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Oral vaccination of *Macrobrachium rosenbergii* with baculovirus-expressed *M. rosenbergii* nodavirus (*Mr*NV) capsid protein induces protective immunity against *Mr*NV challenge

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ABSTRACT

White Tail Disease (WTD) is one of the important viral diseases of fresh water giant prawn *Macrobrachium rosenbergii,* which is caused by *Macrobrachium rosenbergii* nodavirus (*Mr*NV). In the present study, the capsid protein gene of *Mr*NV containing a His-tag was cloned into a baculovirus vector pVL1393 and expressed the recombinant *Mr*NV protein in insect cells, using a baculovirus expression system. A band corresponding to the *Mr*NV protein of 43 kDa was characterized after fractionating the proteins of baculovirus-infected cell lysates by SDS-polyacrylamide gel, and immunostaining with His-tag monoclonal antibody. Furthermore, purified *Mr*NV capsid protein assembled into virus-like particles (VLPs) of ~30 nm in diameter, when examined by transmission electron microscopy (TEM). To vaccinate the larvae by oral route, the recombinant *Mr*NV (r-*Mr*NV) protein was coated with artificial prawn feed and fed to *M. rosenbergii* larvae (90 ± 10 mg) for 60 days. After 30 and 60 days of vaccine treatment, group of prawns were challenged with virulent *Mr*NV orally. Samples were collected at different time intervals to evaluate the survival of larvae and to analyze the presence of *Mr*NV by double-step PCR and expression of immune/ toll-like receptor (TLR) genes. Non-vaccinated group of *M. rosenbergii* larvae succumbed to death and had 90% mortality, whereas the r-*Mr*NV protein treated groups exhibited 65 and 80% survival ($P \leq 0.001$) for 30 and 60 days post-vaccination (dpv), respectively. Double-step PCR diagnosis revealed that there was 100% positive signals observed in non-vaccinated prawn group, whereas the infection was reduced significantly ($P < 0.001$) to 32 and 17% respectively in 30 and 60 dpv. Among the four different immune/ TLR genes such as antimicrobial peptide (*Mr*amp), lysozyme (*Mr*LY), proPhenol Oxidase (*Mr*PPO) and Toll-Like Receptor (*Mr*Toll) expression screening, *Mr*amp was successfully expressed in the *Mr*NV subunit protein vaccinated prawns, whereas the non-vaccinated prawn had no immune/TLR gene expression. Taken together, our results demonstrate that oral vaccination of *M. rosenbergii* larvae with baculovirus-expressed *Mr*NV capsid protein confer up to 78% protection against *Mr*NV infection.

1. Introduction

Macrobrachium rosenbergii, the giant freshwater prawn (GFP), is an economically important crustacean, being farmed on a large scale in many different countries [1], including China, Israel, Japan, India, Latin America, Caribbean and some countries in Africa due to its potential of fast growth, large size, disease tolerance and export market value [2]. China is the top most global producer of GFP and rose to a peak of 111,282 tonnes in 2001, representing 65% of global production [3]. The last decade, there has been significant increase in the global production of the giant freshwater prawn which had exceeded 400,000tonnes in 2010 [4]. Unfortunately, infectious diseases in *M. rosenbergii* culture caused by bacteria and virus constitute main barrier for the development and leading to huge economic losses and severe damages. A new viral disease outbreak occurred in the last decade namely, white tail disease (WTD) to cause immense economic losses in

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hatcheries and farms, with mortalities often reaching 100% within 2 or 3 days [5,6]. The WTD was first identified in Guadeloupe Island in 1995 and followed by Martinique Island of French West Indies [5], China [7] and India [8]. The causative virus for WTD, identified as *M. rosenbergii* nodavirus (*Mr*NV), is a small, icosahedral, non-enveloped virusof 26–27 nm in diameter [5]. The *Mr*NV genome consists of two segments of ssRNA (RNA1 and RNA2) of 2.9 and 1.26 kb, respectively. RNA1 contains the coding sequences of two non-structural proteins; A protein, which is RNA-dependent RNA polymerase (RdRp) of 100 kDa, and B protein with an estimated size of 13 kDa. RNA2 encodes for a single capsid protein (CP) of 43 kDa [6]. The virus replicates in the cytoplasm of connective tissues in the host, and the clinical symptoms are lethargy, anorexia and opaqueness of abdominal muscle in post-larvae and adults. Whitish appearance of the tail is the prominent sign and milky opaqueness gradually expands on anterior and posterior sides of the prawn, and sometimes degeneration found in telson and uropods [8]. The affected prawns are weakening to swimming and feeding ability [9].

