

AN OPTIMIZED LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) ASSAY FOR THE DETECTION OF WHITE SPOT SYNDROME VIRUS (WSSV) AMONG CULTURED SHRIMPS IN THE PHILIPPINES

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ABSTRACT

Loop-mediated isothermal amplification (LAMP) technique is an emerging diagnostic tool for the rapid and sensitive detection of pathogens in aquaculture. In this study, the LAMP assay was evaluated for the detection of the white spot syndrome virus (WSSV), a serious viral pathogen affecting all life stages of penaeid shrimps. The optimum time and temperature conditions for LAMP were 60 min and 65°C, respectively. The assay was highly specific for WSSV and did not cross-react with other shrimp viruses including monodon baculovirus (MBV), infectious hypodermal and hematopoietic necrosis virus (IHHNV) and hepatopancreatic parvovirus (HPV). The limit of detection of the WSSV using the LAMP assay was 5 pg of DNA ml⁻¹ or 5 fg of the genomic DNA per LAMP reaction and was 10 times more sensitive than conventional PCR in detecting the viral pathogen from infected samples. *In vivo*, both LAMP and conventional PCR were able to detect the virus from tissues of infected shrimp postlarvae. Our results demonstrated that LAMP is a simple and sensitive method that has potential application as a diagnostic tool in the field for routine detection of WSSV in shrimp aquaculture in the Philippines.

Key words: WSSV, white spot syndrome virus, loop-mediated isothermal amplification, LAMP, shrimp.

INTRODUCTION

There are more than 20 viruses that infect both wild and cultured penaeid shrimps (Hernandez-Rodriguez *et al.*, 2001). The white spot syndrome virus (WSSV) is one of the widespread and devastating viruses that has affected the shrimp culture industry. This virus does not only infect shrimps but other crustaceans including crabs and crayfish that are found in both freshwater and marine environment (Lo *et al.*, 1996). It affects all life stages of the shrimps (Karunasagar *et al.*, 1997) and mortalities could reach up to 100% in a short period of time, *e.g.*, 3-10 days (Takahashi *et al.*, 1994; Wang *et al.*, 1995). WSSV has been detected in the postlarvae of both wild and hatchery-reared shrimps (Lo *et al.*, 1997; Hao *et al.*, 1999). Thus, the disease caused by this viral pathogen is not only a significant threat to the shrimp culture industry but to the marine ecology as well (Flegel, 1997).

Detection of shrimp pathogens at an early stage is crucial towards effective management in any aquaculture operation. In cases when there is the presence of the pathogen in the cultured stock, it provides an important information in deciding whether there is a need to continue the culture operation or to remedy the situation by several management procedures. Whatever decisions that will be made are all geared towards preventing huge economic losses in a particular aquaculture activity.

The use molecular methods including dot blot assays, *in situ* hybridization and polymerase chain reaction (PCR) has been developed to diagnose a wide array of shrimp diseases caused by different pathogens (Flegel, 2006). In the case of WSSV, the detection of this virus at an early stage has been made possible through conventional PCR, which has been optimized by several research laboratories in shrimp-producing regions (Takahashi *et al.*, 1996; Tapay *et al.*, 1999; Otta *et al.*, 2003). A more sensitive and specific PCR-based technique known as the real-time quantitative PCR has also been developed for shrimp pathogens (Durand *et al.*, 2003).

Despite the advantages on the use of PCR for virus detection in shrimp, there are limitations on the use of this technique such as not being suitable for testing in field conditions, time consuming and the requirement of sophisticated and sensitive equipment that are not readily available in most shrimp farms. Hence, other detection methods have been standardized to overcome the limitations that conventional PCR pose for pathogen detection. A recent molecular detection technique known as the loop-mediated isothermal amplification (LAMP) was developed by Notomi *et al.* (2000). This assay is highly sensitive, specific and can be carried out under isothermal conditions within 1 h. This reaction involves an autocycling strand displacement during DNA synthesis using the *Bst* DNA polymerase and a set of four

primers that recognizes six distinct sequences of the target region. The amplification products consist of stem-loop DNAs having inverted repeats of the target and appear as several bands of different sizes when visualized during gel electrophoresis. Moreover, the presence or absence of the pathogen can be directly observed due to the presence of a white precipitate formed from magnesium pyrophosphate (Mori *et al.*, 2001) or by the addition of some stains (Iwamoto *et al.*, 2003).

