Current Research Journal of Biological Sciences 3(4): 416-420, 2011
ISSN: 2041-0778

Received: May 27, 2011 Accepted: June 28, 2011 Published: July 15, 2011

Development of a Polymerase Chain Reaction (PCR) Assay for the Detection of Philippine Isolates of the *Penaeus monodon*-type Baculovirus (MBV)

[1]Christopher Marlowe A. Caipang, [2]May Flor J. Sibonga,
[2]Mary Jane A. Amar and [3]Jane S. Geduspan
[1]Faculty of Biosciences and Aquaculture, University of Nordland, Bodø 8049, Norway
[2] National Institute of Molecular Biology and Biotechnology, University of the Philippines Visayas, Miag-ao 5023, Iloilo, Philippines
[3]College of Arts and Sciences, University of the Philippines Visayas, Miag-ao 5023, Iloilo, Philippines

Abstract: *Penaeus monodon*-type baculovirus (MBV) is a DNA virus that infects postlarvae and early juveniles of shrimp, *Penaeus monodon*. Several variants of this virus occur through nucleotide analysis of its genomic DNA. In the present study, a one-step PCR method was developed for the detection of the Philippine isolates of MBV by designing PCR primers on the least conserved region of the Philippine MBV. Using genomic DNA of MBV-infected shrimp postlarvae, the PCR assay amplified a 193-bp PCR product. Its sensitivity was comparable to the published PCR assays. The strain-specific primers did not cross-react with other DNA viruses including White Spot Syndrome Virus (WSSV), Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) and hepatopancreatic parvovirus (HPV). This PCR assay could be used for regular monitoring and surveillance of MBV in shrimp as well as tracing the movement of the Philippine MBV in shrimp farms in different geographic regions.

Key words: MBV, monodon baculovirus, PCR, *Penaeus monodon*, shrimp

INTRODUCTION

Viral diseases are among the limiting factors in the success of commercial shrimp culture on a global scale. At present, there are more than 20 viruses that affect penaeid shrimps, and these infect both cultured and wild stock (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. An example of this is the *Penaeus monodon*-type baculovirus (MBV), which is implicated in the stunted growth of the shrimp (Flegel *et al*., 1999).

Shrimps, especially the postlarvae, that are infected with MBV can easily be detected in squash mounts of the hepatopancreas stained with malachite green (Flegel, 2006). Viral inclusion bodies are found in enlarged nuclei and they are stained green upon addition of the chemical. These protein particles are are able to protect or enclose the virus, hence termed as occlusion bodies. Using hematoxylin and eosin staining, virus-infected cells had enlraged nuclei with acidophilic occlusion bodies (Flegel, 2006).

MBV was the cause of the collapse of the shrimp culture industry in Taiwan in the mid-1980's (Lin, 1989), and this virus has since been found in various parts of the world (Fegan *et al*., 1991; Belcher and Young, 1998; Madhavi *et al*., 2002). Despite its occurrence over a large geographical area, 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.

The use of hematoxylin and eosin to stain viral occlusion bodies in infected shrimp is a cheap detection method, but heavy infection must occur before a positive reaction will take place. Alternative methods that are more sensitive in detecting the virus have been developed including in situ hybridization (Poulos *et al*., 1994), polymerase chain reaction (Chang *et al*., 1993; Belcher and Young, 1998; Surachetpong *et al*., 2005), immunohistochemistry (Boonsanongchokying *et al*., 2006) and the loop-mediated isothermal amplification (LAMP) reaction (Chaivisuthangkura *et al*., 2009). At

Corresponding Author: Christopher Marlowe A. Caipang, Faculty of Biosciences and Aquaculture, University of Nordland, Bodø 8049, Norway. Tel.: +47-75517735; Fax: +47-75517349

present, the PCR assays recommended by the Office International des Epizooties (OIE) reference manual are the protocols developed by Belcher and Young (1998) and by Surachetpong *et al.* (2005).

In the Philippines, MBV infections have been found in most shrimp hatcheries and farms. In 1992, there was an incidence of 85-100% MBV infections of shrimp postlarvae that were sampled (Natividad and Lightner, 1992), and in 2004, there was a prevalence rate of 59% of MBV infections based on fry quality assessment tests (Natividad *et al.*, 2006). MBV infections have also been detected from wild shrimp and dual infections with WSSV were recorded, hence, Natividad *et al.* (2006) developed a duplex PCR protocol to diagnose WSSV and MBV infections in shrimp.