Considering the potential threat of diseases on the one hand, environmental issues on the other hand, diseases management should concentrate on environment friendly biotechnological methods, such as efficient vaccine construction and safety delivery protocols. Even though synthetic drugs and antibiotics give positive effects against pathogenic control, they cannot be recommended due to their negative roles like residual problems, higher cost and biomagnifications [10]. Eventhough traditional vaccines gives positive effects and have some demerits, such as weak and shorter immunity, reversion of virulence, some times ineffective, heat liable and need an adjuvant. Efficacy, safety and vaccine delivery route deserve special attention for designing and delivering the vaccines [11]. In addition, novel vaccination methods have to be cost-effective, easy to apply, and if possible less stressful to the animals. Recent advances in recombinant DNA technology have provided a means of economic production of sufficient quantities of immunoprotective antigens in the form of recombinant subunit vaccines, which are safer than traditional vaccines which will provide strong and long-lasting immunity to eradicate the pathogens.

The proposed method of recombinant subunit vaccine production using *Mr*NV-CP-2 gene through baculovirus expression system has the advantages including, strong polyhedrin promoter in the baculovirus vector that gives high-level expression of antigens with post-translational modifications, which evoke effective immune responses in animal systems [12,13]. In addition, oral delivery of *Mr*NV-CP subunit vaccine by top-coating on the feed pellets helps to alleviate stress to the prawns, when compared to bacterially-expressed injectable vaccine which needs to be purified to avoid bacterial endotoxin toxicity [14]. This proposed vaccination approach can elicit strong immune response and offers economic benefit, environmental and safety advantages, which are particularly attractive for the farmers. In this study, we describe the production of the recombinant *Mr*NV capsid protein in insect cells, delivery of this protein orally with the feed to *M. rosenbergii* juveniles, and evaluation of the survival and protective response against *Mr*NV challenge.

2. Materials and methods

2.1. Synthesis of MrNV-CP-RNA-2 gene

The *Mr*NV-CP-RNA-2 gene (1146bp) was synthesized based on the published nucleotide sequence of Indian *Mr*NVRNA-2 segment in the GenBank (Accession No. JQ418298), which contains a BamHI restriction site at the 5′-end, a 6X His-tag at the C-terminal, followed by an EcoRI restriction site at the 3′-end. The gene fragment was cloned into pBME-Amp vector with the size of 4047 bp length (Biomatik, USA).

2.2. Cloning of MrNV-CP-RNA-2 gene into baculovirus vector pVL1393

*Mr*NV-CP-RNA-2 gene from pBME-Amp vector was excised by using the respective restriction enzymes BamHI and EcoRI. Simultaneously, the baculovirus vector pVL1393 was also digested with the same restriction enzymes where these sites are present downstream of polyhedrin promoter. The digested vectors were run in 0.8% agarose gel electrophoresis and the bands were excised, and purified by using QIAquick Gel Extraction Kit (Qiagen). The ligation reaction was performed in 10 µl of ligation mix, which contained 2 µl of digested pVL1393, 5 µl of *Mr*NV-CP-RNA-2 fragment, 1 µl of 10X ligation buffer, 1 µl of nuclease free water and 1 µl of T4 DNA ligase. The mixture was incubated overnight at 4 °C and subsequently used to transform *Escherichia coli* JM109 competent cells (Promega). The transformants were enriched with SOC media (Promega), plated on Luria Bertani (LB) agar plates containing ampicillin and incubated 37 °C for 16 h. Positive bacterial colonies were screened for the presence of *Mr*NV-CP gene in pVL1393, using restriction digestion with BamHI and EcoRI enzymes [15].

2.3. Production of recombinant baculovirus using flashBAC™ system

*Mr*NV- pVL1393 plasmids were precipitated with ethanol, and the DNA was suspended in nuclease-free deionized water and quantified. For viral transfection, *flash*BAC^{TM}baculovirus expression system was used and the protocol was followed based on the instuctions provided by the supplier (Mirus Bio LLC, USA). Briefly, to make tranfection reagent:DNA complexes, one ml of serum and antibiotic free insect culure media (S-900) was placed in a polystyrene sterile tube to which 5 µl of transfection agent (Lipofectin^{R}) was added, followed by addition of 100 ng of *flash*BAC™ DNA. After that, 500 ng of purified *Mr*NV-pVL1393 recombinant plasmid was added, mixed by vortex, and incubated for 15–30 min. Next, this transfection reagent: DNA complexes mixture was added in drop-wise manner to the monolayers of *Spodoptera frugiperda* (*Sf*9) insect cells grown to 80% confluency in a well, and incubated for 5 h at 28 °C. After this incubation period, one ml of S-900 was added and the cells were further incubated for five days.

