



In vivo therapeutic efficacy of recombinant *Penaeus monodon* antiviral protein (rPmAV) administered in three different forms to WSSV infected *Penaeus monodon*

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ABSTRACT

PmAV is the first antiviral gene identified from *Penaeus monodon*. The present study was performed to evaluate the therapeutic role of rPmAV in combating WSSV in *P. monodon*. The protective efficacy of rPmAV was tested in three different forms viz., purified rPmAV protein expressed in a fish cell line, plasmid DNA construct constitutively expressing rPmAV (pCMV-His-PmAV) and rPmAV expressing plasmid DNA encapsulated with chitosan nanoparticles. The challenge studies showed that pCMV-His-PmAV, chitosan-pCMV-His-PmAV nanoparticles and rPmAV protein (23.5 kD) injected intramuscularly into *P. monodon* gave survival rates of 27.8, 83.3 and 66.7%, respectively on fifteenth day post-WSSV challenge. The tissue distribution of the plasmid conjugate (chitosan-pcDNA-His-PmAV), which was injected intramuscularly at the ventral side of the shrimp, was traced at periodical intervals (2, 4, 8, 12 and 16 days post-injection) by PCR. The *cmv* fragment (830 bp) could be amplified from genomic DNA isolated from all the above tissues except gills up to 16th day which was the last day of testing. With this it can be concluded that rPmAV is an effective therapeutic molecule to control WSSV in shrimp provided that detailed studies regarding the best delivery strategy and optimum dosage are carried out.

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1. Introduction

Penaeus monodon is one of the most widely cultured shrimp species in the world. Its aquaculture production during 1981 was merely 21,000 tons and since then there was a gradual increase in production mounting to nearly 7,70,000 tons during the year 2009 with a value of USD 3.65 billion (FAO, 2009). Major producers are Thailand, Vietnam, Indonesia, India, Philippines, Malaysia and Myanmar. It was earlier expected that *P. monodon* aquaculture industry would show a significant growth and expansion all over the world but due to a number of causes it's declining. The major setbacks include viral disease outbreaks, shortage of broodstock, market competition and trade barriers. Amongst the viral threats, WSSV is the major culprit that is crippling the *P. monodon* industry. It is estimated that WSSV caused losses of 300,000 tonne of shrimp, worth more than 1 billion US dollars (Rosenberry, 2001). However, so far there has been no effective therapy to eliminate this deadly disease from shrimp farming industry and it can be attributed to fact that specific immune response system in shrimp is rudimentary and little understood (Söderhäll and Cerenius, 1999).

In recent years there has been an expansion in knowledge pertaining to shrimp defense at molecular level. Some of the antimicrobial defense mechanisms that have been characterized include the prophenoloxidase (proPO) activating system (Cerenius and Soderhall, 2004; Soderhall and Cerenius, 1998) and antimicrobial peptides (Bachere et al., 2004; Destoumieux et al., 2000). But however, only little is known about the possible antiviral factors in shrimp at the molecular level (He et al., 2005; Pan et al., 2000). In this context, understanding the antiviral mechanism of *P. monodon* against WSSV i.e. utilizing of natural host innate immune factors for disease control could be an effective alternative that may greatly help in WSSV disease control.

It is a well known fact that C-type animal lectins exist widely in vertebrates and invertebrates, and they represent an important recognition mechanism for oligosaccharides at cell surfaces (Drickamer, 1999). All known C-type lectins are unique as they possess a carbohydrate recognition domain (CRD), which is known to mediate sugar binding with Ca²⁺ (Feizi, 2000). C-type lectin, coagulation factor binding protein, IgE Fc receptor and NK cell receptor etc are some of the molecules containing CTLD that are known to play an important role in immunity (Drickamer, 1999). PmAV is the first antiviral gene encoding an innate immune protein being identified in shrimp and was observed to be up-regulated in response to viral infection (Luo et al., 2003). It has been cloned from a virus-resistant shrimp *P. monodon* by differential display (DD) and recorded to possess an open reading frame (ORF) encoding a 170 amino acid peptide with a

