Optimized multiplex PCR assays for simultaneous detection of viruses infecting hatchery-reared shrimp, *Penaeus monodon* in the Philippines

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Abstract. Multiple viral infections occur in most shrimp hatcheries and early detection of the pathogens is necessary to ensure efficient management of the hatchery facilities. The presence of the white spot syndrome virus, *Penaeus monodon*-type baculovirus and the infectious hypodermal and hematopoietic necrosis virus in shrimp, *Penaeus monodon* postlarvae was confirmed simultaneously using multiplex PCR assays based on primers from previously published studies. Different primer combinations and the optimum annealing temperatures were standardized that could be applicable for routine diagnoses of these viral diseases in shrimp. The optimized multiplex PCR assays could be used for regular monitoring and surveillance of these viruses in shrimp hatcheries as well as tracing the movement of these viruses in different shrimp-farming regions in the country during transport of shrimp postlarvae for stocking as well as broodstock for hatchery purposes.

Key Words: hatcheries, postlarvae, crustaceans, WSSV, IHHNV, MBV, detection.

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Introduction

There are more than 20 viruses that affect penaeid shrimps, and these infect both cultured and wild stock in various geographical locations worldwide (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. However, there are other viruses, which are considered less virulent but can cause significant reduction in the profitability of the culture operations. Examples of these are the *Penaeus monodon*-type baculovirus (MBV), which is implicated in the stunted growth of the shrimp (Flegel *et al* 1999) and the infectious hypodermal and hematopoietic necrosis virus (IHHNV), which can lead to growth reduction and severe cuticular deformities in the affected shrimp especially *Litopenaeus vannamei* and *Penaeus monodon*, known as the runt deformity syndrome (Kalagayan *et al* 1991). These deformities and decrease in growth can reduce the market value of the shrimp by 10-50%, depending on the severity of the disease (Lightner & Redman 1998).

These viruses are not only prevalent in shrimp farms but also infect shrimp postlarvae in hatcheries. Dual infections of MBV and WSSV have been observed in wild black tiger shrimp from several sampling sites in the Philippines using PCR (de la Peña *et al* 2005). Infections of WSSV and MBV were also seen in *P. monodon* hatcheries in the Philippines (Natividad *et al* 2006). In similar cases, mixed infections of WSSV and MBV were also reported in other Asian countries like Vietnam (Hao *et al* 1999) and Thailand (Flegel *et al* 2004). In India, Manivannan et al (2002) reported that *P. monodon* post-larvae were simultaneously infected by three different viruses including MBV, hepatopancreatic parvovirus (HPV) and WSSV as detected by histology and non-nested PCR.

In an effort to minimize the spread of viruses in shrimp farms and hatcheries, the Philippine government through the Bureau of Fisheries and Aquatic Resources (BFAR) has modified routine fry quality assessment to include screening for WSSV. Definitive diagnosis and certification of the infection status of *P. monodon* broodstock and fry by WSSV, the use of polymerase chain reaction (PCR) assay is recommended (Natividad *et al* 2006). However, the inclusion of other shrimp viruses including MBV and IHHNV during routine PCR analysis is also done because these viruses can result in reduction in growth of infected shrimp and thus decrease productivity. The simultaneous detection of these shrimp viruses in shrimp postlarvae can be done in a single PCR analysis known as multiplex PCR. There have been several multiplex PCR assays that have been developed to detect commercially important viruses of shrimp. Tsai et al (2002) developed multiplex reverse transcription-polymerase chain reaction (mRT-PCR) for simultaneous detection of WSSV and Taura syndrome virus (TSV) in pacific white shrimp P. vannamei. Xie et al (2007) standardized a multiplex RT-PCR assay to detect IHHNV, TSV and WSSV in penaeid shrimp. Recently, Khawsak et al (2008) used multiplex RT-PCR to detect simultaneously six shrimp viruses including, yellow-head virus (YHV), WSSV, TSV, IHHNV, HPV and MBV.

In the Philippines, a duplex PCR assay was developed to detect WSSV and MBV in shrimp postlarvae (Natividad *et al* 2006). However, multiplex PCR assays that can be easily applicable for the simultaneous detection of viruses in shrimp in the country have not been optimized. Hence in this study, both duplex and multiplex PCR assays have been developed from published individual PCR assays that could allow the simultaneous detection of WSSV, IHHNV and MBV in shrimp postlarvae.