2.4. Characterization of recombinant MrNV-CP by Western blot

Recombinant baculovirus harboring the *Mr*NV-CP-2 gene was harvested aseptically from the cell culture flasks and spun at 5000 × *g* for 5 min. Five hundred micro liter of supernatant was transferred to fresh confluent monolayers of *Sf*-9 cells for reinfection. Five days post-infection, *Sf*-9 cells were harvested by centrifugation. After lysis, cellular proteins were resolved by 12.5% SDS-PAGE [16], transferred to a nitrocellulose membrane, reacted with histidine (6xHis) tag primary antibody (1: 1000 dilution) and alkaline phosphatase-conjugated goat-*anti*-mouse secondary antibody (1:2000 dilutions). For His-tag antibody source, 6X His Epitope Tag Antibody (Product # MA1-21315) was obtained from Thermo Fisher, USA. Secondary antibody was goat anti-mouse alkaline-phosphatase conjugated antibody from Thermo Fisher, USA. A single band of recombinant *Mr*NV protein (43 kDa) was detected with streptavidin-alkaline phosphatase and naphthol phosphate fast red color development (Sigma, St. Louis, MO, USA).

The recombinant baculovirus (harboring *Mr*NV gene) infected *Sf*-9 cells were recovered from the cell culture media by centrifugation at 800 × *g* for 5 min, washed twice with PBS, and re-suspended in 10 ml of PBS. The cell suspension was sonicated for 4X for 20 s to disrupt the cells. The sonicated cells were centrifuged at 15,000 × *g* for 15 min at 4 °C. His-tag protein purification mini column (Bio-Rad, USA) was equilibrated with two bed volume of binding buffer and spun at 700 × *g* for 2 min before loading the proteins. One ml of lysate containing recombinant *Mr*NV protein was loaded to the column and placed in the gel rocking shaker for 30 min at room temperature and then washed the resin with two bed volume of wash buffer at 700 × *g* for 2 min (twice).

The target protein, which was bound to the column resin, was eluted using 2 volume of elution buffer, and the fractions were collected and run on SDS-PAGE.

2.5. Virus-like particle (VLP) preparation

Recombinant baculovirus (harboring *Mr*NV-CP gene) infected *Sf*-9 cells were propagated in 1 L of culture for five days and the cells were harvested by centrifugation at 800 × *g* for 5 min to obtain a cell pellet. An aliquot of the cells was tested by Western blotting to confirm the expression of *Mr*NV His-tag protein, following the protocol mentioned in section 2.4. Approximately 14 g of pellet was re-suspended in 30 ml of 0.01 M Tris (pH 8.0), followed by addition of 200 µl of 10% deoxycholate and 10 µl of Benzonase (2500 units). The mixture was incubated on ice for 20 min and then sonicated 4X for 20 s to disrupt the cells. The mixture was extracted with 10 ml of Freon (2 tubes), and clarified by centrifugation (Sorvall, 10,000 rpm for 30 min). The lower phase and interphase was pooled into one tube and 5 ml of PBS was added. The mixture was sonicated again and centrifuged (~33 ml). This mixture was layered into two ultracentrifuge tubes containing 7 ml of 56% sucrose +15 ml of 28% sucrose and ultracentrifuged at 26,000 rpm for 2 h, using SW28 rotor. Visible opaque band was collected and the sucrose was removed using 30 K cutoff filter. Next, the viral suspension material was loaded (~3 ml) into another tube containing CsCl gradient and spun at 36,000 rpm for 5 h, using a SW40Ti rotor in an ultracentrifuge. A single opaque band at 1.3 g/ml CsCl density was collected; desalted and concentrated using a 10 K cutoff filter, and then resuspended in a small volume of nuclease free water. Finally, the suspension (VLPs) was examined under transmission electron microscope (TEM) by applying the sample on a grid that was negatively stained with sodium phosphotungstate.

2.6. Subunit vaccination of M. rosenbergii

2.6.1. Vaccine preparation

*Mr*NV-CP expressing baculovirus infected *Sf*-9 cells pellets were mixed with sufficient quantity of PBS (pH, 7.2) and sonicated to disrupt the cells. The ruptured cells were diluted and the total protein was quantified which was at least 200 µg per ml by nanodrop spectrophotometer (Thermo Scientific, USA). One ml of cell lysate, containing approximately 10 µg of *Mr*NV-specific protein (~5% yield), was mixed with 30 g of artificial diet (Basal ratio of 45.1% protein; 7.2 lipid, 14.6% ash, 7.1% moisture and 3% fibre) and incubated 20 min. Prawn consumes approximately 150 mg of feed daily (average) and therefore every day about 1 µg of total insect protein is consumed by the individual prawn in early stages (up to 20 days), which was increased over the period to 2 µg. At 60 days post vaccination, each prawn would consume about 100 µg of total protein or 5 µg of *Mr*NV-specific protein. Sufficient amount of cod liver oil was mixed with the diets to avoid leaching of *Mr*NV recombinant protein. Control diet was prepared with uninfected *Sf*-9 cells pellet lysate and the diets were stored at −20 °C freezer until use.