The use LAMP assay for diagnosing virus diseases in shrimps has been developed for a number of viruses (Mekata *et al.*, 2006; Pillai *et al.*, 2006; Sun *et al.*, 2006; Kiatpathomchai *et al.*, 2007; Nimitphak *et al.*, 2008; Chaivisuthangkura *et al.*, 2009; Puthawibool *et al.*, 2009; He and Xu, 2011). In the case of WSSV, the LAMP assay was developed by Kono *et al.* (2004). However, strain variations of this virus are present (Molina-Garza *et al.*, 2008; Pradeep *et al.*, 2008; Park and Shin, 2009), thus, it is important that detection assays have to be standardized for a particular geographical isolate of the virus. This has been done in our previous work on the detection of the Philippine isolate of the monodon baculovirus (MBV) (Caipang *et al.*, 2011 b). Hence, in the present study we standardized the LAMP assay for the detection of the Philippine isolate of WSSV using the endonuclease as the target gene. Moreover, we compared the efficiency of this method with conventional PCR in terms of specificity, sensitivity and *in vivo* detection.

MATERIALS AND METHODS

Sources of samples and DNA extraction: Shrimp postlarvae that were infected with WSSV were collected from a hatchery in Iloilo, Philippines (Central Philippines). Individual samples were placed in microfuge tubes containing 1 ml of DNA extraction buffer (Caipang *et al.*, 2004) for the subsequent extraction of genomic DNA.

Genomic DNA of the infected shrimps was extracted following the procedures of Caipang *et al.* (2011 a). The resulting DNA pellet was resuspended in 1x TE buffer (pH 7.5), quantified using a commercial kit following the manufacturer's protocol (Molecular Probes, Invitrogen, USA), aliquoted and stored at -20°C for subsequent LAMP assays.

Design of LAMP primers: LAMP primers targeting the Philippine isolates of WSSV were designed following Notomi *et al.* (2000). The primers were generated using the Primer Explorer software (<http://primerexplorer.jp/e>), each consisting of the sequences: F3 (5'-ATTTCTTTGCTCGAGGCC-3'), B3 (5'-CTAGATATTCCTTCTTCTGTGT-3'), FIP (5'-GGTACTGCATCTCTAGAGCAAAATTTTCGGCTGGAGATTTTTCC-3') and BIP (5'-ATTGAAAATCGTGCA

AGAACTACGTTTTGGTAAAATATTGGTCCAGTCTCA-3'). The outer primers are composed of F3 and the complementary sequence of B3. The forward inner primer, FIP consisted of the complementary sequence of F1, a TTTT-linker and F2. The backward inner primer, BIP is composed of B1, a TTTT-linker and the complementary sequence of B2. Both the FIP and BIP primers structure "the loop" through during the reaction, whereas the F3 and B3 are responsible for the strand displacement during DNA synthesis at the early stages of the LAMP reaction (Notomi *et al.*, 2000).

LAMP assay: The LAMP assay was carried out in a 25 µl reaction volume, consisting of: 12.5 µl reaction mix with 2x Thermopol buffer (New England Biolabs), 8 mMol L⁻¹ MgSO₄, 0.8 mMol L⁻¹ betaine (Sigma), 2 mMol L⁻¹ dNTP (Invitrogen), 2 µl of the FIP and BIP primers (20 pMol), 1 µl of F3 and B3 primers (5 pMol), 1 µl of the *Bst* DNA polymerase (8 U), 2 µl of the DNA template and 3.5 µl of distilled water.

The reaction was carried out under the following conditions: heating at 95°C for 5 min and then incubating at 65°C for 15, 30, 45 and 60 min. The reaction was terminated by heating the samples at 80°C for 2 min. Initial tests at temperatures ranging 60-65°C showed that 65°C was the optimum temperature for the LAMP assay, hence was used in succeeding reactions. The LAMP reaction products were electrophoresed on a 1.5% agarose gel, stained with SYBR Safe (Invitrogen) and photographed using gel documentation system (Biorad). Direct visualization was also done by adding 2 µl of 1:100 dilution of SYBR Safe (Caipang *et al.*, 2011 b) to the LAMP reaction products and placed under a handheld UV densitometer.

