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Macrobrachium rosenbergii nodavirus (*Mr*NV)-CP-RNA-2 DNA vaccine confers protective immunity in giant freshwater prawn *Macrobrachium rosenbergii* against *Mr*NV infection

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ABSTRACT

Macrobrachium rosenbergii Nodavirus (*Mr*NV) causes white tail disease (WTD) in Giant freshwater prawn *Macrobrachium rosenbergii* which leads to immense economic losses in hatcheries and farms. In the present study, we cloned the capsid protein gene of *Mr*NV-CP-RNA-2 (1146 bp) into a DNA vaccine vector pVAX1 to form *Mr*NV-CP-RNA-2- pVAX1. The bacterial transformant, containing the *Mr*NV-CP gene, was coated on the fish diet pellets and fed to juvenile *M. rosenbergii* for 40 days. After the vaccine delivery, group of *M. rosenbergii* were challenged with virulent *Mr*NV on 20 and 40th days post-vaccination (dpv) respectively and monitored for the survival. The non-vaccinated *M. rosenbergii* succumbed to death (100%) within 5 days, whereas the *Mr*NV-CP-RNA-2- pVAX1 treated groups had the survivals of 60 and 80% in 20 and 40 dpv respectively ($P \leq 0.001$). To study the *Mr*NV infection level, double step PCR was performed at different dpv. The results revealed that in 20 dpv group, the infection was decreased to 65% and in 40 dpv group the infection decreased to 69% from control diet fed prawns ($P < 0.001$). Haematological parameters like coagulation time, total haemocyte count (THC) and oxyhaemocyanin levels were performed for the control and vaccinated prawns. The vaccination helped to decrease the time of coagulation, improved THC and oxyhaemocyanin levels at a significant level ($p < 0.001$) when compared to the non-vaccinated group. The immunological parameters like prophenol oxidase (ProPO), superoxide anion and intra-agar lysozyme activity were also performed and the results revealed that the level of proPO, superoxide anion and lysozyme activities were significantly ($P \leq 0.05$) increased in 20 and 40 dpv groups respectively, when compared with the non-vaccinated groups. Based on the vaccination trials, the DNA vaccine construct *Mr*NV-CP-RNA-2-pVAX1 effectively improved the survival against *Mr*NV challenge, helped to decrease viral load and enhanced the immune system to protect the prawn from *Mr*NV infection. This vaccine construct is highly useful to protect the *M. rosenbergii* from *Mr*NV infection.

1. Introduction

Many freshwater prawn species are presently being cultured with major culture farming [1] and among these, *Macrobrachium rosenbergii*, the giant freshwater prawn is one of the commercial important species due to its delicious taste, continuous food supply and valuable export market value [2]. *M. rosenbergii* is widely cultured in Southeast Asian countries, Israel, Japan, Taiwan, PR China, India, Latin America, Caribbean and few African countries [3]. Generally, they are less susceptible for diseases outbreaks in comparison to the farmed penaeid shrimp due to the less intensified culture practices [4]. Unfortunately, white tail disease (WTD) or white muscle disease, caused by *Macrobrachium rosenbergii* nodavirus (*Mr*NV), was first reported in the Guadeloupe Island in the French West Indies in 1997 with mass mortality in hatchery post-larvae [5] and later it was dispersed in different counties, including French West Indies, China, Taiwan and India [3,6]. The virus belongs to the family of Nodaviridae, which is a small icosahedral, non-enveloped virus of 27–30 nm in diameter [6]. The viral genome contains two segments of positive-sense, single-stranded RNA (ssRNA): RNA1 (3.2 kb) and RNA2 (1.25 kb), which encodes a single polypeptide

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of 43 kDa that forms the capsid [7,8]. The important clinical symptoms include the presence of white muscle in the abdominal part which is associated with the decline in feeding and unusual swimming behaviors. The discoloration appears two to three days after infection and these signs continue up to 15 days [5]. Mortalities reaches a maximum level of 95% around fifth day after the first appearance of the gross sign [6].

To control the infectious diseases in aquaculture industry, alternative strategies like vaccine development and immunostimulations are the important approach to solve the problems. Even though synthetic drugs and antibiotics are giving positive effects, unfortunately they cannot be recommended due to their negetive roles like residual problems, biomagnifications, cost effectiveness and non-biosafety [9]. Their negative aspects in aquaculture include their relatively high cost, prohibited or uncertain regulatory status, unfeasible administration routes, poor absorption, toxicity, biomagnification, certain effects on the environment, possibility of consignment rejection and the fact that resistant bacteria may be transferred to humans through food handling and consumption [10]. At the global level, farmers have great concern on the bad effect of antibiotics and they are now shifting over to safety biotechnological eco-friendly approaches, including novel vaccination approaches. Unfortunately application of traditional vaccines gives positive effects and has some demerits, such as weak and shorter immunity, reversion of virulence, ineffective, heat liable and theneed of mixing with adjuvant. Efficacy, safety and vaccine delivery route deserve special attention for designing and delivering the vaccines.

