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Optimization of PCR protocols for the detection of viral pathogens in shrimp aquaculture in the Philippines

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ABSTRACT

Early detection of the causative agent which resulted in massive mortalities is a crucial aspect in the health management of shrimp aquaculture. Several diagnostic techniques have been developed for several pathogenic diseases and the use of molecular approaches is widely accepted. The present study optimized published PCR protocols to detect a number of commercially important viral pathogens, including white spot syndrome virus (WSSV), monodon baculovirus (MBV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) in shrimp as well as to ensure that these PCR assays are suited to Philippine conditions. The optimized PCR protocols for the detection of various viral pathogens in shrimp are sensitive as well as site-specific. These assays can be used as a management tool for the prevention and control of viral diseases in shrimp aquaculture in the country through early detection of the pathogen.

Keywords: diseases, diagnostics, pond culture, virus, MBV, IHHNV, WSSV

INTRODUCTION

Bacterial and viral diseases are major problems in the shrimp aquaculture industry in the Philippines. Disease has caused a significant drop in shrimp production resulting in tremendous losses among shrimp farmers [1, 2]. Among the major causes of shrimp diseases are luminous vibriosis due to *Vibrio* spp. [3] and the white spot syndrome virus (WSSV) [2], which resulted in severe mortality of the cultured stock. Other viral pathogens that led to massive disease outbreaks include monodon baculovirus (MBV) [5], hepatopancreatic parvovirus (HPV[6], infectious hypodermal and hematopoietic necrosis virus (IHHNV) [7], yellow-head virus (YHV), and Taura syndrome virus (TSV) [8]. Diseases due to these pathogens are usually encountered in hatchery and grow-out operations. Dependence of the shrimp industry on *Penaeus monodon* broodstock from the wild or the introduction of *Penaeus vannamei* into the endemic stock are also possible sources of asymptomatic carriers of these pathogens. A number of control measures used to curb these disease problems, including "green water" technology, properly drying the ponds during pond preparation, use of chlorinated reservoirs and treating the pond water prior to stocking, have to some extent helped in controlling diseases in shrimp. However, a major concern for successful shrimp farming operations is a rapid and efficient diagnosis of diseases prior to the onset of heavy infection leading to mass mortality. The use of molecular techniques over traditional methods of diagnosing fish diseases has been widely accepted because of its high degree of specificity and sensitivity, rapidity and its ability to detect the presence of pathogens even in extremely low amounts. Rapid detection can reduce disease risks and in the long run lead to enhanced production.

Conventional PCR assays have been developed to detect pathogens in shrimp [4, 9-15]. Despite its high cost due to expensive reagents and equipment, the amount is still insignificant in comparison to the amount saved when immediate management procedures can be applied to curb the spread of the disease and avoid mass mortality. In addition, PCR assays need to be optimized to suit the prevailing conditions where the biological samples were obtained. This is to prevent variations in the results of the assays, which can lead to misinterpretation, thereby causing severe consequences in the management of the aquaculture facility. Hence, this study was conducted to optimize previously published PCR protocols for the detection of various viral pathogens in shrimp in order that these will be suited to the existing conditions of the aquaculture sites in the Philippines.

MATERIALS AND METHODS

Sampling of shrimp

Different stages of shrimp, *P. monodon* were collected from shrimp farms and hatcheries in various shrimp-producing regions in the Philippines. These places included: Iloilo, Negros Occidental, Negros Oriental, Bohol, Cebu, Leyte, and Samar. The shrimps were immediately dissected and tissue samples were placed in 1 ml DNA extraction buffer at room temperature. Remaining undissected shrimp samples were placed in ice and upon arrival in the laboratory stored at -80 ^{0} C.

Extraction of DNA

Different tissues of shrimps were used for DNA extraction following the procedures described by Caipang et al. [16] with some modifications. Shrimp tissues were dissected and placed in 1 ml of DNA extraction buffer with the following composition; 10 mM Tris, 125 mM NaCl, 10 mM EDTA, 0.5% SDS and 4M Urea. For the postlarvae samples, an additional step of eye stalk removal was employed to remove inhibitors. Proteinase K at a volume of 10 µl (1 mg/ml) was added to the extraction buffer and the solution was incubated for 1 hour at 37^{0}C. Total DNA was extracted using phenol: chloroform: isoamyl alcohol. The aqueous (upper) layer (approximately 500 µl) was transferred to a new tube and mixed with an equal volume of absolute ethanol, 10 µl of 3M sodium acetate and 2 µl of 1% glycogen (1 mg/ml). The mixture containing the DNA was stored in the freezer (-20°C) for 24 hrs and then centrifuged at 12,500 rpm for 10 minutes at 4°C. The supernatant was discarded leaving the visible (precipitate) pellet in the tube. The DNA pellet was washed with 1 ml 70% ethanol and centrifuged at 12,500 rpm for 5 minutes at 4°C. The ethanol was discarded and the pellet was air dried for few minutes. Dried pellet was resuspended in 100 µl of 1X TE buffer and stored at -20°C until use.