2.6.2. Experimental set-up and MrNV challenge

M. rosenbergii post larvae, weighing about 90 ± 10 mg, were purchased from ADAK (Agency for Development of Aquaculture) hatchery, Department of Fisheries, Varkala, Kerala, India. They were stocked in a FRP tanks with the capacity of 5,000 L in wet lab until acclimatization. Uniform size of *M. rosenbergii* was stocked into individual experimental FRP tanks with the capacity of 1000 L experimental and a control groups. Triplicate culture ($n = 25 \times 3 = 75$) was maintained in each group with continuous flow through water and constant aeration system. Prawns were fed thrice a day at 8.00, 13.00 and 18.00 h at 10% of the body weight for maximum of 60 days. Uneaten food and waste matters were removed before feeding. The water quality parameters such as temperature (28 ± 1.0 °C), salinity (3 ± 1.0‰) and pH (8.3 ± 0.1) were maintained. After feeding experiment, group of prawns from each replicates ($n = 20 \times 3 = 60$) of the experimental and control groups were challenged with *Mr*NV filtrate coated diets (200 µg/ g) feeding at 30 and 60 days of post-vaccination (dpv). The *Mr*NV was isolated from infected *M. rosenbergii* post larvae samples collected from Kerala, India during 2016. After confirming the presence of *Mr*NV by diagnostic PCR, the PCR-positive samples of *M. rosenbergii* post larvae were ground with NTE buffer (pH 7.4) and filtered. The filtrate was injected in to the adult *M. rosenbergii* at the rate of 300 µg of total protein for *Mr*NV propagation. After 4–5 days (Moribund condition), the haemolymph was bled from the *Mr*NV filtrate injected *M. rosenbergii.* Also, the different parts of post larvae such as gills and abdominal muscle were dissected out, ground with NTE buffer, filtered using the 0.45 syringe filter and the filtrate was used for challenge studies. Meanwhile, prawns from blank control group were fed with normal diets and no *Mr*NV challenge. The percentage of cumulative mortality was monitored at least for 10 days after challenge.

2.7. Molecular diagnosis of MrNV load

The experimental as well as control prawn samples after *Mr*NV challenge were checked by *Mr*NV diagnostic PCR (two steps) using the primer sets (Forward: 5′ CAAGCGCCGTAAGCGTAATC 3'; Reverse: 5′ GTTGGTGGAACCCAATTGCC 3') to amplify the product size of 750 bp. The DNA extraction and PCR amplification were carried out following the method described by Chang et al. [17]. The DNA samples of experimental and control shrimp were tested by first step PCR. The negative samples detected in the first step were further subjected to second step PCR analysis.

2.8. Immune gene and toll-like receptors (TLR) expression by semi-quantitative PCR

Head region of the control and experimental prawns were aseptically excised and ground with TRI reagent (Sigma Aldrich, Cat: T9424) and the total RNA was isolated. Reverse transcription was performed with 1 µg DNase treated RNA for 3 h at 37 °C. The expression levels of immune genes and Toll-Like Receptor (TLR) were studied. Relative amount of target mRNAs were normalized to β-actin mRNA as internal control. Semi-quantitative PCR was performed in Veriti Thermal Cycler (Applied Biosystems) using Taq DNA Polymerase. The PCR conditions were standardized based on the melting temperature of the primers and the amplicon size.

2.9. Data analysis

One way and Two way Analysis of Variance (ANOVA) were carried out using SPSS statistics data package and Ky plot respectively. Means were compared at 0.01 and 0.001% levels and subsequent post-hoc multiple comparison with SNK test (One way ANOVA).

3. Results

3.1. Cloning of MrNV-CP-RNA-2 to baculovirus vector pVL1393

*Mr*NV-CP-RNA-2 gene fragment, containing desired restriction sites and a His-tag from pBME-Amp vector, was successfully cloned into the baculovirus vector pVL1393. The gene fragment comprised of 1146 bp, included the open reading frame (ORF), restriction enzyme sites and a His-tag. The generated ORF sequence (of *Mr*NV-CP) had the nucleotide length of 1112 bp.

3.2. Baculovirus expression and purification of MrNV-CP

*Mr*NV-CP, with the molecular weight of 43 kDa, was successfully expressed through *flash*BAC™baculovirus expression systemin