Materials and Methods

Sources of samples and DNA extraction. Shrimp postlarvae (PL 15-20) were collected from different hatcheries in Iloilo, Central Philippines. The samples were placed in ice and immediately transported to the laboratory of the National Institute of Molecular Biology and Biotechnology, UP Visayas for processing. The eyestalk was removed prior to placing them in the extraction buffer to prevent the carry-over of inhibitors during the DNA extraction process. The postlarvae were subsequently placed in microfuge tubes containing 1 ml of DNA extraction buffer and kept at room temperature for the extraction of genomic DNA following the procedures described by Caipang *et al* (2004). The resulting genomic DNA pellet was resuspended in 100 µl of 1X TE buffer (pH 7.5) and stored at -20oC until use.
PCR procedures. Duplex PCR conditions were determined for simultaneous detection of WSSV-MBV, WSSV-IHHNV, and MBV-IHHNV. Reactions were performed using primers of Surachetpong et al (2005) and Caipang et al (2011) for MBV, Yang et al (2006) for WSSV and IHHNV and another set of WSSV primers by Flegel (2006). PCR analysis was carried out with same volume of reaction mixture as described previously except that 2 sets of primers were added. In the case of WSSV-MBV duplex, 3 µl of WSSV primer and 2 µl of MBV primer were used. The duplex PCR amplification was performed with initial denaturation at 94°C for 5 min and 35 cycles of amplification with denaturation at 94°C for 30 sec, annealing for 30 sec and elongation at 72°C for 1 min and a final elongation at 72°C for 5 minutes then incubation at 4°C. Optimal conditions for annealing were determined.
Multiplex PCR conditions were developed for simultaneous detection of WSSV, MBV and IHHNV. Reactions were performed using primers of Caipang et al (2011) for MBV resulting in the amplification of a 193 bp PCR product. The primers developed by Yang et al (2006) for the detection of WSSV and IHHNV were used and amplified a PCR product of 824 bp and 703 bp, respectively. PCR analysis was carried out with same volume of reaction mixture as described previously except that 3 sets of primers were added - WSSV, IHHNV, and MBV primers at 2 µl, 3 µl, and 2 µl respectively. The amplification was performed with initial denaturation at 94°C for 5 min followed by 40 cycles of amplification with denaturation at 94°C for 30 sec, annealing for 30 sec and elongation at 72°C for 1 min and a final elongation at 72°C for 5 min then incubation at 4°C. Five microliters of the PCR product were loaded into a 1.2% Agarose gel with ethidium bromide and electrophoresed at 100V for 30-32 minutes. The bands were viewed using Gel Documentation System (Cell Biosciences) and photographed.

Results and Discussion

In this study, the individual PCR assays to detect common shrimp viral pathogens including WSSV, IHHNV and MBV were optimized to be used either as duplex or multiplex PCR. The use of both assays enable to detect the presence of multiple pathogens in shrimp in a single reaction tube; thus, saving both time and resources during analysis. Studies have shown simultaneous presence of two pathogens on *P. monodon* postlarvae (Otta *et al* 2003; Mishra *et al* 2005; Caipang & Aguana 2010). Approximately 70% of the ponds and hatcheries in the Philippines were found to be infected with MBV (Baticados *et al* 1991). Spawners and broodstock of wild black tiger shrimp, *P. monodon* that are used in the hatcheries have been likewise shown to harbor WSSV (de la Peña *et al* 2007). Therefore, there is a possibility of having dual or multiple viral infections of broodstock and postlarvae in ponds and hatcheries. Some samples of postlarvae showed positive results for the presence of two viral pathogens- WSSV and MBV. Protocols for the detection of dual-pathogen infection in hatchery-reared *P. monodon* were developed. WSSV-MBV infection can be simultaneously detected by PCR using a combination of published primers by Yang et al (2006) for WSSV and Surachetpong et al (2005) for MBV (Figure 1). The optimum annealing temperature was observed to be at 56^{0}C.

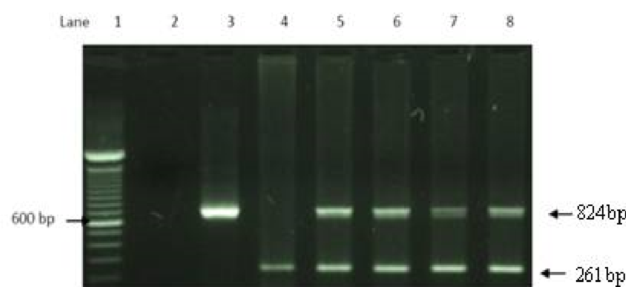

Fig. 1. Simultaneous detection of WSSV-MBV at different annealing temperatures: 52°C, 54°C, 56°C, and 58°C. Lane 1: 100 bp DNA marker; Lane 2: negative control; Lane 3: WSSV-positive; Lane 4: MBV-positive; Lane 5-8: Both positive for WSSV and MBV. WSSV PCR product: 824 bp and MBV PCR product: 261 bp.

Likewise, the protocols for the diagnosis of dual infections in shrimp postlarvae with WSSV and IHHNV as well as MBV and IHHNV were developed. The combination of primers that was used for detection of the viral pathogens was based on the large differences in the sizes of the PCR products. This will facilitate with ease in discriminating the viruses in the organism through amplification of the desired región of the target sequence. The combination of WSSV primers by Flegel (2006) and the IHHNV primers by Yang et al (2006) gave good results (Figure 2a). The presence of WSSV was confirmed through the amplification of a 232 bp PCR product and an amplification of a 703 bp confirmed the presence of IHHNV. The PCR amplifications were carried out at an annealing temperature ranging 540-600C, although good resolution of the bands were obtained at annealing temperatures ranging 540-560C. For the detection of both MBV and IHHNV, the MBV primers by Surachetpong et al (2005) and the IHHNV primers by Yang et al (2006) were

