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Effective health management in shrimp hatcheries: use of loop-mediated isothermal amplification (LAMP) for the rapid detection of Philippine isolates of *Penaeus monodon*-type baculovirus (MBV)

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Abstract. Effective health management in hatcheries is a crucial step towards the prevention of disease outbreaks during the early life stages of the shrimp. Early and sensitive detection of the different pathogens ensure that proper measures are undertaken before massive mortality occurs. In this study, the loop-mediated isothermal amplification (LAMP) assay was evaluated for the detection of the Penaeus monodon-type baculovirus (MBV), a viral pathogen that is common in shrimp hatcheries. The optimized time and temperature conditions for LAMP were 60 min and 63°C, respectively. The assay was highly specific for MBV and did not amplify other shrimp viruses including white spot syndrome virus, infectious hypodermal and hematopoietic necrosis virus (IHHNV) and hepatopancreatic parvovirus (HPV). The sensitivity threshold of LAMP was 10 pg of DNA ml^{-1} or 10 fg of the genomic DNA per LAMP reaction and was 10 times more sensitive than conventional PCR in detecting MBV from infected shrimp postlarvae. As a possible application for field use, both LAMP and conventional PCR were able to detect the virus from tissues of infected shrimp postlarvae. These results demonstrate that LAMP could be used as a simple and sensitive method for the rapid field diagnostic tool for early detection of Philippine isolates of MBV in shrimp hatcheries.
Key Words: LAMP, hatcheries, monodon baculovirus, MBV, shrimp, *Penaeus monodon*.

Introduction. The *Penaeus monodon*-type baculovirus (MBV) was implicated for the collapse of the shrimp culture industry in Taiwan in the mid-1980's (Lin 1989), and has since been found in various parts of the world (Fegan et al 1991; Belcher & Young 1998; Madhavi et al 2002; de la Peña et al 2005). Despite its wide geograhical occurrence, MBV does not pose a serious threat to shrimp culture industry if proper management in the culture site is ensured to prevent secondary infections (Fegan et al 1991; Flegel 2006). However, shrimp farmers are cautious of preventing MBV infections as these could result in the stunting of the growth leading to a reduction in the price during harvest. Hence, various methods have been developed to facilitate the detection of this pathogen during the early stages of infection.

Shrimp postlarvae that are infected with MBV can easily be detected in squash mounts of the hepatopancreas stained with malachite green (Flegel 2006). The viral inclusion bodies are located in enlarged nuclei and they are stained green upon addition of the chemical. These protein particles protect or enclose the virus, hence they are known as occlusion bodies.

Staining the viral occlusion bodies in infected shrimp postlarvae using hematoxylin and eosin is a cheap detection method. The virus–infected cells had enlarged nuclei with acidophilic occlusion bodies (Flegel 2006). However, heavy infection must occur before a

positive reaction could be observed. Alternative methods that are more sensitive in detecting the virus have been developed including *in situ* hybridization (Poulus et al 1994), polymerase chain reaction (Vickers et al 1992; Chang et al 1993; Belcher & Young 1998; Surachetpong et al 2005) and immunohistochemistry (Boonsanongchokying et al 2006). Presently, the PCR assays that are recommended by the Office International des Epizooties (OIE) reference manual are the protocols developed by Belcher & Young (1998) and by Surachetpong et al (2005).

A recent molecular-based diagnostics method known as the loop-mediated amplification (LAMP) reaction has been developed to detect a wide range of pathogens and can amplify the target region with a high degree of specificity, sensitivity and rapidity at isothermal conditions (Notomi et al 2000). This reaction involves an autocycling strand displacement DNA synthesis by the *Bst* DNA polymerase and a set of four primers that recognize six distinct sequences of the target region. The amplification products are composed of stem-loop DNAs with 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 precipiate formed from magnesium pyrophosphate (Mori et al 2001) or by the addition of some stains (Iwamoto et al 2003). The simplicity of the technique has resulted in its use to detect various viral pathogens of shrimp (Kono et al 2004; Mekata et al 2006; Pillai et al 2006; Sun et al 2006; Kiatpathomchai et al 2007; Nimitphak et al 2008; Puthawibool et al 2009; He & Xu 2011). LAMP has also been developed to detect MBV regardless of its geographical isolate (Chaivisuthangkura et al 2009).