Recombinant DNA vaccine technology against viral pathogens is an attractive alternative to traditional vaccines because of certain advantages, which includes straightforward design and construction, heat stability, low production costs, long-term storage capabilities [11,12] and no risk of reversion [13]. The present study intends to construct efficient DNA vaccine using highly immunogenic gene coding the *Mr*NV capsid protein against *Mr*NV infection. This approach can elicit very strong, long-lasting immune response and offers economic benefit, environmental and safety advantages, which are particularly attractive for the farmers.

2. Materials and methods

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

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

2.2. Cloning of MrNV-CP-RNA-2 gene into the DNA vaccine vector pVAX1

*Mr*NV-CP-RNA-2 gene from pBME-Amp vector was restricted by using the respective restriction enzymes *Bam*HI and *Eco*RI. Simultaneously the DNA vaccine vector pVAX1 (Invitrogen, USA) was also digested with the same enzymes which is downstream of CMV promoter. The digested vectors were run in 0.8% agrose gel electrophoresis and the bands were excised using sterile blade and purified by using QIAquick Gel Extraction Kit (Qiagen). The ligation reaction was performed in 20 µl of ligation mix, which contained 1 µl of digested pVAX1, 6.5 µl of *Mr*NV-CP-RNA-2 insert, 2 µl of buffer, 9.5 µl of nuclease free water and 1 µl of DNA ligase enzyme. The mixture was incubated overnight at 4 °C and ligated mixture was subsequently used to transform *Escherichia coli* JM109 super competent cells (Promega). The transformants were enriched with SOC media and plated on Luria Bertani (LB) agar media in the presence of kanamycin and incubated at 37 °C for 16 h. Positive bacterial colonies were screened for the presence of *Mr*NV-CP gene in pVAX1 using restriction digestion with BamHI and EcoRI enzymes [14]. The gene synthesis and construction of DNA vaccine and transformation were performed in molecular virology laboratory, Institute of Marine and Environmental Technology (IMET), University of Maryland Baltimore Country (UMBC), Baltimore, Maryland 21202, USA.

2.3. Experimental diets and vaccine coating

Bacterial transformants harboring *Mr*NV-CP gene in pVAX1 vector were propagated in 1L LB broth (1 × 4 = 4 L) media in the presence of kanamycin for 16 h incubation and the bacterial cells were harvested by centrifuging at 5000 × *g* for 5 min. The bacterial cell pellet was washed with PBS (7.2 pH) and by centrifuged at 5000 × *g* for 5 min. After the wash, cells were inactivated with 0.5% formalin and incubated at 20 °C for 15 min. The formalin was washed away thrice by adding PBS and centrifuging at 5000 × *g* for 5 min at 4 °C. The bacterial cells were suspended and diluted with sufficient PBS and the total DNA content was adjusted by 1000 µg per ml by Nanodrop spectrophotometer (Thermo Scientific, USA). One ml of bacterial suspension was mixed with 1 g of artificial diet (Basal ratio of 45.1% protein; 7.2 lipid, 14.6% ash, 7.1% moisture and 3% fibre) and incubated for 20 min. Sufficient amount of cod liver oil was mixed with the diets to avoid leaching of bacterial cells. Control diet was prepared by normal JM109 cells pellets without the plasmid DNA and the diets were stored at 4 °C until use. The vaccine diet preparation, vaccination and vaccination experiments were performed in Centre for Marine Science and technology (CMST), Manonmaniam Sundaranar University, Rajakkamangalam- 629 502, Tamilnadu, India.

2.4. Experimental set-up and vaccination

M. rosenbergii weighing 12.0 ± 1.0 g 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 5000 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 ($n = 30 \times 3 = 90$) 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 40 days. Uneaten food and waste matters were removed before feeding. The water quality parameters, such as temperature (28 ± 1.0 °C), salinity (27 ± 1.0%) and pH (8.3 ± 0.1), were maintained. After 20 days (20 dpv) feeding experiment, group of prawns from each replicates (n = 15 × 3 = 45) of the experimental and control groups were challenged by feeding of *Mr*NV filtrate (which isolated from *Mr*NV infected prawns) coated diets (200 µg g^{-1}), at three times in a day up to two days. Blank control group was fed with fed JM109 transformants with pVAX and had no *Mr*NV challenge. The remaining prawns were challenged with *Mr*NV after 40 days (40 dpv), till the experiment was terminated. Meanwhile, prawns from blank control group were fed with JM109 transformants with pVAX and had no *Mr*NV challenge. The percentage of cumulative mortality was monitored at least for 10 days after challenge.