Previously, we have sequenced partial fragments of the MBV genomic DNA (Caipang *et al.*, 2011). Structural analysis of these fragments showed that there were differences in 25 nucleotides from the Philippine isolate when compared with the isolates from India and Taiwan. It has been shown that not all MBV isolates could be detected by the primers that have been developed previously (Satidkanitkul *et al.*, 2005), thus Surachetpong *et al.* (2005) designed PCR primers that could detect different geographic isolates of MBV. A "universal" PCR primer set for MBV detection is recommended to determine the immediate cause of the infection, but to find out whether such infections are caused by a certain isolate, then specific PCR primers have to be developed. From the least conserved regions of the partial sequence of the genomic DNA, we designed PCR primers to detect Philippine isolates of MBV. Using the primers of the previous study (Caipang *et al.*, 2011), we developed a PCR assay that targeted a specific geographical isolate of the virus. We have also tested the specificity and sensitivity of these primers for the detection of the pathogen.

MATERIALS AND METHODS

Sources of samples and DNA extraction: The study was conducted at the National Institute of Molecular Biology and Biotechnology of the UP Visayas, from July-October, 2010. Shrimp post-larvae (PL 15-20) were obtained from our previous study (Caipang *et al.*, 2011). The samples were immediately placed in microfuge tubes containing 1 mL of DNA extraction buffer and kept at room temperature for subsequent 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 procedure: Inorder to detect the Philippine isolates of MBV, specific primers (forward: 5'-CTATACTGTTCTATACATTTTGCAAAGC-3'; reverse: 5'-TATATAGCG TTAACACG TTATACAAG-3') were designed from the obtained partial sequence of the MBV genomic DNA (Caipang *et al.*, 2011). These primers amplified a 193-bp fragment of the genomic DNA. The PCR protocol was standardized using different annealing temperatures at 55, 57 and 60ºC and was amplified for 40 cycles.

The PCR reaction mixture consisted of 2 μL of each primer (5 pmol), 2 μL of 10x PCR buffer, 1.5 μL of 2 mM dNTP, 1 μL of 15 μM $MgCl_2$, 0.1 μL of *Taq* DNA polymerase (100 units) (Invitrogen, U.S.A.), 1 μL of the DNA template (1 μg/mL) and scaled up to 20 μL using distilled water. PCR amplification was carried out using the following conditions: initial denaturation at 95ºC for 3 min, followed by 40 cycles of denaturation at 95ºC for 30 sec, annealing at either 55, 57 or 60ºC for 30 sec and elongation at 72ºC for 1 min; then a final elongation at 72ºC for 5 min. PCR products (5 μL) were electrophoresed on a 0.8% agarose gel with 1% TBE electrophoresis buffer (pH 8.0) for 30 min, visualized using a hand-held densitometer and photographed.

Sensitivity and specificity of the PCR assay: To determine the sensitivity of the PCR assay using the newly designed primers, ten-fold serial dilutions (starting dilution of 1 μg/mL genomic DNA) of the genomic DNA were prepared and used as templates for the PCR assay. The limit of detection of this primer set was compared with the primers (forward: 5'-TCCAATCGC GTCTGCGATACT-3'; reverse: 5'-CGCTAATG GGGCACAAGTCTC-3') designed by Belcher and Young (1998), which amplified a 361-bp fragment of the MBV genomic DNA, as well as the primers (forward: 5'-AATCCTAGGCGATCTTACCA-3'; reverse: 5'-CGTTCGTTG ATGAACATCTC-3') developed by Surachetpong *et al.* (2005), which yields an amplified product of 261-bp. All the three primer sets were designed from the same genomic region of the MBV.

The specificity of the primer pair was tested using genomic DNAs of shrimp infected with White Spot Syndrome Virus (WSSV), Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) and Hepatopancreatic Parvovirus (HPV).

RESULTS AND DISCUSSION

Using the primers designed from the least conserved region of the partial sequence of the genomic DNA of MBV isolated from shrimp in the Philippines, a band corresponding to 193-bp was amplified from the genomic DNA of infected post-larvae (Fig. 1). An amplifed product was observed in all annealing tempratures that were tested, namely 55, 57 and 60ºC at 40 cycles of PCR amplification. However, stronger intensity of the