Optimization of the PCR assays

The PCR primers for the detection of MBV, WSSV and IHHNV that were used in the present study were obtained from previously published primer sequences. New primers specific for a Philippine isolate of MBV were designed from the least conserved region of the partial sequence of the MBV genomic DNA [17] and amplified a fragment size of 193-bp.

PCR was performed following published protocol with some modifications. Optimization of the PCR assays was done by testing various annealing temperatures. A 20 µl reaction mixture was prepared containing 2µl of 10X PCR reaction buffer, 1.5µl of 50mM $MgCl_2$, 0.5µl of 10mM dNTP mix (Qiagen), 0.1µl of Taq Polymerase (Invitrogen), 2µl each of the forward and reverse primers, distilled water, and DNA sample . The PCR amplification was performed using MyCycler (Biorad) with an initial denaturation at 95°C for 3 minutes and followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing for 30 seconds at various temperatures for each primer set and elongation at 72°C for 1 minute. The PCR amplification was completed using a final elongation step at 72°C for 5 minutes then incubation at 4°C.

Five microliters (5 µl) of PCR product in 5µl 1X TE Buffer and 1 µl GLB Dye and 0.5µl of 50 bp DNA Ladder in 9.5µl 1X TE Buffer plus Dye 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).

Ten-fold serial dilutions of the genomic DNA were prepared to determine the sensitivity of the PCR assays.

RESULTS AND DISCUSSION

The PCR assays for the detection of the various viral pathogens in shrimp aquaculture were optimized so that these will be applicable to Philippine conditions. Published PCR primers were used for these assays. For the detection of MBV, the primers developed by Surachetpong et al. [18] were initially used for detection of the virus and subsequently modified (Table 1). In a later study, Caipang et al. [17] showed that a partial sequence of a Philippine

strain of MBV had 80% sequence similarity with other MBV strains, indicating the need to develop strain-specific MBV primers for detection of this pathogen.

New set of MBV primers were designed for the specific detection of a local (Philippines) MBV strain (see Table 1; Caipang et al. [19] for the primer sequence). The new primers generated a PCR product of 193 bp. Sensitivity of the new MBV primers was compared to the existing primers used by Surachetpong et al. [18] and Belcher and Young [10]. The new primers were equally as sensitive as the previously published primers in the detection of MBV in shrimp [19]. In addition, the new primers for the detection of the local strain of MBV were highly specific for the virus because no cross amplifications were observed for other shrimp viruses including, WSSV, IHHNV and HPV.

Several published primers were used to detect the presence of WSSV in shrimp samples. For example, the primers developed by Flegel [8] were able to detect the presence of the WSSV in diseased and asymptomatic adult shrimp and post larvae obtained from various localities in the Philippines (Fig. 1). Published protocols for WSSV detection had to be modified when applied to detection of the pathogen in local shrimp samples. A comparison of published protocols and the protocols that were developed in the study for WSSV detection is shown in Table 1. Most of the modifications were done on the annealing temperature and the time for each step during the amplification process. Lower annealing temperatures were used to obtain PCR products except for the primers by Takahashi et al. [20]. These modifications in PCR conditions imply some differences in Philippine strain of WSSV relative to the strains that are found in other countries.

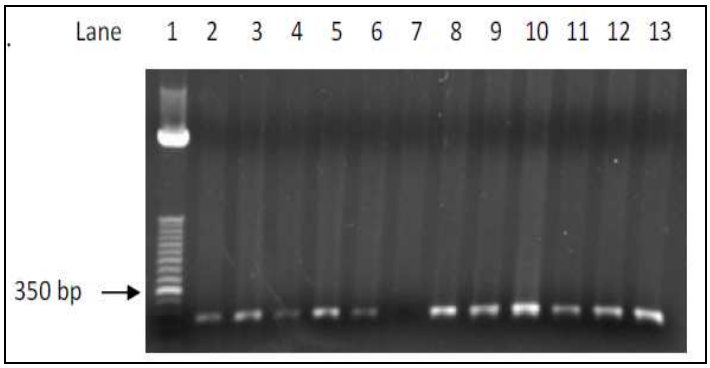

Figure 1. Detection of WSSV in shrimp from various sites in the Philippines using published primers by Flegel [8] at different annealing temperatures