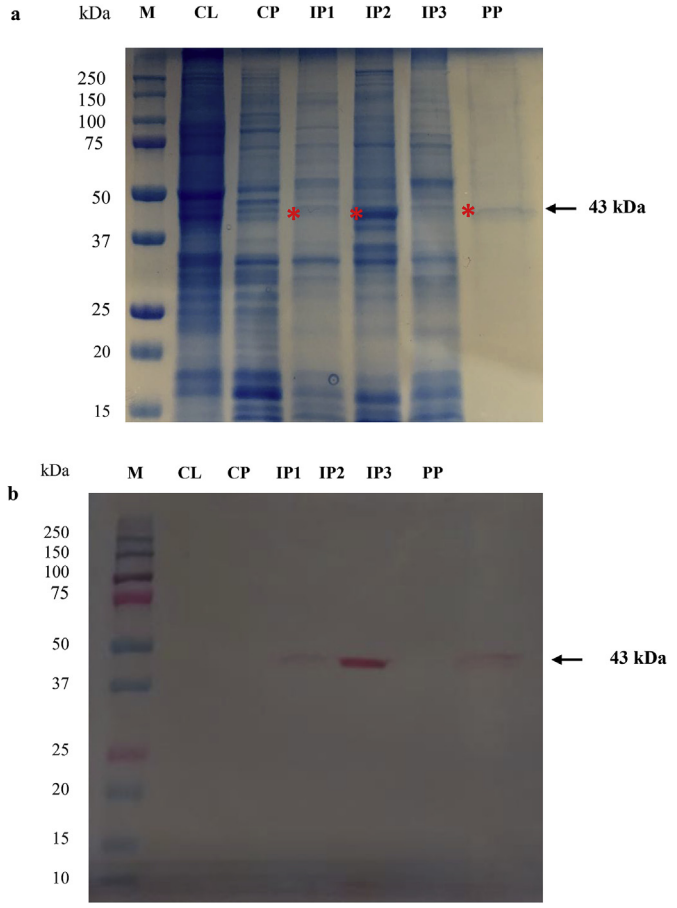

Fig. 1. Coomassie Blue staining of the proteins from baculovirus infected cells and purified *Mr*NV capsid protein. *Spodoptera frugiperda* (*Sf*-9) insect cells were infected with recombinant baculovirus and harvested at 72 h post infection. After lysis, cellular proteins were separated on 12.5% SDS-polyacrylamide gel (a). Western blot analysis of recombinant *Mr*NV capsid protein expressed in *Sf*-9 insect cells. Proteins were fractionated on 12.5% SDS-PAGE, transferred to a nitrocellulose membrane, reacted with histidine (6xHis) tag primary antibody (1: 1000 dilution) and alkaline phosphatase-conjugated goat-*anti*-mouse secondary antibody (1:2000 dilutions) (b). M: Pre-stained low molecular weight protein markers from Bio-Rad; CL: Control *Sf*-9 cell lysate (uninfected); CP: Control cell pellet (uninfected); IP1 to IP3: Infected *Sf*-9 cell pellets 1–3 and PP: purified recombinant *Mr*NV capsid protein by Ni-NTA affinity column chromatography. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Spodoptera frugiperda insect cell (*Sf*-9) lines (Fig. 1a). We screened different infected cell line passages and detected the *Mr*NV-CP with the molecular weight of 43 kDa in the cell pellets of IP1 and IP2 by SDS-PAGE. The lane PP (purified protein) also had the single band of polypeptide with the molecular weight of 43 kDa after His-tag column purification from the infected cell homogenate. The molecular weight of the *Mr*NV-CP, including the His-tag binding protein, was 43 kDa and therefore, the molecular weight of mature peptide of *Mr*NV-CP. Western blot analysis also revealed that the cell pellets of IP1, IP2 and the PP gave positive signals after staining with the histidine-tag antibody (Fig. 1b). In addition, a very strong signal was detected when an aliquot of soluble cell fraction of the large-scale *Sf*-9 cell culture material was tested and used for vaccine and VLP preparation (data not shown).

3.3. Virus-like particle (VLP)

Virus-Like Particles (VLPs) were formed by assembly of r-*Mr*NV capsid protein in the higher density gradient of CsCl. Transmission electron microscopic (TEM) analysis confirmed that the purified *Mr*NV capsid protein assembled into icosahedral structures of approximately 30 nm in diameter, resembling the native *Mr*NV virions (Fig. 2).

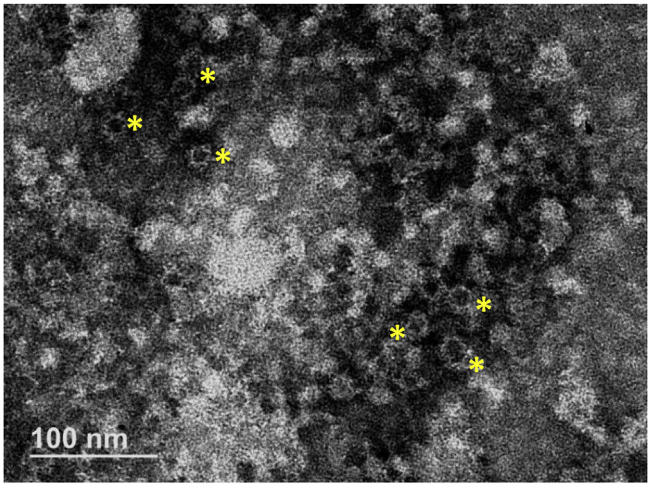

Fig. 2. Transmission electron microscopic (TEM) analysis of CsCl purified recombinant *Mr*NV virus-like particles (VLPs) negatively stained with sodium phosphotungstate. Bars = 100 nm. Asterisks indicate the presence of VLPs which are approximately 30 nm in diameter.