2.5. Molecular diagnosis of MrNV load by double step PCR

On the 8th days of post challenge with *Mr*NV challenge, random prawn samples from blank, control and experimental were checked by *Mr*NV diagnostic PCR (two steps) using the specific primer sets (Forward: 5′ CAAGCGCCGTAAGCGTAATC 3'; Reverse: 5′ GTTGGTGG AACCCAATTGCC 3') to amplify the *Mr*NV gene with the product size of 750 bp. The DNA extraction was carried out by high salt method and the standard PCR amplification protocols was followed for amplifying the *Mr*NV-CP-RNA2 gene. 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.

2.6. Haematological parameters

The haematological analyses measured parameters, such as coagulation time of the haemolymph, total haemocyte count (THC) and oxyhaemocyanin, were calculated for both control and experimental groups. The coagulation time of the haemolymph was determined by the capillary tube method [15]. Burker haemocytometer was used to determine total haemocyte count (THC) (cells ml^{-1}) from the haemolymph. The haemocytes were counted manually in all 25 squares of the haemocytometer using a phase-contrast microscope [16]. The oxyhaemocyanin concentration was calculated following the method of Hagerman [17]. About 100 µl of haemolymph sample was immediately diluted with 900 µl distilled water in a 10-mm quartz cuvette, and its absorbance was measured at 335 nm using a Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan).

2.7. Immunological parameters

Phenoloxidase activity in haemolymph samples were determined spectrophotometrically by recording the formation of dopachrome using L-dihydroxyphenylalanine (L-DOPA) as a substrate [18]. Superoxide anion was quantified by the method of Song and Hsieh [19]. The optical density of the dissolved formazan was read at 630 nm and the effects of different treatments on the generation of O_{2^-} in all the experimental *M. rosenbergii* groups were compared with the control. Intra-agar lysozyme activity was performed against *Micrococcus luteus* culture by diluting the haemocytes cells and adding a drop onto the agar wells. Once the drop was completely absorbed into the agar, the wells were incubated upside down for 24–48 h at 37 °C. The wells were then recorded for the highest dilution of haemocytes capable of lysing the test microbes.

2.8. Data analysis

One way Analysis of Variance (ANOVA) was carried out using the SPSS statistics data package. Means were compared at 0.05, 0.01 and 0.001% levels and subsequent post-hoc multiple comparison with SNK test.

3. Results

3.1. Cloning of MrNV-CP-RNA-2 to pVAX1

The *Mr*NV-CP-RNA-2 gene was cloned in to the *Bam*H1 and *Eco*R1 sites of the DNA vaccine vector pVAX1 (Invitrogen, Carlsbad, CA, USA) downstream of the CMV immediate early promoter (Fig. 1a). The cloning was confirmed by digesting the recombinant plasmid *Mr*NV-CP-RNA-2-pVAX1 by the same restriction enzymes *Bam*H1 and *Eco*R1. The digestion results revealed that the digested *Mr*NV-CP-RNA-2 gene had the size of 1146 bp (Fig. 1b).

3.2. Survival of vaccinated M. rosenbergii after challenge

The prawn group which was fed on JM109 cells with pVAX1 coated diet (without *Mr*NV-CP-RNA-2-gene- control group) succumbed to 95 and 100% death after *Mr*NV challenge until 10th day of post-infection respectively in 20 and 40dpv. The survival was significantly increased in the experimental groups of 20 and 40dpv after *Mr*NV challenge. Among the different period of vaccinations, the 20 dpv helped to improve the survival of 55% and 40 dpv helped to improve the survival of 80% respectively from the control group due to the influence of *Mr*NV-CP-RNA-2-pVAX1 DNA vaccine (Fig. 2). Two way ANOVA revealed that the survival differed significantly from each other (Column: $F = 19.01$; Row: $F = 8.49$; $P \leq 0.001$).

3.3. Molecular diagnosis by double step PCR

The prawn group that was fed with the control diet was positive for *Mr*NV infection at 90–100% level by first and second step PCR detection. In the experimental groups, there were 28 and 7% PCR positive signals observed in the first and second step detections respectively in the 20 dpv group after *Mr*NV challenge (Fig. 3). The prolonged vaccination helped to decrease the infection significantly ($P < 0.001$) by 14 and 7% in first and second step detection respectively in 40 dpv group after *Mr*NV challenge. Overall, in 20 dpv group, the infection was decreased to 65% and in 40 dpv group, the infection was decreased to 69%, when compared with the control group of prawns fed with normal diet ($P < 0.001$).

