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Rapid Detection of the Philippine Isolate of Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) in Shrimp, *Penaeus monodon* Using Loop-Mediated Isothermal Amplification (LAMP)

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Abstract: The aim of this study was to standardize a Loop-Mediated isothermal Amplification (LAMP) assay for the detection of the Philippine isolate of Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) in postlarvae of shrimp, *Penaeus monodon*. The assay was optimized at an incubation time of 1 h at 63°C. The assay was highly specific for IHHNV and did not cross-react with other shrimp viruses including Hepatopancreatic Parvovirus (HPV), Monodon Baculovirus (MBV) and White Spot Syndrome Virus (WSSV). The limit of detection of the IHHNV using the LAMP assay was 10 pg of DNA/mL or 10 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. These results demonstrated that LAMP is a simple and sensitive diagnostic technique that has potential application for routine detection of IHHNV infections in shrimp hatcheries in the Philippines.

Keywords: IHHNV, LAMP, *Penaeus monodon*, shrimp

INTRODUCTION

Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) is a single-stranded DNA virus that belongs to the Parvoviridae family (Bonami *et al*., 1990). Its virions are non-enveloped icosahedrons with size of approximately 22 nm in diameter and has a genome size of 4.1 kb with three Open Reading Frames (ORFs), coding for a non-structural protein, a capsid protein and a third ORF of unknown function (Shike *et al*., 2000). Moreover, this virus is closely related to the mosquito brevidensoviruses.

IHHNV is one of the important viral pathogens in shrimp. It affects two of the most important shrimps species, *Litopenaeus stylirostris* and *L. vannamei*. Infection with this virus can lead up to 90% mortality in *L. stylirostris* juveniles and sub-adults during a disease episode (Lightner *et al*., 1983). Though not lethal to *L. vannamei*, the virus causes growth reduction and severe cuticular deformities in the affected shrimp, which is also known as the runt deformity syndrome (Kalagayan *et al*., 1991); thus, reducing the market value of the shrimp by 10-50%, depending on the severity of the disease (Lightner and Redman, 1998).

This virus is widely distributed in both cultured and wild shrimp populations in the Americas, Asia and Oceania (Nunan *et al*., 2000; Tang and Lightner, 2002).

It was first detected in Hawaii in 1981 from *L. stylirostris* stocks imported from Costa Rica and Ecuador (Lightner *et al*., 1983). Its presence was confirmed in the Gulf of California, Mexico in 1987 (Lightner *et al*., 1992) and has caused epizootics in both wild and farmed populations of *L. stylirostris* (Pantoja *et al*., 1999). The prevalence of this virus among wild populations of *L. stylirostris* in the Gulf of California reached almost 100% by 2005 (Robles-Sikisaka *et al*., 2010), but, massive mortalities due to its infection have not been observed since the epizootics in 1990 (Morales-Covarrubias *et al*., 1999).

In the Philippines, IHHNV has been detected from both *Penaeus monodon* and *L. vannamei* based on the sequences of the IHHNV capsid protein gene that have been deposited in public databases (Caipang *et al*., 2011b). The ban on the importation of *L. vannamei* has been lifted recently as a means to revive the ailing shrimp industry in the country (Aguiba, 2007). The lifting of the ban means that there must be strict monitoring and screening of the imported shrimp stock for the presence of the virus. Hence, rapid and sensitive detection methods of the pathogen must be developed so that proper monitoring of the presence of the virus can be implemented.

A recent molecular-based diagnostics technique known as the Loop-Mediated Amplification (LAMP)

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detects 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) The reaction is carried out through an autocycling strand displacement DNA synthesis by the Bst DNA polymerase and a set of four primers that bind six distinct sequences of the target region. The amplification products consist of stem-loop DNAs with inverted repeats of the target and appear as several bands of different sizes when viewed under UV light following 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 simplicity of the technique has resulted in its use for the detection of 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 and Xu, 2011). LAMP has also been developed to detect MBV regardless of its geographical isolate (Chaivisuthangkura *et al*., 2009). In the present study, the LAMP assay for the detection of the Philippine isolate of IHHNV using the capsid protein gene as target was standardized. Moreover, the efficiency of this method was compared with conventional PCR in terms of specificity and level of sensitivity.

MATERIALS AND METHODS

Sources of samples and DNA extraction: The study was conducted at the laboratory of the National Insitute of Molecular Biology and Biotechnology (NIMBB), UP Visayas on July-September, 2011. Shrimp postlarvae were collected from a hatchery in Iloilo, Philippines (Central Philippines). Individual postlarvae 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 postlarvae was extracted following the procedures described by Caipang and Aguana (2011a). The DNA pellet was resuspended in 1xTE 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 targeted the capsid protein gene of the Philippine isolate of IHHNV from a previous study (Caipang *et al*., 2011b) were designed following Notomi *et al*. (2000). The primers were generated using the Primer Explorer software (http://primerexplorer.jp/e) and consisted of the sequences, F3, B3, FIP and BIP. The sequences of the primers are the following (in the 5'-3' direction): F3-:

TCAATACATGTTACTTTCCAAAC;B3-
ATCCGTAGGTTTTCATCATTG;FIP-
TGGGAGGCAGTATAAATTTTTTCCGTTTTGATA
CTTCGAATTCGACGCT;BIP-
TATCTCTATGGTCTGAAGAGCAGTTTTTTTA
CCATTTTATATCGCTGTGTTTC.