3.4. Vaccination of M. rosenbergii

3.4.1. Survival

The prawn group which was fed normal diet (without coating of r-*Mr*NV protein) succumbed to 90% death after *Mr*NV challenge until 10th day of post-infection. On the other hand, the survival was significantly increased in the prawn groups that were fed with diet containing *Mr*NV protein. The survival of 65 and 80% was observed in 30 and 60 dpv prawn groups respectively after *Mr*NV challenge. Two way ANOVA revealed that the survival differed significantly from each other (Column: $F = 23.15$; $P \leq 0.001$ and Row: $F = 4.28$; $P \leq 0.01$). Among the different intervals of vaccinations, the 30 dpv group had the survival of 55% and the 60 dpv group had the survival of 70% due to the influence of *Mr*NV recombinant subunit vaccines (Fig. 3).

3.4.2. Molecular diagnosis by double-step PCR

Double-step PCR diagnosis for *Mr*NV in control and vaccinated *M. rosenbergii* are presented in Fig. 4. All the screened prawns from unvaccinated group (control) were *Mr*NV positive in the first and second step detection. The infection was gradually reduced when the vaccination time was prolonged. There were 23% and 9% PCR-positive signals observed in the first and second step detection in respectively 30 dpv after *Mr*NV challenge. In 60 dpv group, 12 and 5% PCR-positive signals were observed in first and second steps, respectively. In overall status, the infection observed of 32% in 30 dpv and it was significantly ($P < 0.001$) decreased to 17% in 60 dpv after *Mr*NV challenge. The vaccination period of 30 dpv helped to reduce the infection by 68% and 60dpv helped to reduce the infection by 83%, respectively.

3.4.3. Immune gene and toll-like receptors (TLR) expression

Even though we screened different immune genes, such as *Mr*amp, *Mr*LY, *Mr*PPO and the TLR gene *Mr*Toll, with β- Actin control by semi-quantitative levels in the control and experimental prawn samples after *Mr*NV challenge, only *Mr*amp was expressed in the *Mr*NV vaccine coated diet fed prawn group. Unfortunately, the other genes like *Mr*Toll, *Mr*LY and *Mr*PPO were not amplified by the semi-quantitative PCR amplification. The immune gene *Mr*amp was successfully amplified with the size of 453 bp in *Mr*NV vaccine coated diet fed prawn group after 60 dpv, whereas no expression was obtained in the control diet fed prawns (Fig. 5). The internal control β- Actin was expressed in both

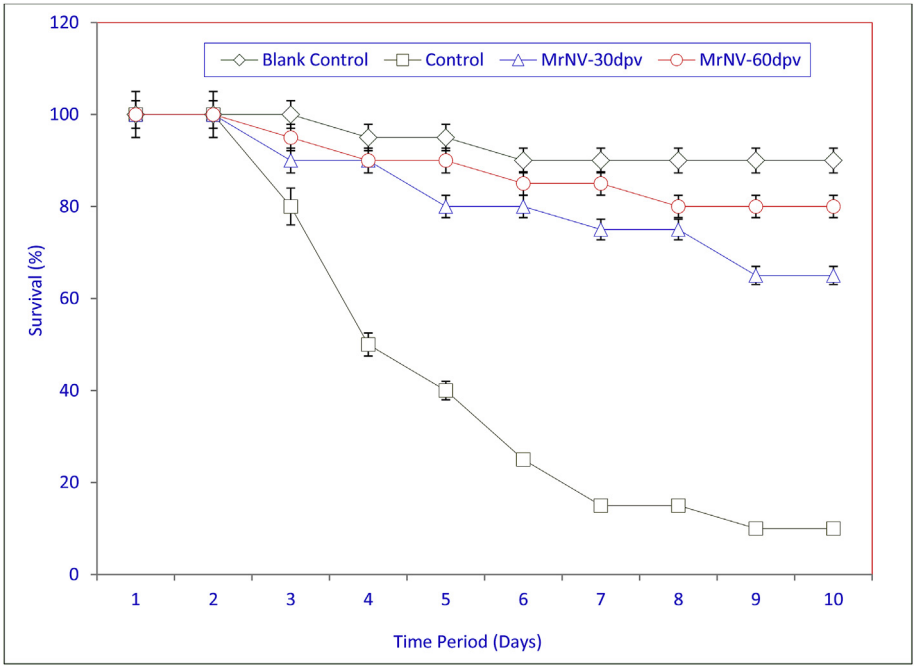

Fig. 3. Survival (%) of giant freshwater prawn *Macrobrachium rosenbergii* fed on recombinant *Mr*NVcapsid protein vaccine coated diet and challenged with *Mr*NV. The values are significantly different from each other (Column: $F = 23.15$; $P \leq 0.001$ and Row: $F = 4.28$; $P \leq 0.01$) -Two way ANOVA.

control and experimental prawns with the size of 686 bp after 60 dpv.

