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Research Article

STRAIN VARIATIONS IN MONODON BACULOVIRUS (MBV) INFECTING *PENAEUS MONODON* (TIGER SHRIMP) IN INDIA

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ABSTRACT
Viral diseases are the most serious problem in shrimp culture as they result in severe production and economic losses. Monodon baculovirus (MBV) is a member of nucleopolyhedrosis virus (NPV) group that infect penaeid shrimps. Polymerase chain reaction (PCR) assays have been reported for specific and accurate detection of MBV. As variations in the strains of MBV would greatly affect the detection efficiency of PCR assays, the objective of this study was to understand the existence of strain variations in MBV infecting *Penaeus monodon* in India. Shrimp samples collected from the shrimp farms and hatcheries of India during the period from March 2004 to March 2012 were screened for MBV infection by wet mount squash method and PCR. MBV infected samples collected during different years and from different sources were used to study the strain variations by PCR amplification of polyhedrin gene. Nucleotide sequencing and aminoacid analyses showed that the isolate, MBV9/ SDDL/04 was distinctly different among the nine isolates obtained from shrimp samples which confirmed the existence of variation in the strains of MBV in India.

Key Words: *Monodon Baculovirus (MBV), Polymerase Chain Reaction (PCR), Strain Variations*

INTRODUCTION
Monodon baculovirus (MBV) causes disease and mortality in post-larvae and juveniles of cultured shrimp and predispose them to secondary infections (Ramasamy *et al*., 1995). MBV infections have been reported to be transmitted both vertically and horizontally in shrimp (Karunasagar and Karunasagar, 1995). The clinical symptoms of the disease caused by MBV in shrimps include discoloration from pale bluish-grey to dark blue-black coloration, retarded growth, sluggish and inactive swimming movements, loss of appetite and yellowish-white hepatopancreas (OIE, 2003). Conventionally, MBV infection is diagnosed by the presence of occlusion bodies in wet mount squash preparations of hepatopancreas (Lightner, 1996) and by histopathology (Lightner *et al*., 1983). As detection of MBV occlusion bodies by microscopic method in shrimp requires high level of infection, Polymerase chain reaction (PCR) assays have been developed (Hsu *et al*., 2000, Surachetpong *et al*., 2005) and are widely being used as the diagnostic tool for MBV detection.

MATERIALS AND METHODS
Shrimp Samples
Samples of post-larvae and juveniles of *Penaeus monodon* (Tiger shrimp) were collected from shrimp farms and hatcheries located in and around Tamilnadu, India. The samples were screened for MBV by wet mount squash method (Lightner, 1996). Briefly, the hepatopancreas of shrimp samples were separated and stained with malachite green (0.5%). Wet mount squash of hepatopancreas tissue was prepared by placing a cover slip and pressing the tissue and observed under the microscope. MBV infection was also confirmed in samples by PCR. MBV infected samples thus confirmed were fixed in 70% ethyl alcohol and used for the study. The detail of the shrimp samples collected and used in the study is presented in Table 1.

Table 1: Details of the MBV infected samples used in the study

No.	Sample Code	Date and year of sample collection
1	MBV 9/SDDL/04	16/03/2004
2	MBV 6/SDDL/05	15/02/2005
3	MBVS1/SDDL/06	01/02/2006
4	MBV12/SDDL/07	13/09/2007
5	MBV1/SDDL/08	24/08/2008
6	MBV 8/SDDL/09	07/03/2009
7	MBV F9/SDDL/10	28/04/2010
8	MBV A9/SDDL/11	16/02/2011
9	MBV 2/SDDL/12	24/03/2012

DNA Extraction

DNA was extracted from the hepatopancreas of MBV infected shrimp samples (Lo *et al*., 1996) Hepatopancreas were separated and homogenized in NTE buffer (0.2M Nacl, 0.02M Tris Hcl, 0.02M EDTA, pH 7.4). The homogenate was centrifuged at 3000xg (4°C) and the supernatant (200µl) was added with 600µl digestion buffer (100mM Nacl, 10mMTris Hcl, 50mM EDTA, 0.5% sodium dodecyl sulfate, 0.1mg/ ml proteinase K, pH 8.0). After 2h incubation at 65°C, the digest was deproteinized by successive phenol/chloroform/isoamyl alcohol extractions. The DNA was ethanol precipitated and dried. The dried DNA pellets were resuspended in 50µl sterile nuclease-free water.