In the Philippines, MBV infections have been diagnosed in most shrimp hatcheries and farms, with an incidence rate of 85-100% of the shrimp postlarvae that were sampled in 1992 (Natividad & Lightner 1992). By 2004, a prevalence rate of 59% was observed in the postlarvae based on fry quality assessment tests (Natividad et al 2006). We have sequenced a fragment of the MBV genomic DNA and showed structural differences between the Philippine isolate with the other geographical isolates (Caipang et al 2011). In order to facilitate the rapid detection of Philippine isolates of MBV from shrimp hatcheries, we designed LAMP primers and optimized the assay that has the potential to be used under field conditions. The isolate-specific detection of MBV from shrimp postlarvae will provide useful information as to the likely origin of the virus and thus effective screening and health management procedures could be employed to prevent the spread of the virus from the hatcheries to the culture site.

Materials and Methods

Sources of samples and DNA extraction. MBV-infected shrimp postlarvae 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) and kept at room temperature for subsequent extraction of genomic DNA.

Genomic DNA of the infected shrimps was extracted following the procedures described by Caipang et al (2011). The resulting DNA pellet was resuspended in 1x TE buffer (pH 7.5), quantified using a commercial kit (Molecular Probes, Invitrogen, USA) and stored at -20^{o}C until use for the LAMP assays.

Primer design. LAMP primers that are specific for the Philippine isolates of MBV were designed following Notomi et al (2000). The primers were constructed based on the genomic DNA sequence of MBV that was previously described (Caipang et al 2011). The primers were generated using the Primer Explorer software (http://primerexplorer.jp/e), each consisting of the sequences, F3, B3, FIP and BIP (Table 1). The outer primers are composed of F3 and the complementary sequence of B3. The forward inner primer or the FIP, consisted of the complementary sequence of F1 and F2. The backward inner primer, BIP is composed of B1 and the complementary sequence of B2. Both the FIP and BIP primers structure "the loop" through 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). In addition, a TTTT-linker was added within the sequence of both the FIP and BIP primers.

Optimization of the 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} $MgSO_4$, 0.8 mMol L^{-1} betaine (Sigma), 2 mMol L^{-1} dNTP (Invitrogen), 2 μl of the FIP and BIP primers (20 pMol), 1 μl of F3 and B3 primers (5 pMol), 1 μl of the *Bst* DNA polymerase (8 U), 2 μl of the DNA template and 3.5 μl of distilled water.

Table 1

Primers for the LAMP assay of Philippine isolates of MBV designed from its partial genomic sequence

Primers	*Sequence (5' – 3')*
F3	TGTTCTATACATTTTGCAAAGC
B3	AAAGGAGTGCAGATCTTGA
FIP	AAGGTCAGCAAAAAACACTCAATTTTTTCCTCTACTGATATGGTATCAATG
BIP	AAGAATCACCGGGATCCTTCATTTTAAATCTATATAGCGTTAACACGT

The reaction was carried out under the following conditions: heating at 95°C for 5 min and then incubating at 63°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 63°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 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 white spot syndrome virus (WSSV), 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 MBV adjusted to an initial concentration of 1 μg 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 at 60 min. The resulting products were subjected to both gel electrophoresis and direct visualization.

Detection of MBV from infected samples. The LAMP assay was carried out on genomic DNA samples of shrimp larvae that were infected with MBV. This was to determine the effectivity of this assay in monitoring the status of infection in hatcheries. 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 five samples and the results of the LAMP assay were compared with conventional PCR.

Results and Discussion. The LAMP primers targetting a fragment of the genomic DNA of Philippine MBV resulted in the detection of the virus both by gel electrophoresis (Fig. 1a) and visualization under ultra-violet light (Fig. 1b). The LAMP products showed laddering patterns in the gel electrophoresis, and under UV light the positive samples stained brightly in the presence of the dye in contrast to the faint coloration of the samples negative for the presence of MBV.

The LAMP assay was optimized at 63°C, and different incubation times were tested to determine the appearance of the LAMP products. Faint laddering patterns in the gel electrophoresis were observed at an incubation time of 45 min, while distinct patterns were seen at 60 min of incubation (Fig. 2). *Bst* DNA polymerase has been shown to

exhibit optimum activity at an incubation temperature of 65°C (Li et al 2010), however several LAMP assays demonstrated that this enzyme is able to amplify the target sequence at lower temperatures (Parida et al 2004; Kulkarni et al 2009). An efficient amplification of this enzyme at a lower temperature range (60-64°C) enures that during the application of this assay in the field, the occurrence of slight temperature variations will not adversely affect the reaction. This is especially true when water bath apparatus or block heaters are used where precision in temperature varies considerably (Li et al 2010).