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. The FIP and BIP primers structure "the loop" during the LAMP reaction, whereas the F3 and B3 are responsible for the strand displacement during DNA synthesis (Notomi *et al*., 2000). 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, composed of: 12.5 µL reaction mix containing 2x Thermopol buffer (New England Biolabs), 8 mMoL/L MgSO4, 0.8 mMoL/L betaine (Sigma), 2 mMoL/L 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), 1 µL of the DNA template and 4.5 µL of distilled water.

The assay was carried out by heating the samples at 95^{o}C for 5 min and then incubating for 60 min for 63^{o}C. The reaction was terminated by heating at 80^{o}C for 2 min. Initial tests at temperatures ranging 60-65^{o}C showed that 63^{o}C was the optimum temperature for the LAMP assay of IHHNV, 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 a gel documentation system (Biorad). Direct visualization was also done following the protocol of an earlier study (Caipang *et al*., 2011c).

The specificity of the LAMP assay was assessed using DNA samples of shrimp postlarvae infected with other shrimp viruses such as Monodon Baculovirus (MBV), White Spot Syndrome Virus (WSSV) and Hepatopancreatic Parvovirus (HPV). The sensitivity of the assay was done using 10-fold serial dilutions of the genomic DNA infected with IHHNV adjusted to an initial concentration of 0.1 µg/Ml in 1x Phosphate Buffered Saline (PBS) solution. The serially diluted

samples were used as templates (2 µL) for the LAMP assay following the conditions described above. The sensitivity of the LAMP assay was compared with conventional PCR using the primers and thermal cycling conditions of Yang *et al*. (2007). The resulting LAMP and PCR products were subjected to gel electrophoresis. Direct visualization of the LAMP products was also carried out.

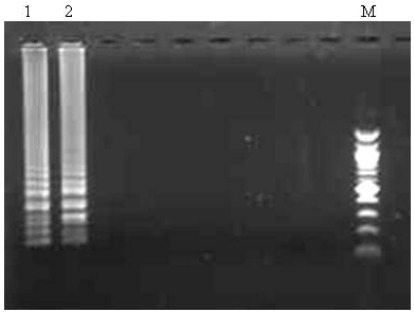
RESULTS AND DISCUSSION

The LAMP primers that targeted a fragment of the capside protein gene of a Philippine isolate of IHHNV resulted in the detection of the viral pathogen both by gel electrophoresis (Fig. 1a) and visualization under ultra-violet light (Fig. 1b). During gel electrophoresis, the LAMP products showed laddering patterns and under UV light the positive samples showed intense staining in the presence of the dye in contrast to the faint coloration observed in the negative samples.

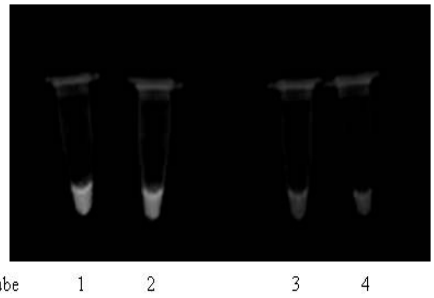
The LAMP assay was optimized at 63^{o}C for 1 h. It has been shown that Bst DNA polymerase exhibited optimum activity at an incubation temperature of 65 ^{o}C (Li *et al*., 2010), but several LAMP assays demonstrated that this enzyme is able to amplify the target sequence at lower temperatures (Parida *et al*., 2004; Kulkarni *et al*., 2009; Caipang *et al*., 2011c). The efficient amplification of this enzyme at a lower temperature range, e.g., 60-64^{o}C ensures that 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 temperature varies considerably (Li *et al*., 2010). The significance of this efficient enzyme activity at a wide temperature range enables the LAMP assay to have practical application for field use, where fluctuations in temperature could likely take place due to the use of low precision equipment.

The advantages of using LAMP for rapid detection of pathogens over other detection assays are the following: it does not require expensive equipment and the reaction is carried out at isothermal conditions (Notomi *et al*., 2000). In the case of IHHNV, the LAMP assay can be completed in 1 h, which was considerably faster than conventional and nested-PCR assays. 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.