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possible C-type lectin-like domain (CTLD). Also the recombinant PmAV protein purified displayed a strong antiviral activity against virus-induced cytopathic effect in a grouper fish cell line *in vitro* (Luo et al., 2003). In this study, an attempt was made to explore the antiviral activity of PmAV *in vivo* in black tiger shrimp against WSSV. In order to arrive at a better strategy to administer rPmAV to shrimp, different forms *viz.*, recombinant protein expressed from a fish cell line, plasmid DNA construct constitutively expressing the rPmAV in the shrimp body and the rPmAV expressing plasmid DNA encapsulated with chitosan nanoparticles were evaluated for their efficacy.

2. Material and methods

2.1. Shrimp rearing

Black tiger shrimp, *P. monodon* weighing 20 ± 2 g were procured from Pancham Aquafarm, Maharashtra, India and maintained in 1000 l FRP tanks (50 shrimp/tank) with natural sea water (25–28 ppt) and aeration. The animals were fed *ad lib* with an artificial pellet feed (CP, Thailand) thrice a day. Prior to use, the experimental animals were checked for the presence of any external abnormalities, disease symptoms, erratic swimming and lethargy. Samples were screened using Single-tube WSSV detection kit (Bangalore Genei, India) to detect any possible WSSV infection. Only the healthy shrimps were used for all the experiments. Experiments were conducted in plastic crates (Nilkamal, India) of dimension $75 \times 50 \times 40$ cm (6 shrimps/tank) and hide outs were provided to avoid cannibalism. A static seawater system with sufficient aeration and a temperature of 28 ± 1 °C was used.

2.2. Construction of expression plasmids

The total RNA was isolated from hepatopancreas of tiger shrimp and cDNA was prepared following standard protocols (Sambrook et al., 2001). The complete open reading frame of PmAV gene was amplified from the cDNA using specific primers PmAV-F and PmAV-R (Table 1). The PCR cycling conditions included an initial denaturation at 94 °C for 6 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 64 °C for 30 s and an extension at 72 °C for 1 min. Final extension was done for 8 min. The PmAV amplicon was cloned into eukaryotic expression vector pcDNA4-His-Max-A (Invitrogen) downstream to the CMV promoter using PstI and XhoI restriction sites and transformed into *Escherichia coli* (DH5 α) cells. The recombinant plasmid was confirmed by DNA sequencing and named pCMV-His-PmAV. The recombinant plasmid was isolated using EndoFree plasmid purification Giga kit (Qiagen, Germany), according to manufacturer's instruction and stored at -20 °C until further use.

2.3. Production of rPmAV protein in FHM cell line

Fat Head Minnow (FHM) cell line (NCCS, Pune) was regularly sub-cultured in MEM media following supplier's protocol. The pCMV-His-PmAV pDNA was transfected into FHM cells using TurboFect™ transfection reagent according to manufacturer's protocol. The expression of rPmAV protein was analyzed 48 h after transfection by

Table 1

Primers used in this study.

Name	Sequence (5' to 3')	Ta (°C)
PmAV-PstI-F	CGCTGTCAGACAATGCGTCATACAATCCTAG	64
PmAV-XhoI-R	CCC CTCGAGGATTAATGTGTCCTGCTTTCAC	
VP19-F	AAAGCTAGCCGATGGGTCTCTTTTGAT	55
VP19-R	AAAGAAITCCGTCATATCCTGGTCTGT	
EF1 α -F	GGTGTCTGGACAAGCTGAAGGC	60
EF1 α -R	CGTTCGGTGATCATGTTCTTGATG	
CMVpro-F	AAAGCACGCAATCTGCTGGGTTAGG	55
CMVpro-R	AAATCTAGAAATTCGATAAGCCAGTAAGC	