PCR Amplification

Confirmation of MBV infected samples was carried out by PCR using self-designed, MBV- specific PCR primers (MBV 250 F and 250 R). Strain variation in these samples was studied by PCR amplification of polyhedrin gene of MBV using self- designed PCR primers (MBV/SDDL/1358 F and MBV/SDDL/1358 R). The details of the primers used in the study are presented in Table 2.

Table 2: Primers used in this study

S. No.	Primers Codes	Details
1	MBV/SDDL/1358F MBV/SDDL/1358 R	Designed based on nucleotide sequence information of polyhedrin gene (Acc. No. EU251062).
2	MBV/SDDL/250F MBV/SDDL/250R	Designed based on the genomic sequence of MBV. Acc. No. AY819785

PCR was performed in a PCR thermal cycler (Eppendorf, Germany) with an initial denaturation at 95ºC for 5min; 30 cycles of denaturation at 94ºC for 1min; annealing at 57ºC for 1min (MBV/SDDL/1358F and MBV/SDDL/ 1358R), and 52ºC for 30sec (MBV 250F and MBV 250 R); extension at 72ºC for 1min and a final extension at 72ºC for 5min. PCR amplified products were resolved in a 2% agarose gel at 80v for 45 min. The separated PCR products were visualized under UV after stained with ethidium bromide and the results were documented in a gel documentation system (BioRad INC., USA). The PCR products were purified using a commercial PCR product purification kit (RBC, Canada).

Nucleotide Sequence Analysis

PCR products of polyhedrin gene of nine MBV isolates obtained from samples were sequenced using commercial nucleotide sequencing services (MWG, Bangalore, India). The nucleotide sequence information of the MBV isolates were submitted in the Genbank (www.ncbi.nlm.nih.gov) for obtaining accession numbers. The nucleotide sequence information was translated to the corresponding amino acids using an online translation tool (www.expasy.org). The homology and variations in the aminoacid sequences of the nine MBV isolates were compared with the polyhedrin protein sequences available in the Genbank database (www.bcb.lon.ac.uk)

RESULTS AND DISCUSSION

Microscopic observation of wet mount method squash preparations from hepatopancreas of MBV infected samples showed characteristic MBV occlusion bodies (Figure 1). PCR confirmation of MBV infected samples by PCR using MBV 250F and MBV 250R primers showed specific amplification of 250bp fragments (Figure 2). PCR amplification of polyhedrin gene in the MBV infected samples using polyhedrin-specific PCR primers, MBV/SDDL/1358F and MBV/SDDL/1358R resulted in PCR products of 1358bp size (Figure 3).

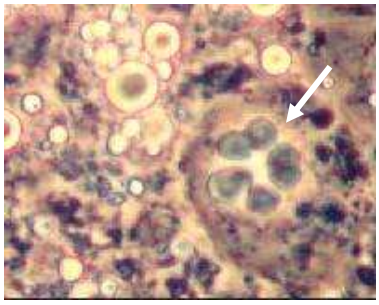

Figure 1: MBV Occlusion bodies in the hepatopancreatic cells of tiger shrimp

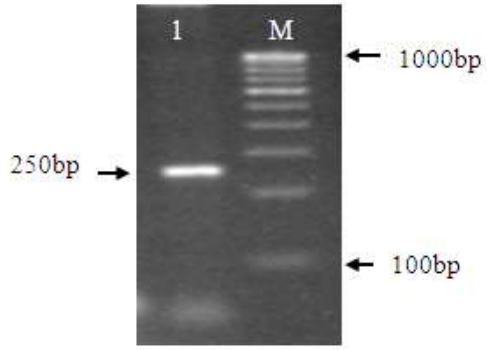

Figure 2: PCR amplified products of MBV isolates using MBV250F and MBV 250R

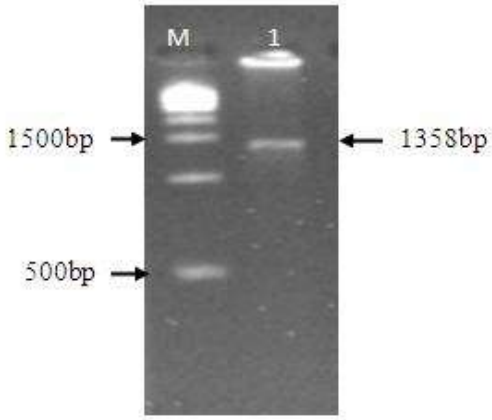

Figure 3: PCR amplified products of MBV isolates using MBV1358F and MBV 1358R

The nucleotide sequences of the MBV isolates were analysed using BLAST n analysis to understand the similarities with the available sequences in the Genbank database (blast.ncbi.nlm.nihgov). The nucleotide sequence of the isolate MBV9/ SDDL/2012 showed variations when comopared to other sequences of the isolates obtained in this study and with the existing sequence information in the Genbank database. The translated aminoacid sequence information of the nine MBV isolates obtained from samples showed that the isolate, MBV 9/SDDL/04 varied at three positions in 452 residue long polyhedrin protein. The