The LAMP assay was only specific for the detection of IHHNV and no cross-reactions were



(a) Gel electrophoresis



(b) Visualization under UV light

Fig. 1: Detection of IHHNV by LAMP reaction through (a) gel electrophoresis and (b) visualization under UV light after the addition of SYBR safe dye Lanes 1 and 2: Correspond to IHHNV-positive samples; Lane M: 100 bp-DNA marker; Tubes 1 and 2: Correspond to the IHHNV-positive samples; Tubes 3 and 4: The negative control

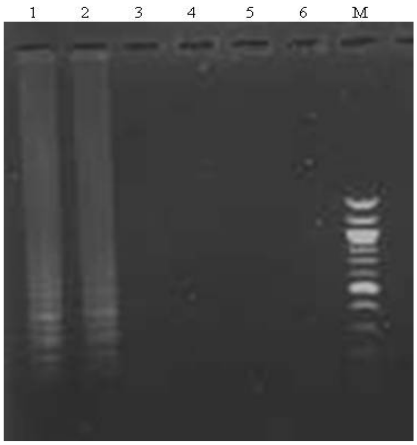
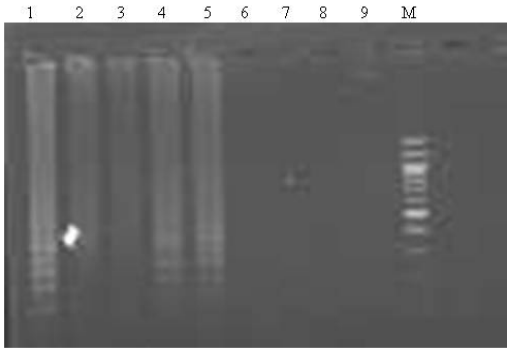
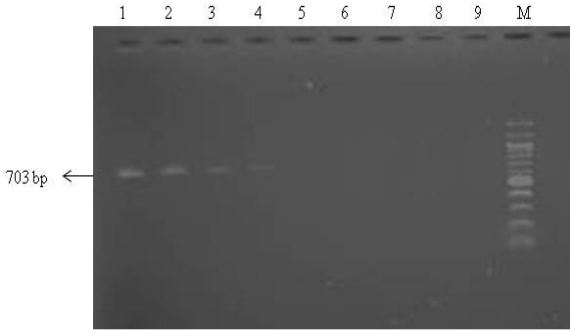


Fig. 2: Specificity of the LAMP assay for the detection of IHHNV by gel-electrophoresis Lanes 1-2: IHHNV; Lane 3: MBV; Lane 4: WSSV; Lane 5: HPV; Lane 6: Negative control (distilled water); Lane M: 100-bp DNA marker



(a)LAMP assay



(b)Conventional PCR

Fig. 3: Sensitivity of (A) LAMP assay and (B) conventional PCR for the detection of IHHNV. Lane 1: 0.1 ug DNA/mL, Lane 2: 10 ng DNA/mL, Lane 3: 1 ng DNA/mL, Lane 4: 100 pg DNA/mL, Lane 5: 10 pg DNA/mL, Lane 6: 1 pg DNA/ mL, Lane 7: 100 fg DNA/mL, Lane 8: 10 fg DNA/mL, Lane 9: negative control (distilled water), Lane M: 100-bp DNA marker.

observed with other viral pathogens of shrimp, including MBV, WSSV and HPV (Fig. 2). In terms of sensitivity, the LAMP assay was able to detect IHHNV in infected samples at a concentration of 10 pg/mL or 10 fg of DNA per PCR reaction (Fig. 3a). On the other hand, using the published PCR primers of Yang *et al*. (2007) to detect IHHNV isolates, the limit of detection was 100 pg/mL or 100 fg of DNA per PCR reaction as shown by the presence of the 703 bp that was amplified during the reaction (Fig. 3b). The LAMP assay was 10 times more sensitive than conventional PCR in detecting the Philippine isolate of IHHNV in shrimp larvae. For comparison, an earlier LAMP assay for the different isolates of IHHNV showed that this method was 100 times more sensitive than conventional PCR in detecting the virus in infected shrimps (Sun *et al*., 2006). As to other shrimp viruses, LAMP assay was 10 times more sensitive than conventional PCR in detecting WSSV (Kono *et al*., 2004) and HPV (Nimitphak *et al*., 2008) Using a Philippine isolate of MBV, the LAMP assay was 10 times more sensitive in detecting the virus compared with conventional PCR (Caipang *et al*., 2011c) and a similar level of sensitivity was obtained in the present study.

CONCLUSION

This study has optimized a Loop-Mediated isothermal Amplification (LAMP) assay to detect the presence of a Philippine isolate of Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) in shrimp postlarvae. The assay is highly specific and has a sensitivity limit of 10 fg of DNA per PCR reaction. The simplicity, high level of sensitivity, accuracy and speed of the assay make the LAMP useful for field use especially during routine diagnosis of IHHNV infections in shrimp hatcheries, aquaculture sites and in the wild.

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