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Partial sequence of the genomic DNA from a Philippine isolate of the *Penaeus monodon*-type baculovirus (MBV) and comparison with other geographical isolates

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Abstract. Partial genomic DNA sequences of a Philippine isolate of the *Penaeus monodon*-type baculovirus (MBV) was identified. The two partial sequences of the MBV genomic DNA from the Philippines showed 100% homology at the nucleotide level, indicating the occurrence of a single strain. It shared at least 87% homology of their nucleotides with the partial sequences of MBV isolates from Taiwan and India. Moreover, the sequences of the genomic DNA from the Philippine isolates clustered together and were distinct from either the Taiwan and Indian isolates. A set of primers that will yield an amplicon size of 193-bp, was designed from the least conserved region of the partial sequence for the specific detection of the Philippine isolates of this virus.
Key Words: monodon baculovirus, MBV, shrimp, *Penaeus monodon*.

Introduction. *Penaeus monodon*-type baculovirus (MBV) was first identified in 1977 from shrimps in Taiwan (Lightner & Redman 1981). It was detected from shrimps in Thailand in 1990 (Fegan et al 1991) and in the Philippines, this virus was prevalent in 85-100% of the shrimp postlarvae based on fry quality assessment tests (Natividad & Lightner 1992). Although MBV is not as lethal compared with white spot syndrome virus (WSSV), MBV is considered a serious viral pathogen in shrimp aquaculture. This virus can cause significant reduction in the length of the infected shrimp (Flegel et al 2004) and can lead to significant economic loss due to slow growth of the shrimp stock (Flegel et al 1999). In addition, shrimps infected with MBV are susceptible to secondary infections caused by *Vibrio* spp and protozoans at poor environmental conditions (Ramasamy et al 2000; Vaseeharan & Ramasamy 2003). In addition, stress and overcrowding can also aggravate the severity of MBV infections (Lightner et al 1983). At present, MBV is found in a wide range of penaeid shrimp species (Manivannan et al 2004) in various parts of the world (Lightner 1996; Belcher & Young 1998; Fegan et al 1991; Vaseeharan & Ramasamy 2003; Uma et al 2005; Natividad et al 2006).

MBV is listed by the OIE as a category 2 shrimp pathogen (OIE 2003). Diagnosis of MBV infection depends on the presence of occlusion bodies in hypertrophied nuclei of the anterior portion of the gut epithelium and hepatopancreatic cells by light microscopy or by staining with hematoxylin and eosin (Lightner & Redman 1998; Flegel 2006). However, the detection of these occlusion bodies in shrimp requires a high level of infection; thus molecular-based methods have been developed to provide an accurate and sensitive detection of the virus during the early stages of infection. As such, the use of *in situ* hybridization (Poulos et al 1994) and polymerase chain reaction (PCR)-based methods such as conventional PCR (Belcher & Young 1998) and the loop-mediated isothermal amplification (LAMP) (Chaivisuthangkura et al 2009) for the diagnosis of MBV infections is routinely used in most shrimp health laboratories.

Surachetpong et al (2005) developed a PCR assay to detect MBV isolates from various geographical regions. This was based on earlier observations that strain differences in MBV might exist (Lightner et al 1985; Doubrovsky et al 1988). The PCR protocol was able to detect the virus that was isolated from Taiwan, Thailand, Malaysia, Hawaii, the Philippines, Ecuador, Mexico and Madagascar. Despite of this PCR protocol that was developed for MBV detection from various locations, there have been limited studies done on the characterization of the MBV genome to determine whether genetic organization is related to virulence as shown in WSSV (Zwart et al 2010). Hence, this study was conducted to determine a sequence of a genomic DNA from MBV that was isolated from the Philippines and compared these sequences from isolates of other geographical locations. This will provide a better understanding of the virulence of MBV by establishing relationships among these MBV isolates at the molecular level.