3.4. Haematological changes after challenge

The haemolymph took 97.18 s to coagulate when the prawns were fed normal diet without *Mr*NV challenge. After *Mr*NV challenge, the coagulation time was prolonged to 167.64 s due to high viral load in haemolymph. The vaccination helped to significantly decrease ($p < 0.001$) the time of coagulation of 117.83 and 103.59 s in 20 and 40 dpv groups, respectively in MrNV-CP-RNA-2-pVAX1DNA vaccine treated prawns. THC is drastically down to 19.05 and 21.73×10^5 cellsml^{-1} in the control diet fed prawn groups after 20 and 40 days of culture respectively from the blank control group (37.84×10^5 cellsml^{-1}). The improved THCs, 32.18 and 36.05×10^5 cells ml^{-1}observed in the DNA vaccine treated prawns after 20 and 40 dpv respectively did not vary significantly ($p < 0.01$). The oxyhaemocyanin level observed was increased from 0.93 to 0.98mmoll^{-1}in the control diet fed *M. rosenbergii* at 20 and 40 dpv respectively after *Mr*NV challenge, whereas the level was significantly ($p < 0.05$) increased to 1.68 and and 1.94 mmol l^{-1}respectively in *Mr*NV-CP-RNA-2-pVAX1 vaccinated diet fed *M. rosenbergii* groups (Table 1).

3.5. Immunological changes after challenge

Prophenol oxidase activity (proPO) observed was found to varied from 0.097 to 0.136 OD at 240 min incubation in the heamolymph of control diet fed *M. rosenbergii* after 5th day post *Mr*NV challenge. The proPO level was improved to 1.745 and 2.361 OD respectively in *Mr*NV-CP-RNA-2-pVAX1 DNA vaccinated *M. rosenbergii* in 20 and 40 dpv groups respectively after 240 min incubation. Two way ANOVA revealed that the proPO value were significantly (Column: $F = 26.59$; $P \leq 0.001$ and Row: $F = 3.46$; $P \leq 0.05$) different among the control and vaccinated groups (Fig. 4). Intracellular superoxide anion production also reflected the same way like proPO. The improved significant ($P \leq 0.001$) production of proPO observed were 0.097 and 0.19 OD in the *Mr*NV-CP-RNA-2-pVAX1 vaccinated *M. rosenbergii* in 20 and 40 dpv respectively after *Mr*NV challenge (Fig. 5). The intra-agar lysozyme activity of the haemolymph of control and vaccinated *M. rosenbergii* is shown in Table 2. The zone of inhibition was found to be 2.18 and 1.54 mm in the control groups at 10^{-1} and 10^{-2} dilutions respectively. This was significantly ($P < 0.05$) increased to 8.82 and 11.36 mm of zone of inhibition respectively in the *Mr*NV-CP-RNA-2-pVAX1 vaccinated *M. rosenbergii* at 20 and 40 dpv after *Mr*NV challenge.

4. Discussion

Adaptive immunity has been assumed to be absent from invertebrates because they lack the immunoglobulin (Ig), T cell receptor (TCR) and major histocompatibility complex (MHC) high diversity molecules [20]. The defense mechanisms of crustaceans depend completely on the innate immune system that is activated when pathogen-associated molecular patterns are recognized by soluble or by cell surface host proteins, such as lectins, antimicrobial, clotting, and