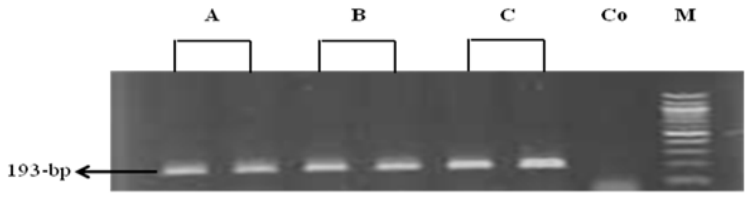

Fig. 1: PCR amplification of a 193-bp DNA fragment of MBV from genomic DNA infected shrimp postlarvae at various annealing temperatures. (A) 55ºC, (B) 57ºC and (C) 60ºC. Amplification was done at 40 cycles in a 20 μL PCR reaction. Lane Co: negative control (distilled water), Lane M: 100 bp-DNA marker

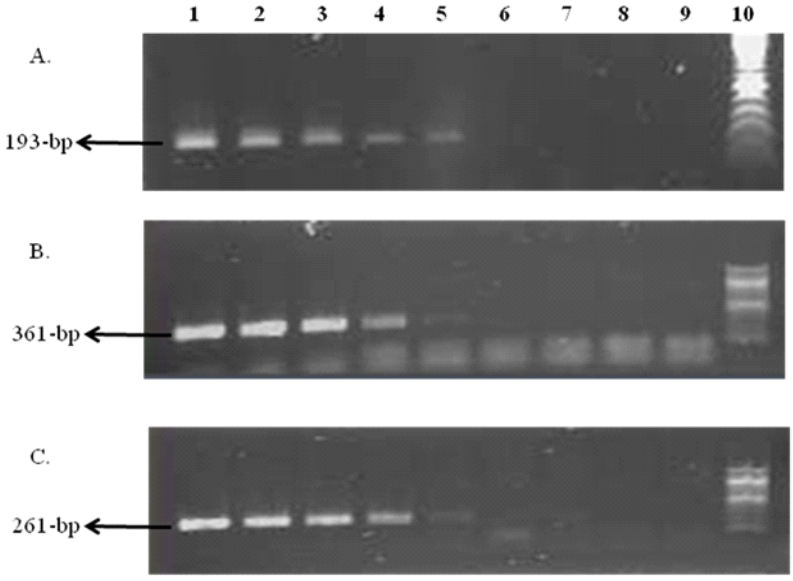

Fig. 2: Sensitivity of the PCR assay using different primers for the detection of MBV in infected shrimp postlarvae. (A) primers of the present study, (B) primers of Belcher and Young (1998) and (C) primers of Surachetpong *et al*. (2005). Amplification was done at 40 cycles at an annealing temperature of 57ºC in a 20-μL PCR reaction. 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 10: 100-bp DNA marker. This is a representative of three independent PCR reactions

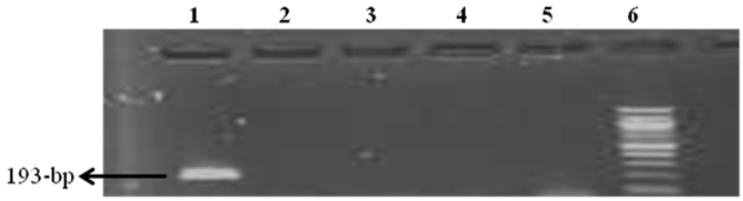

Fig. 3: Specificity of the PCR primers to detect Philippine isolate of MBV. Lane 1: MBV, Lane 2: WSSV, Lane 3: IHHNV, Lane 4: HPV, Lane 5: negative control (distilled water), Lane 6: 100-bp DNA marker. This is a representative of two independent PCR reactions

amplified producs was observed at an annealing temperature of either 57 or 60ºC. It is therefore recommended that in the PCR assay for the detection of this virus, an annealing temperature of at least 57ºC at 40 PCR cycles would result in good amplification efficiency. For comparison, in a nested PCR assay developed by Belcher and Young (1998), they used an annealing temperature of 65 and 60ºC for the first and second PCR,

respectively. On the other hand, the one-step PCR developed by Surachetpong *et al.* (2005) had an annealing temperature of 60ºC. Both of these PCR assays, had at least 35 PCR cycles.

In terms of sensitivity, the PCR assay that we developed was able to detect the MBV in infected samples at a concentration of 100 pg/mL or 100 fg of DNA per PCR reaction (Fig. 2). Surachetpong *et al.* (2005) obtained a limit of detection of 13.42 pg total DNA using the primers they designed to detect MBV and also obtained the same detection limit when they tested the primers of Belcher and Young (1998). Because we tested the genomic DNA of shrimp larvae infected with the virus, we could not determine the sensitivity in terms of viral copies. Hence, using the DNA template in this study, we tested the amplification efficiency of the published primers of Belcher and Young (1998) and Surachetpong *et al.* (2005). Results showed that the limit of detection of published primers was comparable with the PCR primers that we designed (Fig. 2a). In succeeding studies, we aim to purify the virus from infected samples and test the efficiency of the PCR assay on DNA extracted from purified virions.