Materials and Methods

Sources of samples. Shrimp post-larvae (PL 15-20) were collected from different hatcheries in Iloilo, Philippines (Central Philippines). These were immediately placed in microfuge tubes containing 1 ml of DNA extraction buffer and kept at room temperature for subsequent extraction of genomic DNA. The composition of the DNA extraction buffer is as follows: 10 mM Tris, 125 mM NaCl, 10 mM EDTA, 0.5% SDS and 4M Urea at pH 7.5 (Caipang et al., 2004).

Extraction of genomic DNA. Genomic DNA from infected post-larvae was extracted following the procedures described by Caipang et al (2004) with some modifications. Briefly, the DNA extraction buffer containing the post-larvae samples was added 10 μl of Proteinase K and incubated for 1 hour at 37°C. This was followed by the addition of 500μl of the phenol:chloroform:isoamyl (PCI) followed by vigorous shaking and further incubation for an hour at 37°C. The solution was centrifuged at 12,500 rpm for 10 minutes at 4°C. The aqueous (upper) layer was transferred to a new tube and mixed with an equivalent volume of absolute ethanol. The sample was centrifuged at 12,500 rpm for 10 minutes at 4°C. The supernatant was then discarded leaving the visible (precipitate) pellet in the tube. The DNA pellet was washed with 1ml 70% ethanol and centrifuged at 12,500 rpm for 5 minutes at 4°C. The ethanol was discarded and the pellet was air dried. The dried pellet was resuspended in 100 μl of 1X TE buffer (pH 7.5) and stored at -20°C until use.

PCR amplification and sequence analyses. Two PCR primer sets were used to amplify the fragment of the genomic DNA of MBV. The primers (forward: 5'-TCCAATCGCGTCTGCGATACT-3'; reverse: 5'-CGCTAATGGGGCACAAGTCTC-3') designed by Belcher & Young (1998) amplified a 361-bp fragment of the MBV genomic DNA. On the other hand, the primers (forward: 5'-AATCCTAGGCGATCTTACCA-3'; reverse: 5'-CGTTCGTTGATGAACATCTC-3') developed by Surachetpong et al (2005) amplified a 261-bp fragment of the MBV genome. These primers have been tested previously and were specific for the detection of the target pathogen.

The reaction mixture consisted of 2 ml of each primer (5 pmol), 2 ml of 10x PCR buffer, 1.5 μl of 2 mM dNTP, 1 μl of 15 mM $MgCl_2$, 0.1 μl of *Taq* DNA polymerase (100 units) (Invitrogen, U.S.A.), 2 μl of the DNA template (1 μg ml^{-1}) and scaled up to 20 μl using distilled water. PCR amplification was carried out using the following conditions: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 sec and elongation at 72°C for 1 min; then a final elongation at 72°C 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.

The PCR products from the amplification of the partial sequence of the MBV genomic DNA were treated with shrimp alkaline phosphatase to remove traces of unbound phosphates during the PCR reaction. These were sequenced using Big Dye Terminator ver 3.1 (Applied Biosystems, CA, USA) and comparative sequence analyses were performed by the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/). Multiple alignment

of the partial sequences of MBV (Indian isolates: Genbank accession numbers AY494595, AY494593, AY494592, DQ494443, DQ494441; Taiwan isolate: EU246944) was done using CLUSTAL W (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Phylogenetic tree was constructed using the Neighbor Joining Method with 1000 boostrap values of MEGA 4 (Tamura et al 2007).

Results and Discussion. Using previously published primer sets to amplify a fragment of the MBV genomic DNA, we were able to obtain partial sequences of the genomic DNA of a Philippine isolate of MBV. One sequence was 308 bp in length, while the other one has 316 bp. Both shared 100% homology at the nucleotide level, hence there is a possibility that there is a single strain of MBV at the particular geographical area where the shrimp larvae were obtained. Alignment of the partial sequences of the Philippine isolate together with the isolates from India (5 sequences) and from Taiwan (1 sequence) is shown in Figure 1. The homology of the Philippine isolates with the other isolates from India and Taiwan was at least 87%. The partial sequence of the MBV genomic DNA was highly conserved among the isolates, having differences in 25 bases.