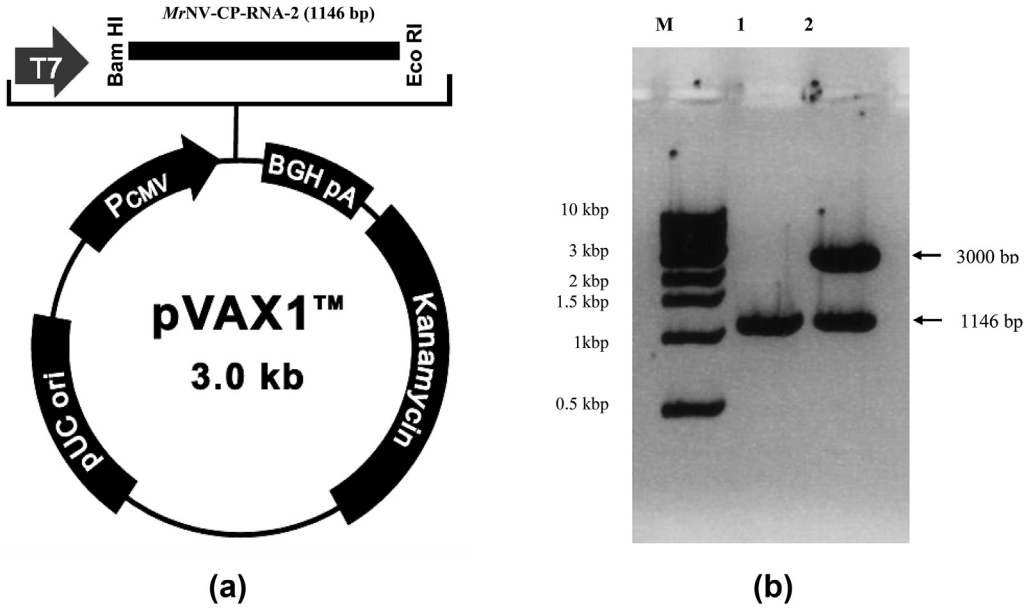

Fig. 1. (a). Schematic structure map of DNAvaccine vector pVAX1 carrying *Mr*NV-CP-RNA-2 gene with the size of 1146 bp. Vector elements include the human cytomegalovirus (CMV) immediate early promoter; the bovine growth hormone polyadenylation signal (BGH pA); the pMB1 origin of replication (pMB1ori); and the kanamycin resistance gene (KanR). *Mr*NV-CP-RNA-2 gene was inserted between the *Bam*H1 and *Eco*R1 sites present in the multiple cloning site of the vector. **(b).** Cloning of *Mr*NV-RNA-2 gene into DNA vaccine vector pVAX1 (3000bp). *Mr*NV-CP-RNA2 gene was digested from pBME-Amp plasmid (4047 bp) using Bam-HI and EcoRI restriction enzymes and the fragment was cloned into Bam-HI and EcoRI digested pVAX1 vector. M: Marker, 1 kb DNA Ladder from New England Biolabs; Lane 1: Digested and purified *Mr*NV-CP-RNA-2 gene from pBME; Lane 2: Digested clone of *Mr*NV-CP-RNA-2 - pVAX1.

pattern recognition proteins, which, in turn, activate cellular or humoral effector mechanisms to destroy invading pathogens [21]. In this study, efficacy of vaccines was tested by cloning the *Mr*NV capsid protein gene into the DNA vaccine vector pVAX1, which helped to improve the survival of prawn up to 60–80% against the *Mr*NV challenge. The results herein demonstrate that *Mr*NV-CP-RNA-2-pVAX1 DNA vaccine effectively elicit the protective immune responses in *M. rosenbergii* against *Mr*NV challenge. Capsid or envelope proteins are highly immunogenic and hence it is used for efficient vaccine construction [22]. In our study, the *Mr*NV-CP DNA vaccine effectively reduced the *Mr*NV load and boosted the immune system in *M. rosenbergii* after 40 days delivery. This is in support that the *Mr*NV capsid protein treated with *M. rosenbergii* by 24 h immersion, followed by *Mr*NV challenge boosted the relative percent survival of 76.03% [23]. Naveen Kumar et al. [24] immunized *M. rosenbergii* through oral administration of inactivated bacteria encapsulated dsRNA of *Mr*NV, where a post-feeding virus challenge showed promising results. The *Mr*NV challenge at 24 h and 72 h post-feeding showed relative high