The specificity of the LAMP assay was assessed using DNA samples of shrimps infected with other shrimp viruses including monodon baculovirus (MBV), infectious hypodermal and hematopoietic necrosis virus (IHHNV) and hepatopancreatic parvovirus (HPV). The sensitivity of the assay was done using 10-fold serial dilutions of the genomic DNA infected with WSSV adjusted to an initial concentration of 50 ng ml⁻¹ using phosphate buffered saline (PBS) solution. The serially diluted samples were used as templates (2 µl) for the LAMP assay following the conditions described above and incubated for 60 min. The resulting products were subjected to both gel electrophoresis and direct visualization.

Detection of WSSV from infected samples: The LAMP assay was carried out using genomic DNA samples of shrimp larvae that were infected with WSSV. The genomic DNAs from whole larvae were isolated and quantified as described previously. Samples that were not infected with the virus were used as controls. Each group is composed of seven samples and the results of the

LAMP assay were compared with conventional PCR for WSSV detection.

Conventional PCR. Genomic DNA of WSSV-infected samples that were used for the LAMP assays were also subjected to conventional PCR for the detection of the viral pathogen. The primers, (WSSV-forward: 5'-GTACGGCAATACTGGAGGAGGT-3' and WSSV-reverse: 5'-GGAGATGTGTAAGATGGACAAG-3') (Flegel, 2006) were used for the assay. These primers amplified a 232-bp fragment of the WSSV genomic DNA.

The PCR reaction mixture consisted of 2 μ l of each primer (5 pmol), 2 μ l of 10x PCR buffer, 1.5 μ l of 2 mM dNTP, 1 μ l of 15 mM MgCl₂, 0.1 μ l of *Taq* DNA polymerase (100 units) (Invitrogen, U.S.A.), 2 μ l of the DNA template and scaled up to 20 μ l using distilled water. PCR amplification was carried out using the following conditions: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55 for 30 sec and elongation at 72°C for 1 min; then a final elongation at 72°C for 5 min. PCR products (4 μ l) were electrophoresed on a 1.0% agarose gel with 0.5% TBE electrophoresis buffer for 30 min, visualized using a densitometer (Biorad) and photographed.

RESULTS AND DISCUSSION

The LAMP primers targetting a fragment of the endonuclease gene of the Philippine isolate of WSSV resulted in the detection of the virus as visualized under ultra-violet light (Fig. 1). The positive samples stained brightly when viewed using UV in contrast with the faint/absence of the coloration in samples negative for WSSV.

The LAMP assay was optimized at 65°C, and different incubation times were tested to determine the appearance of the LAMP products. Laddering patterns in the gel electrophoresis were observed at an incubation time of at least 45 min but distinct patterns were seen at an incubation time of 1 h (Fig. 2). The polymerase that was used for the LAMP assay was *Bst* DNA polymerase, which has a strand displacement activity. This enzyme has an optimum activity at an incubation temperature of 65°C (Li *et al.*, 2010) and the LAMP assay that we developed for the detection of WSSV in shrimps has an optimum temperature at this value. However several LAMP assays for other fish and shrimp pathogens also showed that this enzyme could amplify the target sequence at lower temperatures (Parida *et al.*, 2004; Kulkarni *et al.*, 2009; Caipang *et al.*, 2011 b). An efficient amplification of this enzyme at a wide temperature range, *i.e.*, 60-65°C ensures that during the application of this assay in the field, the occurrence of slight temperature variations will not have adverse effects on efficiency of the reaction. This situation

usually takes place when water bath apparatus or block heaters are used where precision in temperature varies considerably (Li *et al.*, 2010).

The use LAMP for rapid detection of pathogens has the following advantages, including: it does not require expensive equipment and the assay is carried out at isothermal conditions (Notomi *et al.*, 2000). For the detection of WSSV, the results could be obtained as early as 45 min after incubation, and this was considerably faster than either conventional or nested-PCR.

In terms of specificity, the LAMP assay was only specific for the detection of WSSV and not the other viral pathogens of shrimp, including MBV, IHNV or HPV (Fig. 3a). Conventional PCR also had the same level of specificity in detecting WSSV (Fig. 3b). No cross-reactions were observed with the unrelated shrimp viruses when using either LAMP assay or conventional PCR.