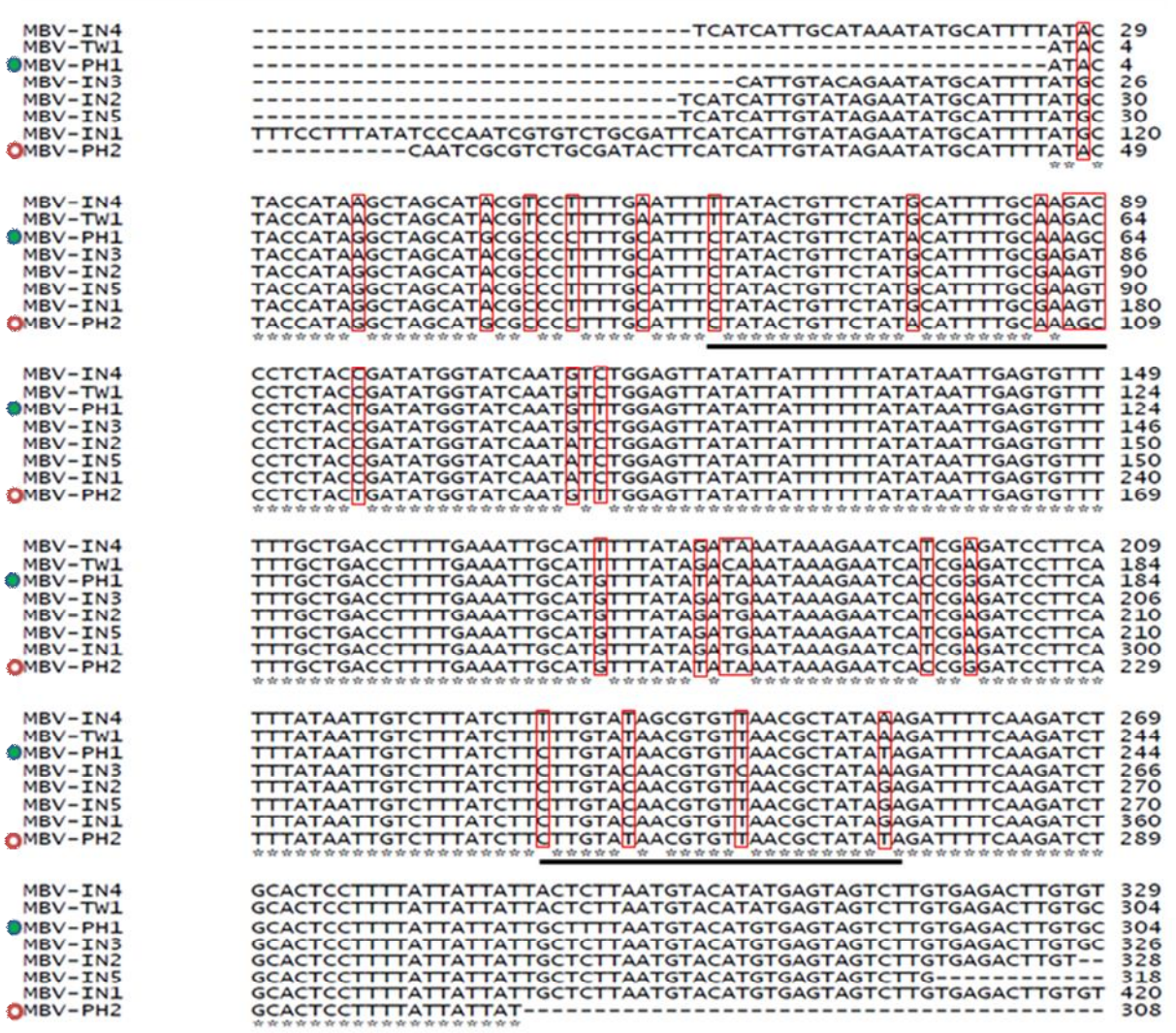

Figure 1. Nucleotide alignment of the partial sequences of the MBV genomic DNA from Philippines, India and Taiwan. Nucleotides that are inside red boxes indicate differences in the nucleotide composition. An asterisk indicates conserved nucleotide composition. The sequences with underline indicate the region of the primers for the detection of the Philippine isolates of MBV. The sequences of the Philippine isolates are indicated with colored circles.