Figure 3 shows the specificity of the PCR primers that we developed for detecting Philippine isolates of MBV. The primer set only amplified the sample that was positive for MBV but not on genomic DNA samples of shrimps infected with White Spot Syndrome Virus (WSSV), infectious hypodermal and hematopoietic necrosis virus (IHHNV) and hepatopancreatic parvovirus (HPV). Other PCR assays that are used for the detection of MBV in infected stock were also specific for this virus (Belcher and Young, 1998; Surachetpong *et al*., 2005).

In this study, we developed a PCR assay using specific primers for the detection of Philippine isolates of MBV. Surachetpong *et al.* (2005) developed a set of PCR primers that could detect MBV isolates from four geographic locations including Thailand, Taiwan, Malaysia and Hawaii. Considering that there is high rate of mobility of shrimps, particularly in *Penaeus monodon*, among shrimp-producing nations through importation of broodstock, there is a greater possibility of introducing strains of MBV from other countries. As such, it is important to know if the disease due to MBV is caused by a local strain or not. Previous studies on the PCR detection of certain strains of MBV have been on Australian MBV (Belcher and Young, 1998) and on Taiwan MBV (Chang *et al.*, 1993; Lu *et al.*, 1993; Hsu *et al.*, 2000). By developing strain-specific PCR assays for MBV detection, it is possible to trace the spread of this virus among countries and strict monitoring as well as surveillance could be carried out during importation of broodstock and postlarvae. It will also provide information as to the prevalence or occurrence of certain strains of this virus in a certain georaphic location. For example, we have shown previously that based on nucleotide level there are at least three strains of MBV that are found in Indian shrimp farms, namely, a MBV Indian strain, a Taiwanese strain and an intermediate strain (Caipang *et al.*, 2011) through bioinformatics analysis of the different sequences of the MBV genomic DNA, which have been deposited in Genbank.

The disease caused by MBV is not as serious as compared to the disease caused by WSSV in shrimps. Once infected with MBV, there is no mass mortality of the shrimp stock if culture conditions are effectively managed (Flegel, 2006). However, the unintentional introduction of other strains of this virus through importation of broodstock and postlarvae, might cause genetic recombination in the local strain of the virus, which could lead to the emergence of highly virulent form of the virus. As such it is important to develop strain-specific PCR methods of detecting MBV in both cultured and wild shrimp stock in order to ensure effective surveillance of this virus in shrimp aquaculture.

CONCLUSION

We have developed a PCR assay for the specific detection of Philippine MBV. The assay was highly sensitive and specific. Future studies shall focus on collecting samples from other shrimp-producing areas and developing strain-specific PCR primers in detecting MBV.

ACKNOWLEDGMENT

This study was supported in part by the project, " Biotechnology for Shrimp: Utilization of Molecular Technologies to Elucidate Shrimp Immunity and Develop Disease Diagnostics" funded by the Department of Science and Technology (DOST), Philippines. The authors also gratefully acknowledge the research staff of the National Institute of Molecular Biology and Biotechnology (NIMBB), University of the Philippines Visayas and the Aquatic Animal Health Research Group, Faculty of Biosciences and Aquaculture, University of Nordland for technical support.

REFERENCES

Belcher, C.R. and P.R. Young, 1998. Colourimetric PCR-based detection of monodon baculovirus in whole *Penaeus monodon* postlarvae. J. Virol. Methods, 74: 21-29.

Boonsanongchokying, C., W. Sang-oum, P. Sithigorngul, S. Sriurairatana and T.W. Flegel, 2006. Production of monoclonal antibodies to polyhedrin of monodon baculovirus (MBV) form shrimp. Sci. Asia, 32: 371-376.

Caipang, C.M.A., I. Haraguchi, T. Ohira, I. Hirono and T. Aoki, 2004 Rapid detection of a fish iridovirus using loop-mediated isothermal amplification (LAMP). J. Virol. Methods, 121: 155-161.

Caipang, C.M.A., M.F.J. Sibonga and J.S. Geduspan, 2011. Partial sequence of the genomic DNA from a Philippine isolate of the *Penaeus monodon*-type baculovirus (MBV) and comparison with other geographical isolates. AACL Bioflux, 4: 387-393.