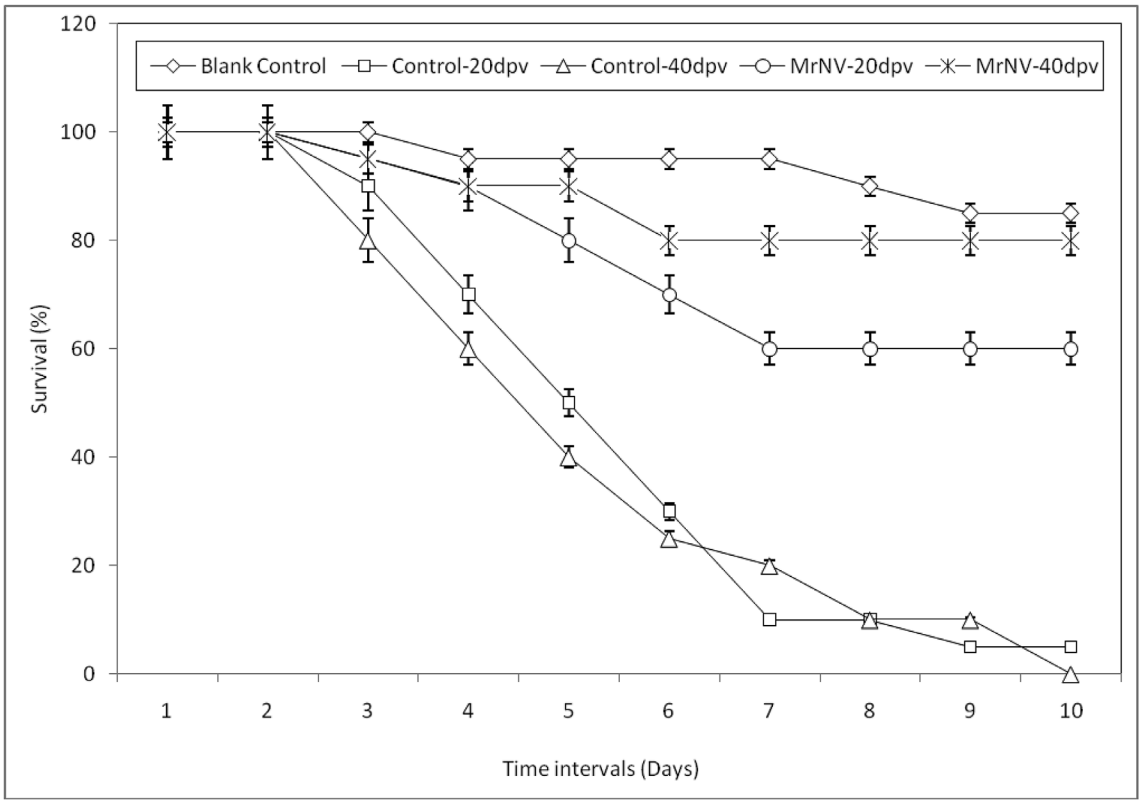

Fig. 2. Survival (%) of giant water prawn *Macrobrachium rosenbergii* treated with *Mr*NV-CP-RNA-2 pVAX1 DNA vaccine and challenged with *Mr*NV after 20 and 40 dpv. The values are significantly different from each other (Column: $F = 19.01$; Row: $F = 8.49$; $P \leq 0.001$) -Two way ANOVA.

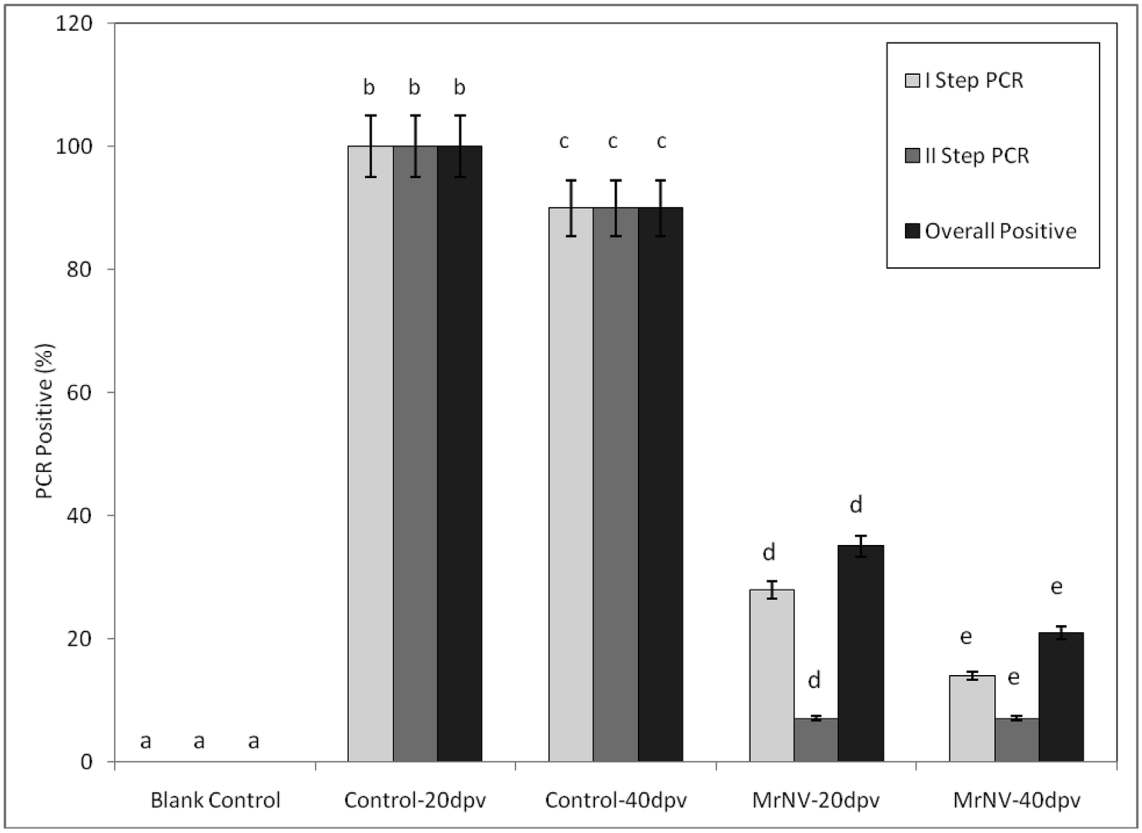

Fig. 3. Percentage PCR detection in giant water prawn *Macrobrachium rosenbergii* treated with *Mr*NV-CP-RNA-2 pVAX1 DNA vaccine and challenged with *Mr*NV after 20 and 40 dpv. Bars with different lowercase letters are statistically different from each other (one-way ANOVA, $P < 0.001$ and subsequent post-hoc multiple comparison with SNK test).

percentage of survival at 80% and 75%, respectively. DNA construct containing extra small virus antisense (XSVAS) gene of nodavirus encapsulated with chitosan nanoparticles treated with *M. rosenbergii* was found to be more effective in increasing the survivability [25]. Zhong-Yuan [26] reported that the DNA vaccine containing *Siniperca chuatsi* rhabdovirus (SCRV) glycoprotein gene (pcDNAG) delivered to mandarin fish exhibited protective effect against SCRV challenge with a relative percent survival of 77.5%.