In terms of sensitivity, the LAMP assay that we developed was able to detect the WSSV in infected samples at a concentration of 5 pg ml⁻¹ or 5 fg of DNA per PCR reaction (Fig. 4a). On the other hand, conventional PCR has limit of detection until 50 pg ml⁻¹ or 10 fg of DNA per PCR reaction (Fig. 4b). The LAMP assay was 10 times more sensitive than conventional PCR in detecting WSSV in shrimp larvae. For comparison, the LAMP assay that we developed for WSSV was comparable to an earlier study done by Kono *et al.* (2004) using the same virus but different isolates. It has also the same level of sensitivity in detecting HPV (Nimitphak *et al.*, 2008) and MBV (Caipang *et al.*, 2011 b) but the assay was 100 times higher in detecting IHNV (Sun *et al.*, 2006).

Both the LAMP (Fig. 5a) and conventional PCR (Fig. 5b) were able to detect the viral pathogen in the genomic DNA of all infected shrimps. This indicates that the viral load in all infected samples was within the sensitivity limit for both assays, hence, it facilitated the detection of the virus.

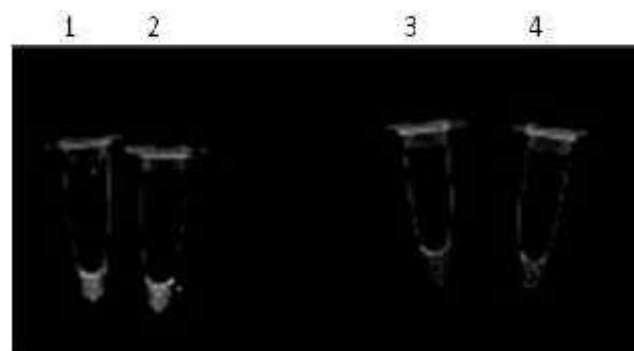


Fig. 1. Detection of WSSV by the LAMP reaction through visualization under UV light after staining with SYBR Safe dye. Tubes 1 and 2 correspond to the WSSV-positive samples, while tubes 3 and 4 are the negative control.

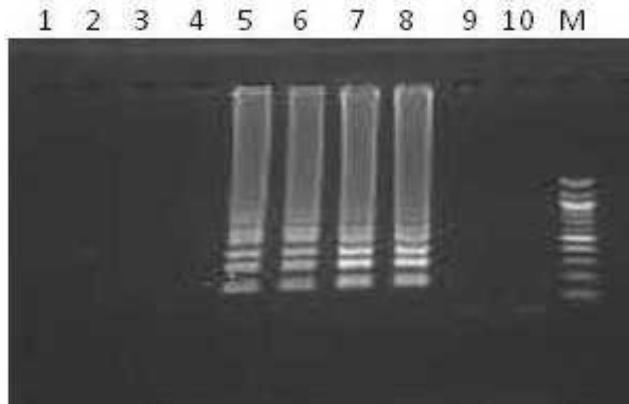
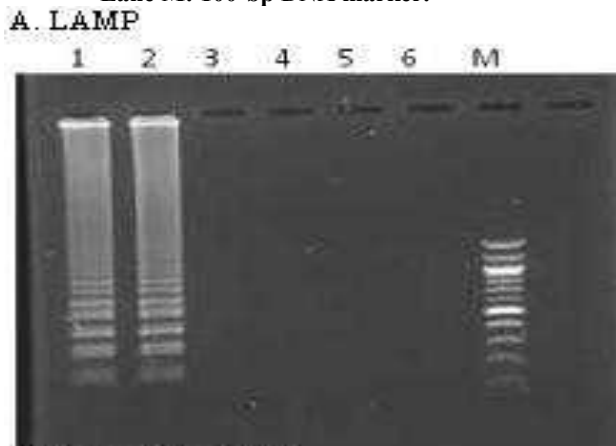


Fig. 2. Optimization of the amplification time for the LAMP assay at an incubation temperature of 65°C in agarose gels. Lanes 1-2: 15 min, Lanes 3-4: 30 min, Lane 5-6: 45 min, Lane 7-8: 60 min, Lane 9-10: negative control (distilled water) and Lane M: 100-bp DNA marker.