4. Discussion

Infectious diseases caused by viruses and bacteria constitute the main barrier to the development and continuation of crustacean aquaculture; each cultivated species being sensitive to several types of pathogens [18]. The current treatment protocols are not very effective and hence alternative effective approaches are needed to solve the disease outbreak problems and improve the economic outputs. Among the alternative practices, vaccinations are one of the suitable tools to control the pathogens. In the development of new generation of subunit vaccines for various pathogens, VLPs-based technology has made great strides in the field of vaccinology in the last three decades [19–22]. VLPs have several advantages for vaccinations strategies over the conventional vaccines. Since VLPs are composed of the capsid protein (s) without the genomic material of the pathogen(s), they are non-infectious and represent a safer alternative to attenuated viruses. In addition, VLPs mimic the natural configuration of authentic virus and they are highly effective in eliciting both humoral and cell-mediated immune responses. To date, several VLP-based vaccines are commercially available, which includes hepatitis B virus, human papilloma virus, malaria, and several others undergoing preclinical and clinical development [21,22].

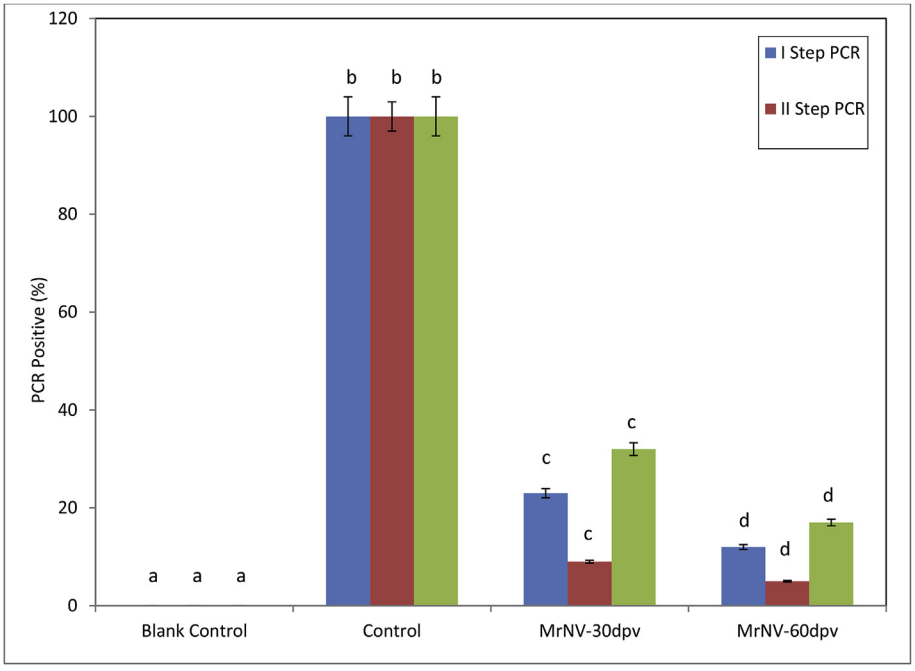

Fig. 4. Percentage PCR detection in giant freshwater prawn *Macrobrachium rosenbergii* fed on recombinant *Mr*NVcapsid protein vaccine coated diet and challenged with *Mr*NV. Bars with different lowercase letters are statistically different from each other (one-way ANOVA, $P < 0.001$ and subsequent post-hoc multiple comparison with SNK test).

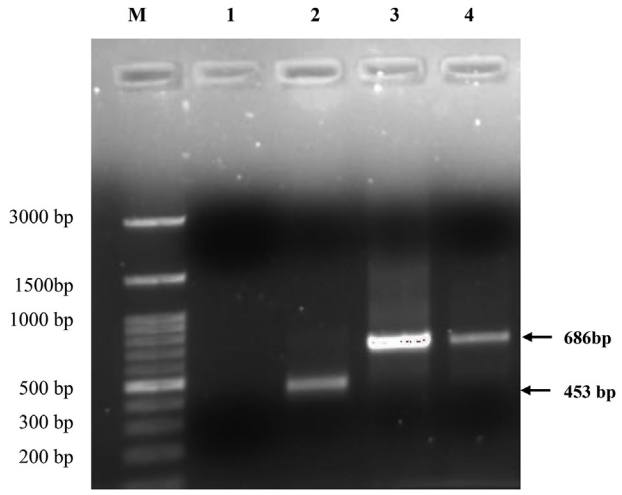

Fig. 5. Semi-quantitative PCR for expression of the *Mr*amp and β-actingene products in the control and experimental *M. rosenbergii* post-larvae that were challenged with *Mr*NV after 60 dpv. Lanes: M: marker (100 bp ladder H3 RTU, GeneDireX, India); 1: *Mr*amp from control; 2: *Mr*amp from *Mr*NV capsid protein vaccine coated diet fed *M. rosenbergii* (453 bp); 3: β-actin (686 bp) from control group and 4: β-actin from *Mr*NV capsid protein vaccine coated diet fed *M. rosenbergii* (686 bp).