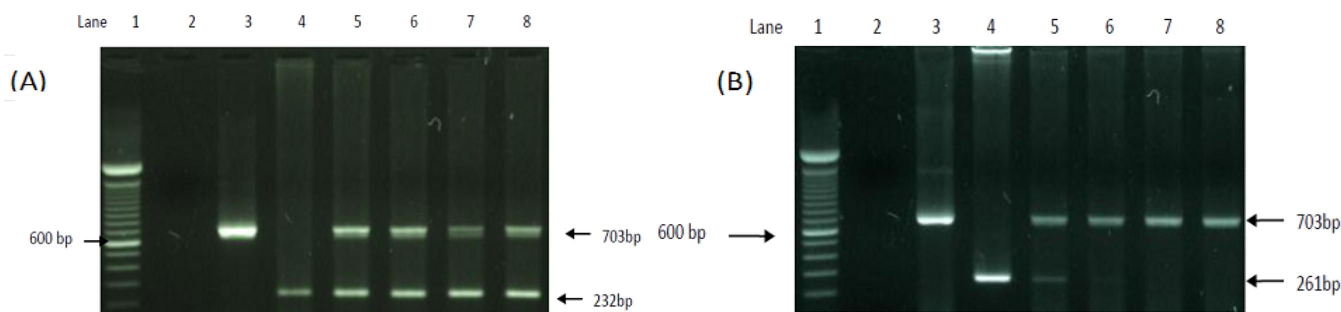

Figure 2. Results of simultaneous detection of (A) WSSV and IHHNV and (B) MBV and IHHNV in *P. monodon* postlarvae. In (A), Lane 1: 100 bp DNA ladder; Lane 2: negative control; Lane 3: IHHNV-positive; Lane 4: WSSV-positive ; Lanes 5-8: Both positive for IHHNV and WSSV, using different annealing temperatures: 54°C, 56°C, 58°C, 60°C respectively. In (B) Lane 1 : DNA marker; lane 2: negative control; Lane 3: IHHNV-positive; Lane 4: MBV-positive; Lanes 5-8: Both positive for IHHNV and MBV, using different annealing temperatures: 56°C, 58°C, 60°C and 63°C. WSSV PCR product: 232 bp; IHHNV PCR product: 703 bp; MBV PCR product: 261 bp.

used. The presence of PCR bands at sizes of 261 bp and 703 bp confirmed the presence of MBV and IHHNV, respectively. Best results were obtained at an annealing temperature of 560C because the PCR bands had good intensity during gel electrophoresis (Figure 2b).

Various studies have addressed the diagnosis of multiple viral infections by developing protocols for simultaneous detection of these pathogens (Khawsak *et al* 2008; Xie *et al* 2007). In our study, we have obtained shrimp postlarvae samples that were positive for all three pathogens-WSSV, MBV and IHHNV, as determined by individual PCR analysis. A PCR protocol was developed to detect the presence of WSSV, MBV and IHHNV simultaneously. The primers that were used for the assays had been developed by Caipang et al (2011) for MBV and Yang et al (2006) for WSSV and IHHNV. Result of the work on multiplex PCR for simultaneous detection of WSSV, MBV, and IHHNV is shown in Figure 3. Annealing temperature at 580C yielded the best results for the assay based on the intensity and clarity of the products (bands) that were produced as visualized during gel electrophoresis. The PCR products of sizes 824 bp, 703 bp and 193 bp confirmed the presence of WSSV, IHHNV and MBV in the postlarvae, respectively. The IHHNV band (703 bp) is diffused with a slightly smaller band, which may be due to insufficient elongation time.

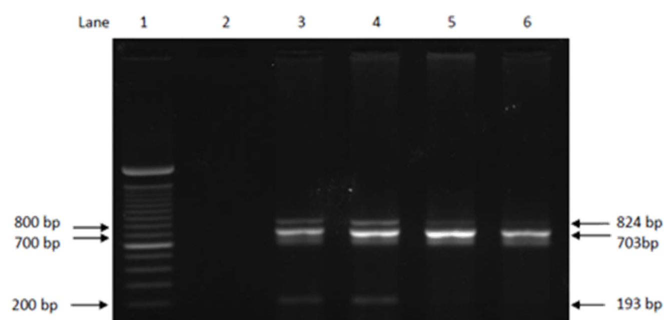

Figure 3. Simultaneous detection of WSSV, MBV, and IHHNV in *P. monodon* postlarvae at different annealing temperaturas. Lane 1: DNA 100 bp ladder; Lane 2: negative control; Lanes 3-4: 58°C and Lanes 5-6: 60°C. WSSV PCR product: 824 bp; IHHNV PCR product: 703 bp, and MBV PCR product: 193 bp and.

In summary, this study has optimized a multiplex PCR assay that can simultaneously detec the presence of three viruses in shrimp postlarvae. This assay is an effective method for rapid detection of these major viruses that cause death and retard the growth of penaeid shrimp. It can be used for screening the shrimp larvae for viral infection before stocking them in grow-out ponds; thus, preventing huge economic losses due to these viruses. In addition, this multiplex PCR assay is useful for epidemiological study of the viruses in shrimp and carriers in the country.

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