variations were observed at 132nd (Arginine-R in the place of proline-P), 304th (Asparagine-(N) in the place of Threonine-(T)), and 330th (Isoleucine-I in the place of Methionine-M) as shown in Figure 4.

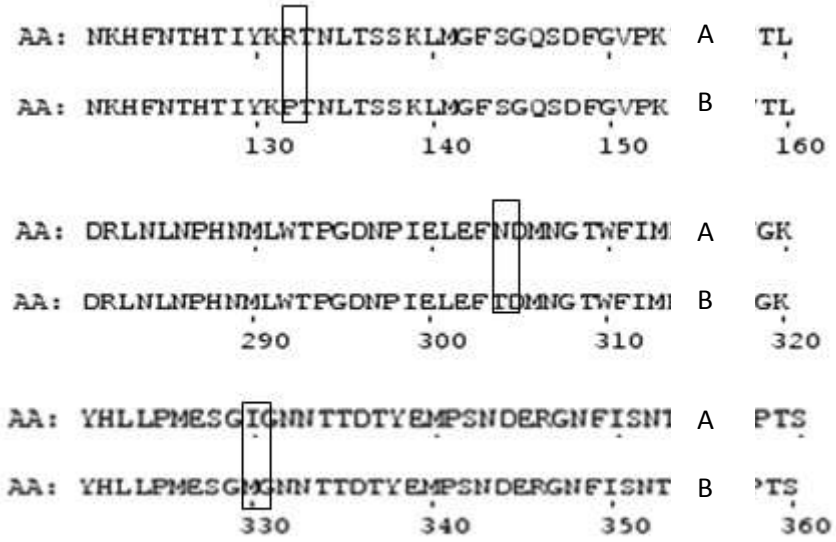

Figure 4: MBV9/SDDL/04 isolate showing variation at 132nd, 304th and 330th positions of polyhedrin gene of MBV. A denotes the amino acid sequences of the polyhedron gene of MBV isolates: MBV6/SDDL/05, MBVS1/SDDL/06, MBV12/SDDL/07, MBV1/SDDL/08, MBV8/SDDL/09, MBV F9/SDDL/10, MBV A9/SDDL/11, MBV 2/SDDL/12 and Thailand isolate, from Gen Bank database (Acc. No. EU251062). **B denotes**-MBV isolates, MBV 9/SDDL/04.

Molecular diagnostic tools such as Polymerase chain reaction (PCR) and gene probes help in rapid and accurate diagnosis of MBV in shrimp (Lightner and Redman, 1998). PCR assays have been developed for diagnosis of MBV by various researchers (Lu *et al*., 1993; Chang *et al*., 1993; Hsu *et al*., 2000). Other molecular methods like loop mediated isothermal amplification (LAMP) methods have also been reported (Chaivisuthangkura *et al*., 2009). Strain variations have been reported in various shrimp viruses like HPV (Karunasagar *et al*., 2008), WSSV (Natividad *et al*., 2006), IHHNV (Karunasagar *et al*., 2008) and MBV (Natividad *et al*., 2006). Analysis of the complete genome sequence of different strains of MBV isolated from different shrimp species and different geographical regions showed that they are genetically different (Surachetpong *et al*., 2005; Natividad *et al*., 2006). Existence of strain variations have been attributed due to the genetic adaptation of the virus to the different environmental factors in different geographical areas (Surachetpong *et al*., 2005). The variations in the nucleotide sequences affect PCR amplification and ultimately the results on the infection status of the sample. Strain variations in MBV would lead to PCR -negative results in MBV infected samples due to the loss of primer sites because of nucleotide sequence variations. This would result in the danger of using MBV infected shrimp for breeding and culture and subsequently the production and economic losses due to disease outbreak. The result of this study gives the information on the existence of the variation in the MBV strains in India which will be important in the development diagnostics for MBV infecting penaeid shrimps.

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