Lane 1: 100 bp DNA marker; lanes 2 and 8: 55°C; lanes 3 and 9: 56°C; lanes 4 and 10: 58°C; lanes 5 and 11: 60°C; lanes 6 and 12: 61°C; lanes 7 and 13: 63°C

Local samples of *Penaeus monodon* postlarvae (PL15) were subjected to PCR to detect the presence of IHHNV using published primers by Yang et al. [7]. The said primers amplified a 703bp fragment of the capsid protein gene of IHHNV. Amplification at different annealing temperatures showed the most distinct band, was obtained at 65^0C (Fig. 8). The PCR conditions that were used are shown in Table 1. The IHHNV primers were able to detect the presence of IHHNV in 10 pg DNA samples, although the electrophoresis band was very light. This DNA concentration was much higher than the results obtained by Yang et al. [7] using the same set of primers. The difference in the sensitivity of the assays can be attributed to the fact that Yang et al. [7] used purified IHHNV DNA for the assay while our study used total DNA from shrimp postlarvae that were infected with the virus. It was noted however that annealing temperature used in the study at 65^0 C was more stringent than the annealing temperature that was used by Yang et al. [7] at 57^0 C. Tang and Lightner [21] have shown low sequence variability of IHHNV isolates, and the strain that is prevalent in the Americas is closely related to the strain that was detected in the Philippines, hence the published primers were suited to detect the local strain of the virus.

Attempts to detect the presence of HPV in shrimp samples using the published primers did not produce any PCR product. This may imply that the shrimp in the wild and in the farms or hatcheries could be free from this virus or the viral load is too low to allow detection of the pathogen.

Table 1. PCR primers and optimized amplification conditions for the detection of various viral pathogens of shrimp in the Philippines

Pathogen/Primer Sequences	References	Published PCR Conditions	PCR Conditions for Philippine samples
White Spot Syndrome Virus (WSSV) WSSV F- 5'-GTACGGCAATACTGGAGGAGGT-3' WSSV R 5'-GGAGATGTGTAAGATGGACAAG-3' Product : 232 bp	[8]	No initial denaturation 40 cycles 95^{0}C, 30s 60^{0}C, 30s 72^{o}C, 30s Final elongation: 72^{o}C, 7min Incubation 4^{o}C	Initial denaturation 95^{o}C, 3min 40 cycles 95^{o}C, 30s 58^{0}C, 30s 72^{o}C, 1min Final elongation: 72^{o}C, 5min Incubation 4^{o}C
WSSV1 5'-TGA TTC TGC ATC CAG CTC-3' WSSV2 5'- GCA CGG TCA ACA TGT CT-3' Product : 824 bp	[22]	Initial denaturation 94^{o}C, 5 min 30 cycles 94^{o}C, 30s 56^{o}C, 30s 72^{o}C, 1min Final elongation: 72^{o}C, 5min Incubation 4^{o}C	Initial denaturation 95^{o}C, 5 min 30 cycles 95^{o}C, 30s 52^{o}C, 30s 72^{o}C, 1min Final elongation: 72^{o}C, 5min Incubation 4^{o}C
WSSV F 5'- ACCTCTTTACTCCCTCGACT-3' WSSV R 5'-TTGTAGAGGGCATGAGGGAT-3' Product : 330 bp	[20]	30 cycles 95 ^{0}C, 30s 58^{0}C, 1 min 72^{0}C, 1 min Final elongation: 72^0 C, 5 min	Initial denaturation 95^{o}C, 3min 40 cycles 95^{o}C, 30s 58^{0}C, 30s 72^{o}C, 1min Final elongation: 72^{o}C, 5min Incubation 4^{o}C
Monodon Baculovirus (MBV) MBV F 5'-AATCCTAGGCGATCTTACCA-3' MBV R 5'-CGTTCGTTGATGAACATCTC-3' Product : 261 bp	[18]	94^{o}C, 5min (hot start) 35 cycles 94^{o}C, 30s 60^{o}C, 30s 72^{o}C, 30s Final elongation: 72^{o}C, 7min Incubation 4^{o}C	Initial denaturation 95^{o}C, 3min 40 cycles 95^{o}C, 30s 55^{0}C, 30s 72^{o}C 1min Final elongation: 72^{o}C, 5min Incubation 4^{o}C
MBVF 5'-CTATACTGTTCTATACATTTTGCAAAGC-3' MBVR 5'-TATATAGCGTTAACACGTTATACAAG-3' Product : 193 bp	[19]	Initial denaturation 95^{o}C, 3min 40 cycles 95^{o}C, 30s 55^{0}C, 30s 72^{o}C, 1min Final elongation: 72^{o}C, 5min Incubation 4^{o}C	Initial denaturation 95^{o}C, 3min 40 cycles 95^{o}C, 30s 55^{0}C, 30s 72^{o}C 1min Final elongation: 72^{o}C, 5min Incubation 4^{o}C
Pathogen/Primer Sequences	**References**	**Published PCR Conditions**	**PCR Conditions for Philippine samples**
MBV F 5'-TCCAATCGCGTCTGCGATACT-3' MBV R 5'-CGCTAATGGGGCACAAGTCTC-3' Product size: 361bp	[10]	Initial denaturation 96^{o}C, 5min 40 cycles 94^{o}C, 30s 65^{0}C, 30s 72^{o}C 1min Final elongation: 72^{o}C, 7min Incubation 4^{o}C	Initial denaturation 95^{o}C, 3min 40 cycles 95^{o}C, 30s 57^{0}C, 30s 72^{o}C 1min Final elongation: 72^{o}C, 5min Incubation 4^{o}C
Infectious hypodermal Hematopoietic Necrosis Virus (IHHNV) IHHNV F 5'-TAATGAAGACGAAGAACACGCCGAAGG-3' IHHNVR 5'-TGGGTAGACTAGGTTTCCAAGGGATGGTT-3' Product : 703 bp	[7]	94^{o}C, 5min (hot start) 40 cycles 94^{o}C, 1min 57^{o}C, 1min 72^{o}C, 1min Final elongation: 72^{o}C, 5min Incubation 4^{o}C	Initial denaturation 95^{o}C, 5min 40 cycles 94^{o}C, 1min 65^{o}C, 1min 72^{o}C, 1min Final elongation: 72^{o}C, 5min Incubation 4^{o}C