Molecular detection of microbial infection with PCR is one of the efficient tools to confirming the infection by amplifying the specific genes. Decreased WSSV load was quantified in cultivable shrimp species treated with antiviral herbal extracts, microbial secondary metabolites and herbal immunostimulants after WSSV challenge [27-29]. Our present study revealed that the prolonged vaccination period helps to reduce the *Mr*NV load at a significant level. In 20 dpv group, 65% of *Mr*NV load was reduced and in 40 dpv group, it was reduced to 69% due to the prolonged period of vaccination. These results indicated that protection offered by DNA vaccination was effective after 20–40 days of dpv. With combined results of *Mr*NV load and survival, we achieved good protection and the prawn survived 60 and 80% after 10 days post-challenge. Sahoo et al. [30] studied the immunomodulatory effect of recombinant RNA-dependent RNA polymerase protein (RdRp) of *Mr*NV in *M. rosenbergii* and their study revealed that *Mr*NV was detected only in control prawn after 14 days, while both RdRP injected groups were *Mr*NV-negative. Rout et al. [22] studied the expression of WSSV genes including pVP15, pVP28, pVP35 and pVP281 by semi quantitative PCR amplification in the DNA vaccines encoding viral envelope proteins delivered to shrimp *P. monodon*. The mRNA expression was observed for the four genes in the abdominal muscle of the shrimp injected with DNA vaccine.

Haematological parameters are one of the important indicators of immune enhancement in crustaceans. Haemocytes are responsible for clotting, exoskeleton hardening and elimination of foreign materials [19]. Mean THCs of healthy penaeid shrimps ranged from 20 to 40×10^6 cells ml^{-1} [31]. Molting, development of organs, reproductive status, nutritional condition and disease have been shown to

Table 1
Haematological changes in the haemolymph of *M. rosenbergii* treated with *Mr*NV-CP-RNA-2 pVAX1 DNA vaccine and challenged with *Mr*NV after 20 and 40 dpv.

Treatments	Haematological changes		
	Coagulase activity(Sec) *	Total Haemocyte Count($\times 10^5$ cells ml^{-1}) **	Oxyhaemocyanin(m mol l^{-1}) ***
Blank Control	$97.18^a \pm 2.34$	$37.84^a \pm 1.04$	$0.77^a \pm 0.03$
Control - 20 dpv	$167.64^b \pm 3.23$	$19.05^b \pm 0.85$	$0.93^b \pm 0.01$
Control - 40 dpv	$174.05^c \pm 1.95$	$21.73^b \pm 1.14$	$0.98^b \pm 0.05$
*Mr*NV-CP-RNA2-pVAX1- 20 dpv	$117.83^d \pm 2.89$	$32.18^c \pm 1.14$	$1.68^c \pm 0.02$
*Mr*NV-CP-RNA2-pVAX1- 40 dpv	$103.59^e \pm 2.72$	$36.05^a \pm 2.76$	$1.94^d \pm 0.04$

Means with different lowercase letters are statistically different from each other (one-way ANOVA, (*$p < 0.001$; **$p < 0.01$; ***$p < 0.05$ and subsequent post-hoc multiple comparison with SNK test).

