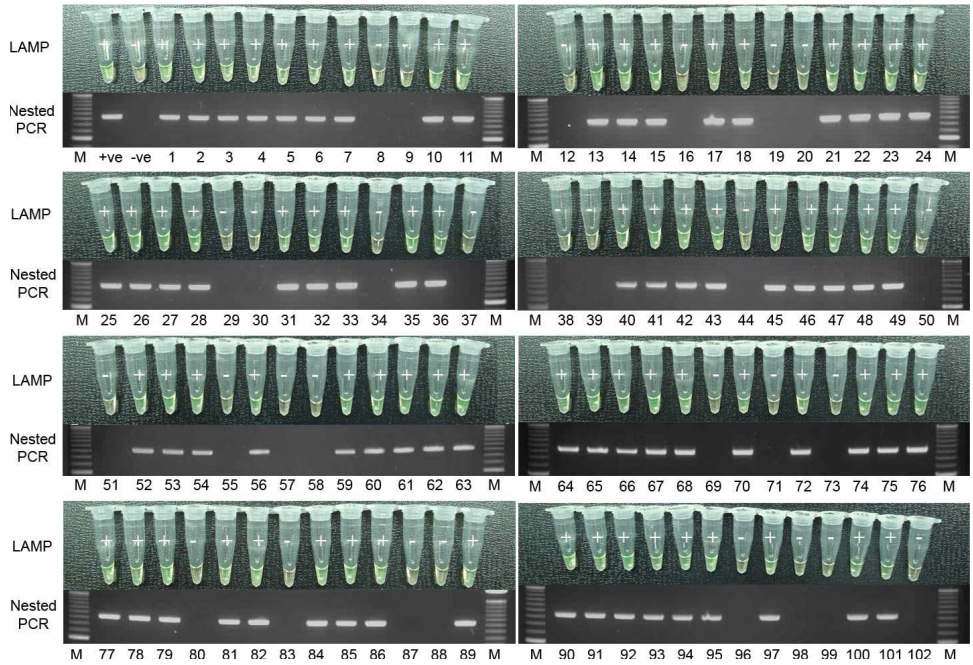


Fig. 8. Field sample detection with LAMP and nested PCR. A total of 102 shrimp samples were examined for WSSV by visualized LAMP and nested PCR. LAMP positive tubes show green fluorescence, whereas negative tubes remain orange. Nested PCR product for WSSV DNA is 941 bp. M: 100-bp DNA molecular weight marker; +ve: positive control; –ve: negative control; 1–102: shrimp DNA samples; (+): the color change to green, i.e. positive reaction of the visualized LAMP; (–): the starting orange color remains unchanged.



การตรวจวินิจฉัยโรคดวงขาวในกุ้งทะเลอย่างรวดเร็ว ด้วยเทคนิค loop-mediated isothermal amplification

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บทคัดย่อ

โรคดวงขาว (white spot disease) เกิดจากเชื้อไวรัส white spot syndrome virus (WSSV) ซึ่งก่ออันตรายร้ายแรงที่สุดในอุตสาหกรรมการเลี้ยงกุ้งทะเลทั่วโลกโดยทำให้มีอัตราการตายสูงมาก ในการศึกษาครั้งนี้ ได้พัฒนาการตรวจเชื้อ WSSV อย่างรวดเร็วด้วยเทคนิค loop-mediated isothermal amplification (LAMP) โดยใช้ไพรเมอร์ 6 ไพรเมอร์ ที่ออกแบบให้มีความจำเพาะ สามารถจับได้กับสารพันธุกรรม 8 กลุ่มเป้าหมาย การเพิ่มจำนวนสารพันธุกรรมเกิดขึ้นพร้อมกันกับการเติมสารเรืองแสง (fluorescence reagent) ได้แก่ calcein และ $MnCl_2$ ลงไปในหลอดทดสอบเดียวกัน ก่อนวางหลอดทดลองในอ่างน้ำที่อุณหภูมิ 65°C คงที่เป็นเวลา 60 นาที เมื่อปฏิกิริยาสิ้นสุดสมบูรณ์จะปล่อยสารเรืองแสงสีเขียวทันที สามารถเห็นผลบวกได้ด้วยตาเปล่า โดยไม่ต้องอาศัยอุปกรณ์หรือรีเอเจนต์เสริมใดๆ กระบวนการตรวจวินิจฉัยโดยวิธีนี้พบว่ามีความจำเพาะสูงต่อ WSSV แต่ให้ผลลบต่อเชื้อไวรัสก่อโรคอื่นๆ ในกุ้ง ได้แก่ Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV), Hepatopancreatic Parvovirus (HPV), Monodon-type Baculovirus (MBV), Baculovirus penaei (BP), Taura Syndrome Virus (TSV), Yellow Head Virus (YHV), และ Infectious Myonecrosis Virus (IMNV) และสารพันธุกรรมของกุ้งเอง นอกจากนี้ยังมีความไวเทียบเท่ากับการตรวจด้วยวิธี nested polymerase chain reaction (nested PCR) แต่นับได้ว่าเป็นวิธีที่มีข้อได้เปรียบมากกว่า nested PCR เพราะนอกจากมีความจำเพาะมีราคาถูกแล้ว ยังปราศจากอันตรายจากการสัมผัสสาร ethidium bromide ซึ่งเป็นสารก่อมะเร็ง ที่ใช้ในขั้นตอน electrophoresis ของวิธี nested PCR

คำสำคัญ : โรคดวงขาว White spot syndrome virus Loop-mediated isothermal amplification กุ้งทะเล