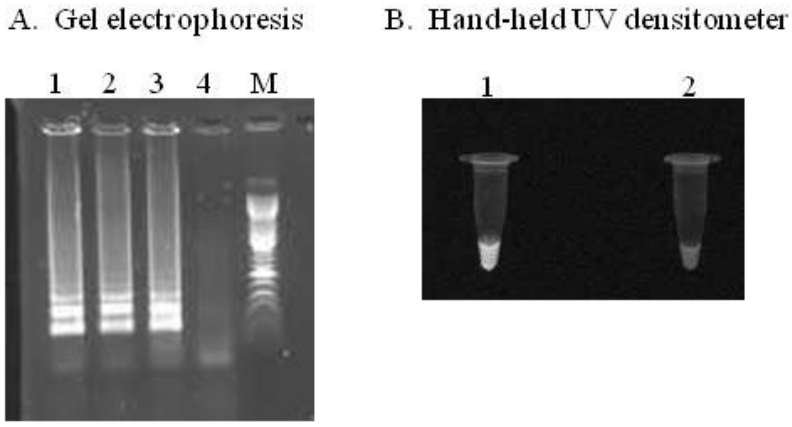

Figure 1. Detection of MBV by the LAMP reaction using (A) gel-electrophoresis and (B) visualization under UV light after staining with SYBR Safe dye. In Figure 1a, lanes 1-3 are MBV-positive samples, lane 4 is the negative control, distilled water and Lane M is the 100-bp DNA marker. In Figure 1b tube 1 corresponds to the MBV-positive sample, while tube 2 is the negative control.

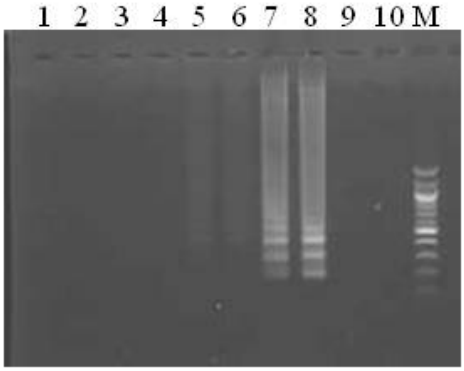

Figure 2. Determination of the amplification time for the LAMP assay at an incubation temperature of 63°C in agarose gels. Lanes 1-2: 15 min, Lanes 3-4: 30 min, Lane 5-6: 45 min, Lane 7-8: 60 min, Lane 9-10: negative control (distilled water) and Lane M: 100-bp DNA marker.

The advantages of using LAMP for rapid detection of pathogens are the following: it does not require expensive equipment and the assay is done at isothermal conditions (Notomi et al 2000). In the case of MBV, the LAMP assay was able to detect the pathogen as early as 45 min after incubation, and this was cosiderably faster than conventional and nested-PCR. The speed and accuracy of detection have implications in providing effective management procedures especially in cases when crucial decisions have to be made in order to prevent massive mortalities.

In terms of specificity, the LAMP assay was only specific for the detection of MBV and not the other viral pathogens of shrimp, including WSSV, IHHNV or HPV (Fig. 3). In

terms of sensitivity, the LAMP assay that we developed was able to detect the MBV in infected samples at a concentration of 10 pg ml^{-1} or 10 fg of DNA per PCR reaction (Fig. 4a). On the other hand, using the published PCR primers to detect Philippine isolates of MBV (Caipang et al 2011), the limit of detection was 100 pg ml^{-1} or 10 fg of DNA per PCR reaction. The LAMP assay was 10 times more sensitive than conventional PCR in detecting MBV in shrimp larvae. For comparison, the LAMP assay developed for WSSV was 10 times more sensitive than conventional PCR in detecting WSSV (Kono et al 2004) and HPV (Nimitphak et al 2008) as well as 100 times higher in IHHNV (Sun et al 2006). The assay was also 100 times more sensitive than nested PCR in detecting MBV regardless of the source of the isolate (Chaivisuthangkura et al 2009).

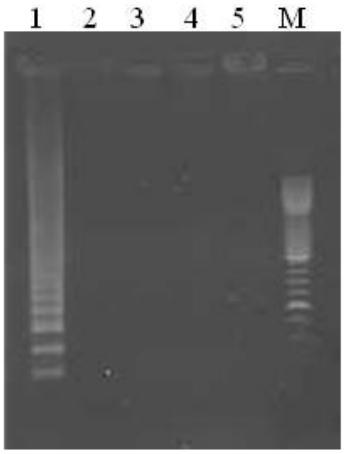

Figure 3. Specificity of the LAMP assay for the detection of MBV by gel-electrophoresis. Lane 1: MBV, Lane 2: WSSV, Lane 3: IHHNV, Lane 4: HPV, Lane 5: negative control (distilled water) and Lane M: 100-bp DNA marker.