Chaivisuthangkura, P., C. Srisuk, S. Rukpratanporn, S. Longyant, P. Sridulyakul and P. Sithigorngul, 2009 Rapid and sensitive detection of *Penaeus monodon* nucleopolyhedrovirus by loop-mediated isothermal amplification. J. Virol. Methods, 162: 188-193.

Chang P.S., C.F. Lo, G.H. Kou, C.C. Lu and S.N. Chen, 1993 Purification and amplification of DNA from *Penaeus monodon*-type baculovirus (MBV). J. Invert. Pathol., 62: 116-120.

Fegan, D.F., T.W. Flegel, S. Sriurairatana and M. Waiakruta, 1991. The occurrence, development and histopathology of monodon baculovirus in *Penaeus monodon* in Southern Thailand. Aquaculture, 96: 205-217.

Flegel, T.W., 2006 Detection of major penaeid shrimp viruses in Asia, a historical perspective with emphasis on Thailand. Aquaculture, 258: 1-33.

Flegel, T.W., V. Thamavit, T. Pasharawipas and V. Alday-Sanz, 1999. Statistical correlation between severity of hepatopancreatic parvovirus infection and stunting of farmed black tiger shrimp (*Penaeus monodon*). Aquaculture, 174: 197-206.

Hernández-Rodriguez A., C. Alceste-Oliviero, R. Sanchez, D. Jory, L. Vidal and L.F. Constain-Franco, 2001. Aquaculture development trends in Latin America and the Caribbean, In: Subasinghe, R.P., P. Bueno, M.J. Phillips, C. Hough, S.E. McGladdery and J.R. Arthur (Eds.). Aquaculture in the Third Millenium. Technical Proceedings of the Conference on Aquaculture in the Third Millenium. Bangkok, Thailand, 20-25 February 2000, NACA, Bangkok and FAO, Rome, pp: 317-340.

Hsu, Y.L., K.H. Wang, Y.H. Yang, M.C. Tung, C.H. Hu, C.F. Lo, C.H. Wang and T. Hsu, 2000 Diagnosis of *Penaeus monodon*-type baculovirus by PCR abd by ELISA of occlusion bodies. Dis. Aquat. Org., 40: 93-99.

Lin, C.K., 1989 Prawn culture in Taiwan, What went wrong? World Aquac., 20: 19-20.

Lu, C.C., K.F.J. Tang, G.H. Kou and S.N. Chen, 1993. Development of a *Penaeus monodon*-type baculovirus (MBV) DNA probe by polymerase chain reaction and sequence analysis. J. Fish Dis., 16: 551-559.

Madhavi, R., P. Janakiram, L. Jayasree and P.S.S. Murthy, 2002 Occurrence of concurrent infections with multiple viruses in *Penaeus monodon* from culture ponds of north coastal Andhra Pradesh. Res. Comm., 82: 1397-1400.

Natividad, J.M. and D.V. Lightner, 1992 Prevalence and Geographic Distribution of MBV and Other Diseases in Cultured Giant Tiger Prawns (*Penaeus monodon*) in the Philippines, In: W. Fulks and Main K. (Eds.), Diseases of Cultured Penaeid Shrimp in Asia and the United States. The Oceanic Institute, Makapuu Point, Honolulu, Hawaii, USA, pp: 139-160.

Natividad, K.D.T., M.V.P. Migo, J.D. Albaladejo, J.P.V. Magbanua, N. Nomura and M. Matsumura, 2006. Simultaneous PCR detection of two shrimp viruses (WSSV and MBV) in postlarvae of *Penaeus monodon* in the Philippines. Aquaculture, 257: 142-149.

Poulos, B.T., J. Mari, J.R. Bonami, R. Redman and D.V. Lightner, 1994. Use of non-radioactively labeled DNA probes for the detection of a baculovirus from *Penaeus monodon* by in situ hybridization on fixed tissue. J. Virol. Methods, 49: 187-194.

Satidkanitkul, A., P. Sithigorngul, W. Sang-oum, S. Rukpratanporn, S. Sriurairatana, B. Withayachumnarnkul and T.W. Flegel, 2005. Synthetic peptide used to develop antibodies for detection of polyhedrin form monodon baculovirus (MBV). Dis. Aquat. Org., 65: 79-84.

Surachetpong, W., B.T. Poulos, K.F.J. Tang and D. Lightner, 2005 Improvement of PCR method for the detection of monodon baculovirus (MBV) in penaeid shrimp. Aquaculture, 249: 69-75.