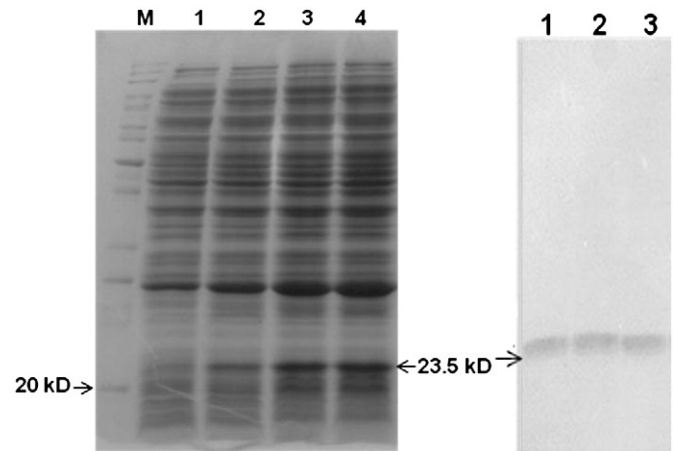


Fig. 1. SDS-PAGE analysis and Western blotting of FHM cells transfected with pCMV-His-PmAV construct, lane M: Protein ladder, lane 1: Control cells with empty vector, lane 2: Cells harvested at 16 h post-transfection, lane 3: Cells harvested at 24 h post-transfection, and lane 4: Cells harvested at 48 h post-transfection.

SDS-PAGE and Western blotting and then the His tagged rPmAV protein was purified from the FHM cells using Ni-NTA agarose (Qiagen, Germany) following manufacturer's instructions. The specificity of the eluted protein was also confirmed by Western blotting using anti-His antibodies (Fig. 1). The concentration of the eluted protein was estimated using Nanodrop.

2.4. Preparation of chitosan-pCMV-His-PmAV nanoparticles

The pCMV-His-PmAV plasmid isolated using EndoFree plasmid purification Giga kit (Qiagen, Germany), was encapsulated with chitosan nanoparticles for increasing the stability of the DNA vaccine. The chitosan-pDNA conjugate was prepared following standardized protocol (Rajeshkumar et al., 2009). Chitosan and pDNA complex formation was confirmed by agarose gel electrophoresis. The mean particle size and size distribution of chitosan-pDNA nanoparticles were determined by photon correlation spectroscopy with Beckman Coulter Delsa Nano Particle Size analyser (Fig. 2). The zeta potential was studied by electrophoretic light scattering (ELS) technique.

2.5. In vivo titration of viral dose

WSSV viral inoculums were prepared from the WSSV infected shrimps following the method of Xie and Yang (2005). Infectivity and optimum dosage of the prepared WSSV stock was determined by administering it to the healthy shrimps. Briefly, the inoculum was diluted into different concentrations *viz.*, 1:1, 1:2 and 1:3 using phosphate buffered saline and one group was given the crude inoculum as

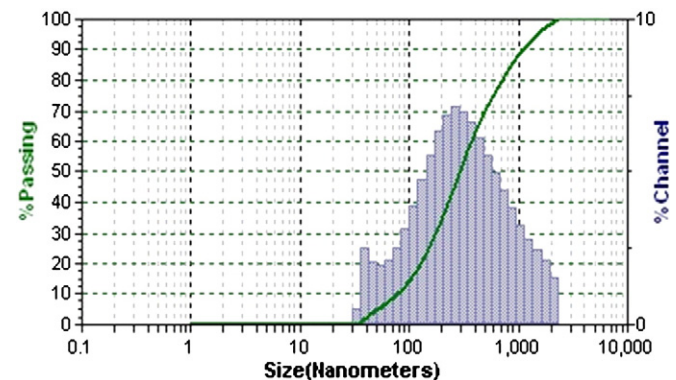


Fig. 2. The mean size of chitosan-pcDNA-His-PmAV conjugate as observed in Beckman Coulter Delsa Nano Particle Size analyzer.

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