Using genomic DNA from shrimp larvae infected with MBV as well as from non-infected samples, both the LAMP (Fig. 5a) and the conventional PCR (Fig. 5b) assays were able to detect the viral pathogen in all infected shrimp. This indicates that the viral load in all infected samples was within the sensitivity limit for both assays, hence, the virus was detected.

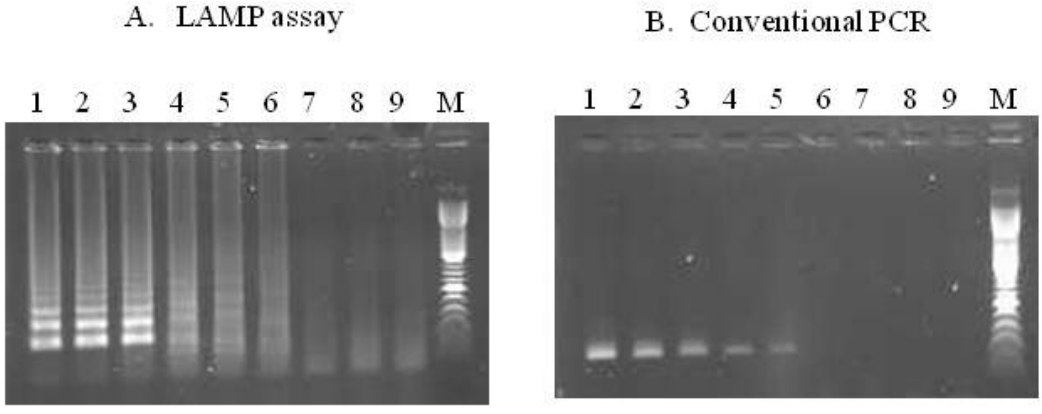

Figure 4. Sensitivity of (A) LAMP assay and (B) conventional PCR for the detection of MBV in infected shrimp postlarvae. Lane 1: 1 μg DNA ml^{-1}, Lane 2: 0.1 μg DNA ml^{-1}, Lane 3: 10 ng DNA ml^{-1}, Lane 4: 1 ng DNA ml^{-1}, Lane 5: 100 pg ml^{-1}, Lane 6: 10 pg DNA ml^{-1}, Lane 7: 1 pg DNA ml^{-1}, Lane 8: 100 fg DNA ml^{-1}; Lane 9: negative control (distilled water), Lane M: 100-bp DNA marker.

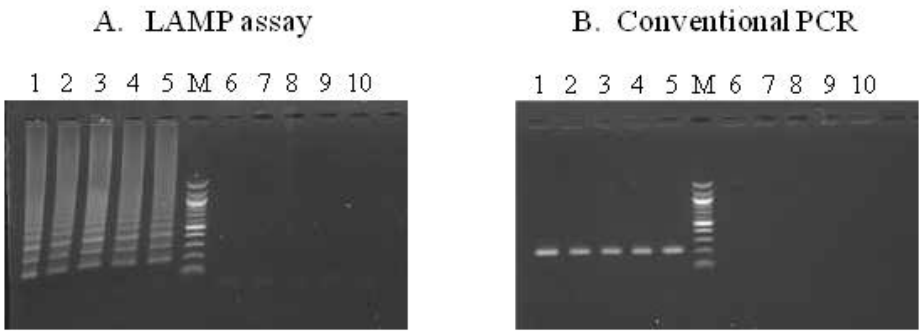

Figure 5. Detection of MBV from infected shrimp postlarvae using (A) LAMP assay and (B) conventional PCR. Lanes 1-5: MBV-infected larvae, Lanes 6-10: non-infected larvae and Lane M: 100-bp DNA marker.

Conclusions. This study has demonstrated an effective detection method for Philippine isolates of the *Penaeus monodon*-type baculovirus (MBV) in infected shrimp postlarvae using loop-mediated isothermal amplification (LAMP) reaction. The assay is highly specific and has a sensitivity threshold of 10 fg of DNA per PCR reaction (2 μl of DNA template in a PCR reaction). Due to its simplicity, high degree of sensitivity, accuracy and speed of the assay, LAMP has tremendous potential for field use particularly in the routine diagnosis of MBV infections in shrimp hatcheries in the Philippines.

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