Phylogenetic tree of the different MBV isolates showed distinct clades (Figure 2). The Philippine isolates clustered in one group as well as the Indian isolates. There is an isolate from India that formed a distinct clade, and another isolate that is grouped with the Taiwan isolate. It can be hypothesized that there could be a single strain of MBV in the Philippines. The existence of different clades in the Indian isolates could mean that aside from the presence of ”distinct” MBV strains in India, MBV strain from Taiwan could have been introduced to India through importation of shrimp broodstock and/or post-larvae. The presence of an intermediate clade could be a result of small mutations in the MBV genomic DNA as a result of the interaction among the different strains.

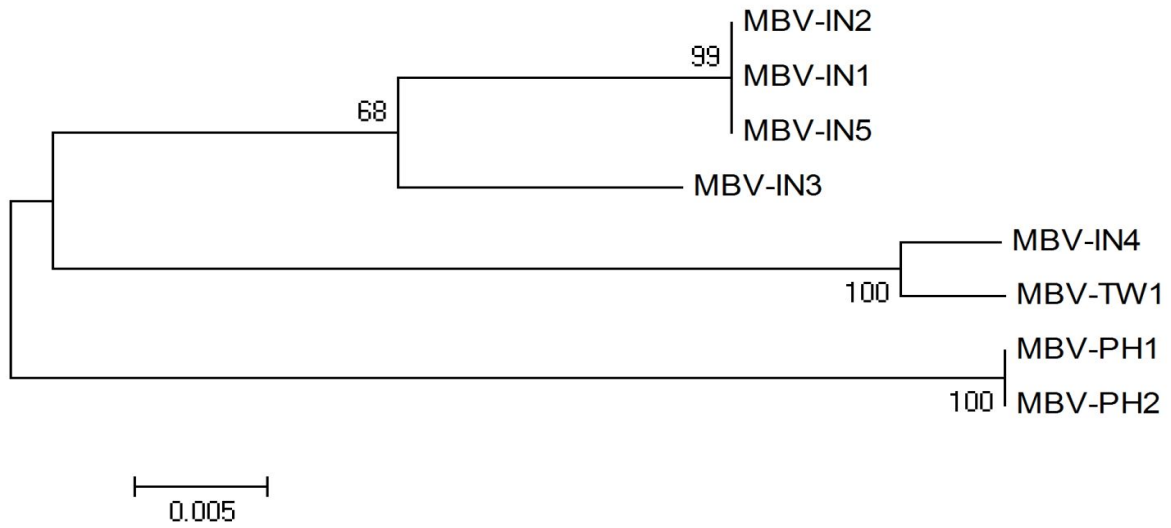

Figure 2. Phylogenetic tree of the different MBV isolates using partial nucleotide sequence of its genomic DNA. The Neighbor Joining method with 1000 bootstrap values was used for the analysis.

Inorder to detect the Philippine isolates of MBV, specific primers (forward: 5’-CTATACTGTTCTATACATTTTGCAAAGC-3’; reverse: 5’- TATATAGCGTTAACACGTTATACAAG-3’) were designed from the obtained partial sequence of the MBV genomic DNA in the present study (Figure 1). These primers were selected from the region of the sequence that contains a number of nucleotide mismatch with the other MBV isolates. This is to ensure that the resulting amplification will have a higher degree of specificity. The newly designed primer set targets a 193-bp fragment of the genomic DNA of the MBV.