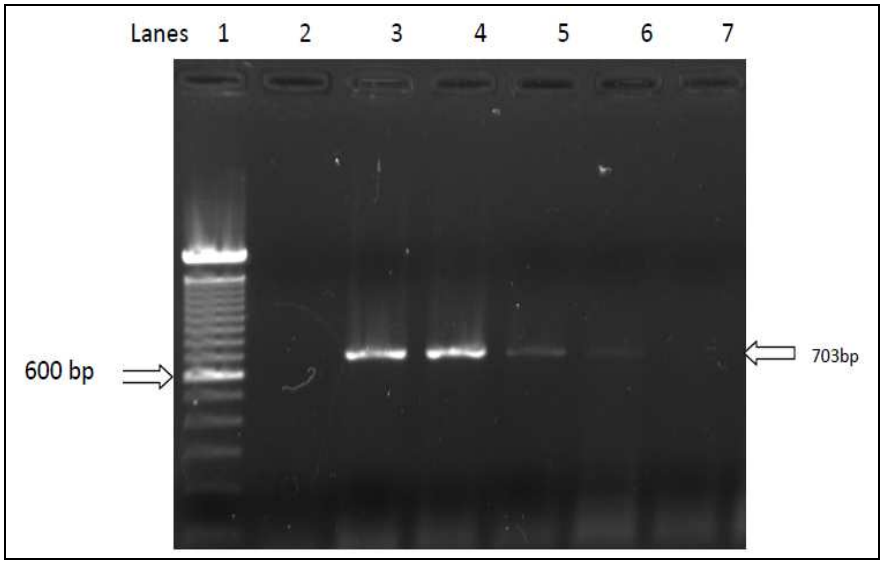

Figure 2. Detection of IHHNV from shrimp samples and determination of the optimum annealing temperature
Lane 1: 100 bp DNA ladder; lane 2: negative control; lane 3: 57°C; lane 4: 60°C; lane 5: 63°C; lane 6: 65°C

CONCLUSION

In conclusion, this study has optimized protocols for the PCR detection of the commercially important viral pathogens of shrimp in the Philippines. Each PCR assay has been tested and is deemed to be sensitive as well as site-specific. These assays can be used for routine procedures in shrimp health or diagnostic laboratories as a management tool for the prevention and control of viral diseases in shrimp aquaculture in the country through early detection of the pathogen.

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