A. LAMP



B. Conventional PCR

Fig. 3. Specificity of the LAMP assay for the detection of WSSV by gel-electrophoresis. Lanes 1-2: WSSV, Lane 3: MBV, Lane 4: IHHNV, Lane 5: HPV, Lane 6: negative control (distilled water) and Lane M: 100-bp DNA marker.

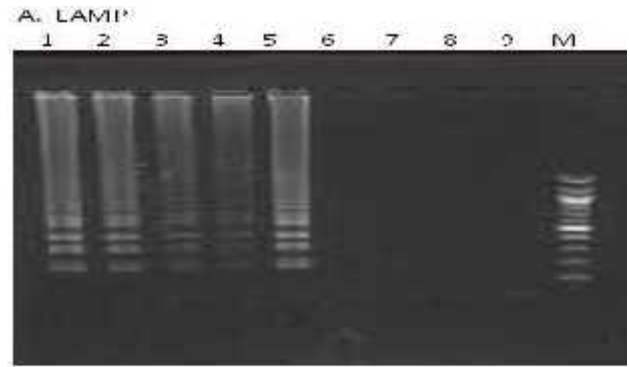


Fig. 4. Sensitivity of (A) LAMP assay and (B) conventional PCR for the detection of WSSV in infected shrimp postlarvae. Lane 1: 50 ng DNA ml⁻¹, Lane 2: 5 ng DNA ml⁻¹, Lane 3: 500 pg DNA ml⁻¹, Lane 4: 50 pg DNA ml⁻¹, Lane 5: 5 pg ml⁻¹, Lane 6: 500 fg DNA ml⁻¹, Lane 7: 50 fg DNA ml⁻¹, Lane 8: 5 fg DNA ml⁻¹; Lane 9: negative control (distilled water), Lane M: 100-bp DNA marker.

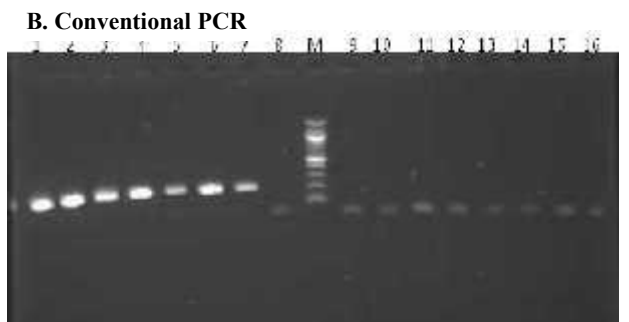
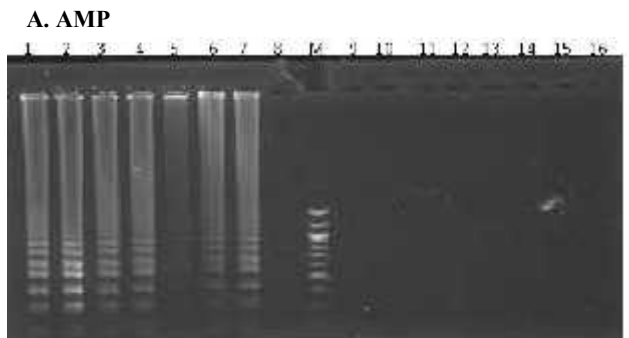


Fig. 5. Detection of WSSV from infected shrimp postlarvae using (A) LAMP assay and (B) conventional PCR. Lanes 1-7: WSSV-infected postlarvae, Lanes 9-15: non-infected postlarvae, Lanes 8 and 16: negative control (distilled water) and Lane M: 100-bp DNA marker.

In conclusion, this study has demonstrated an efficient detection method for the Philippine isolates of white spot syndrome virus (WSSV) in infected shrimp postlarvae using loop-mediated isothermal amplification (LAMP) reaction. The assay is highly specific and has a sensitivity threshold of 5 fg of DNA per PCR reaction. The speed and accuracy of detection have implications in providing effective management procedures in aquaculture especially in cases when crucial decisions have to be made during the early stages of infection. Due to its simplicity, high degree of sensitivity, accuracy and speed of the assay, LAMP has a tremendous potential for field use particularly in the routine diagnosis of WSSV infections in shrimp aquaculture in the Philippines.

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