In the present study, we have shown evidence at the molecular level that there are strain differences of MBV in relation to their geographical location. These findings support the earlier observations of Lightner et al (1985) and Doubrovsky et al (1988), wherein they stated that strain differences in MBV may exist. It was reported that the earliest outbreak of MBV was reported in Taiwan (Lightner & Redman 1981). An Indian isolate (MBV-IN4) was grouped together with the Taiwan isolate, and this could mean that the Taiwan strain could have been introduced to India either by importation of shrimp broodstock, which are carriers of the virus or through the introduction of shrimp postlarvae. Horizontal transmission is a common mode of transmitting the virus (Flegel 2006), and by using broodstock that are asymptomatic carriers of MBV increases the possibility of having offspring that are infected with the virus. We have also found a ”distinct” Indian strain of MBV and another Indian strain (MBV-IN3) that is an intermediate between the Indian strain and the Taiwan strain. We can only speculate that this intermediate strain might be a result of mutations within the genomic DNA and could be due to selection pressures from the host and from the environment. Analysis of the partial sequences from the genomic DNA of the MBV from the Philippines showed that there is likely a single strain of MBV that is pathogenic to shrimps. Further it showed that

the Philippine isolate is a ”distinct” strain. The analysis was done on a particular genomic sequence and additional studies involving the coding and non-coding regions of MBV are necessary to clearly establish whether the same pattern of strain differences occur in this virus.

Until now, there has been no complete sequence information for MBV that has been deposited in the Genbank database (Surachetpong et al 2005). A comprehensive sequence information of MBV has been done by Mari et al (1993), but not the entire genome. This is not surprising because there have been few research studies on this virus and the reason for this is the fact that this virus does not pose serious threat to the shrimp aquaculture industry as long as rearing conditions are optimum (Flegel 2006). In contrast with the white spot syndrome virus (WSSV), which causes severe mortality of the shrimp stock, a number of research studies have been done on the characterization of its genome (Yang et al 2001; van Hulten et al 2001). Using sequences from the different coding and non-coding regions of WSSV, several researchers (Shekhar & Ravichandran 2007; Molina-Garza et al 2008; Park & Shin 2009; Pradeep et al 2008; John et al 2010; Zwart et al 2010) have established strain differences of WSSV by analyzing the gene structure nad organization. Among these studies, the findings of Zwart et al (2010) showed that by analyzing the genomic structure of WSSV, they found a strong correlation between genome shrinkage and virus fitness. This means that the smaller the genome size of the virus, the better is the viral fitness. This was established using mathematical computations as well as virus challenge experiments. In the case of MBV, no such studies have been conducted and it is imperative to identify coding and non-coding regions of this virus and determine whether MBV exhibits the same phenomenon as what is observed in WSSV. At present, MBV may seem not pathogenic to shrimps but if we are to accept the phenomenon taking in place in WSSV and apply it to MBV, the virulence of MBV might increase in the future and could lead to serious mortalities in shrimp aquaculture.

The presence of the least conserved regions among the different MBV isolates facilitates the design of primers for the specific detection of a particular isolate. Surachetpong et al (2005) developed a PCR protocol for the detection of MBV among the different geographical isolates. Among bacterial pathogens of shrimp, conventional PCR assays have been developed and standardized to detect Philippine isolates of *Vibrio* spp (Caipang & Aguana 2011). In the present study, we have designed a primer set that will detect Philippine isolates of MBV, and is the first of such kind for the detection of viruses infecting shrimp aquaculture in the Philippines. There might be differences in the virulence of this pathogen in shrimp, hence specific detection of a particular geographical isolate is deemed necessary. In addition, the development and effectivity of recombinant vaccines or antibodies targeting specific coding regions of the virus may only be necessary by using a particular isolate.

Conclusions. In summary, based on partial sequences of the genomic DNA of MBV, there is likely a single strain of this virus in the Philippines. Primers have been designed from the least conserved regions of the sequence to enable specific detection of the Philippine isolates. Studies are underway to develop and standardize PCR protocols for the early and rapid detection of this particular geographical isolate.

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