In earlier studies, several researchers have cloned and produced the *Mr*NV capsid protein in prokaryotic cells. Goh et al. [23] reported the self-assembly of *Mr*NV capsid protein into virus-like particles (VLPs) in bacterial cells, whereas, Jariyapong et al. [24] also produced the *Mr*NV VLPs in *E. coli* and used it as a carrier to deliver double stranded RNA to protect shrimp against white spot syndrome virus (WSSV). In another study, Yong et al. [25] used the *Mr*NV VLPs as a platform to display a foreign epitope of hepatitis B virus and showed induction of humoral and cell mediated immune responses in mice. Although these researchers did not test the efficacy of *Mr*NV VLPs as a vaccine against WTD, Farook et al. [14] did produce the *Mr*NV capsid protein (r-MCP) in bacterial cells and investigated their ability to protect against WTD. In one of the experiments, purified r-MCP protein was injected into the adult prawns (mean body weight: 14.3 g) and they observed moderate immune response in samples collected at different time intervals (1, 3, 5, and 7 day) that were analyzed by the hematological and immunological parameters. In second experiment, when the post-larvae (10–12 days) of *M. rosenbergii* were exposed to purified r-MCP protein by immersion for 24 h and challenged with *Mr*NV and XSV inoculum, they observed 76% survival rate of post-larvae group on 15th day post-immersion. In addition, their results revealed enhanced expression of the immune genes, such as SOD, proPO, crustin, peroxinectin, ALPs, and lysozyme which modulated the immune response [14].

In our present work, we produced *Mr*NV VLPs vaccine using a baculovirus/insect cell expression system, delivered the protein orally with the feed to *M. rosenbergii* juveniles, and demonstrated that this protein treatment was effective in conferring 78% protection against the *Mr*NV challenge. The results of our study are comparable to the immersion vaccine method mentioned above [14], however, the oral vaccination is more convenient and inexpensive method, which does not require purification of the r-*Mr*NV protein from insect cells. Hence, the ability to deliver potential vaccines to aquatic animals makes baculovirus-expressed protein a better vaccine candidate than other forms of vaccination.

The protective effect was also corroborated in the present study by double step PCR detection in the control and experimental prawn larvae. The vaccination in prawn larvae help to reduce the infection at 68% in 30 dpv and 83% in 60 dpv authenticated by double step PCR detection. The *Mr*NV recombinant proteins may stimulate the immune system and activate the immune cells leading to developing the resistance against the *Mr*NV infections. The prolonged vaccination period (60 dpv) also had higher resistance than 30 dpv due to the shorter treatment.

Many immune responses of *M. rosenbergii* have been reported where hepatopancreas and hemocytes are the main tissues involved in the immune response. These tissues are the major sites for the synthesis of immune defense molecules which are involved in eliminating pathogens [26,27]. Toll receptors are cell surface molecules acting as pattern recognition receptors (PRRs) that have been implicated in the signaling pathway of innate immune responses [28]. The immune parameters like alkaline phosphatase (AKP), phosphatase (ACP), superoxide dismutase (SOD) and catalase (CAT) activity and the immune genes like lipopolysaccharide and β-1,3-glucan-binding protein (LGBP), peroxinectin (PE), α2-macroglobulin was up regulated in *M. rosenbergii* after the exposure of pathogen Spiroplasma [29]. In our study, we tried to express three immune genes of *Mr*amp, *Mr*LY, *Mr*PPO and a toll *Mr*Toll from the control and vaccinated prawns. Unfortunately *Mr*Toll, *Mr*LY and *Mr*PPO were not expressed, probably due to small size of the experimental animals from which we could not extract enough hemocytes to perform the test. The *Mr*NV vaccine helped to induce the immune system by directly interacting with the serine protease cascade and activated the prophenoloxidase system and toll-like receptors (TLR), leading to the production of antimicrobial peptides, antioxidant enzyme production, and immune gene expression. Our findings revealed that the immune gene *Mr*amp might be involved in innate immune response against *Mr*NV infection.

The findings of the present study revealed that the baculovirus expression system is highly useful to produce the recombinant subunit vaccine using the *Mr*NV-CP-RNA2 gene. The *Mr*NV subunit vaccine stimulates the innate immunity in *M. rosenbergii* larvae by improving survival, viral load reduction, up regulation of an immune gene against *Mr*NV infection. The expression of *Mr*amp might be involved innate immune response against *Mr*NV infection. By utilizing the *Mr*NV subunit vaccine, it will mitigate the disease outbreaks, improve the economy, avoiding environmental damage and cease the consignment rejection.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.12.010.

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