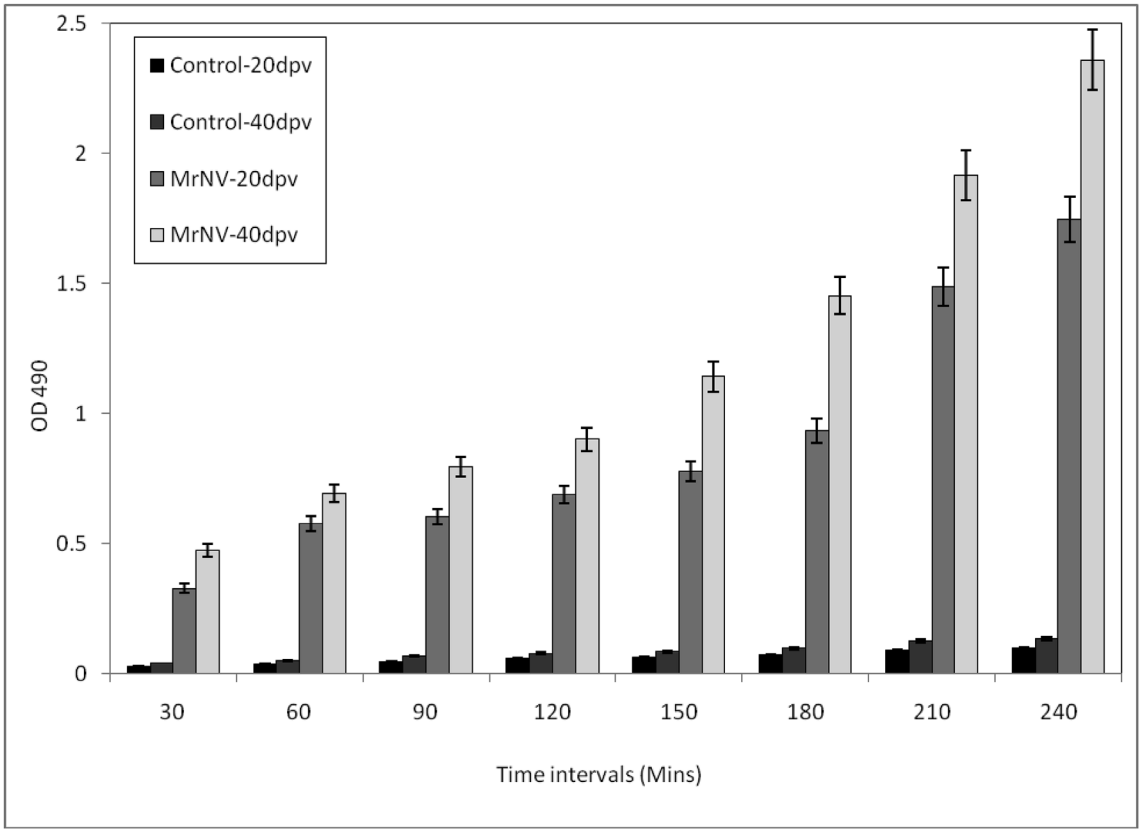

Fig. 4. Prophenol Oxidase activity of haemocytes of *M. rosenbergii* treated with *Mr*NV-CP-RNA-2 pVAX1 DNA vaccine and challenged with *Mr*NV after 20 and 40 dpv. The values are significantly different from each other (Column: $F = 26.59$; $P \leq 0.001$ and Row: $F = 3.46$; $P \leq 0.05$).

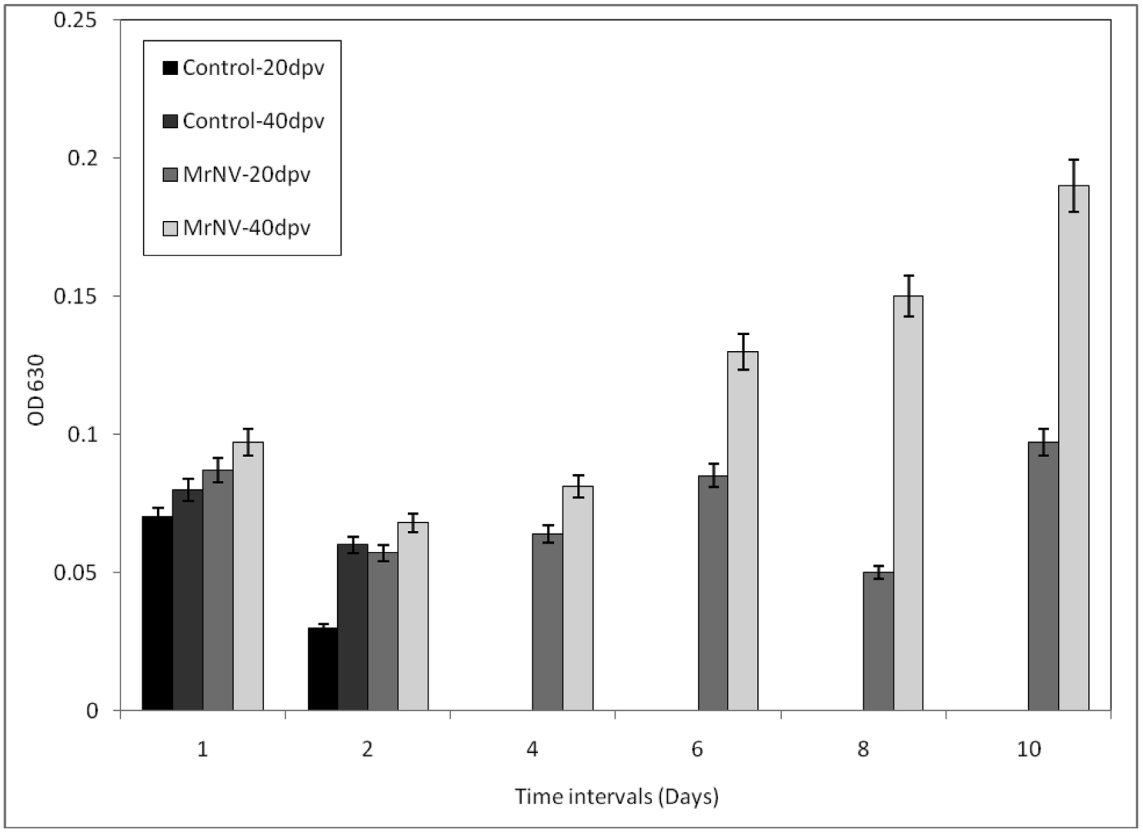

Fig. 5. Intracellular superoxide anion production (NBT assay) of *M. rosenbergii* treated with *Mr*NV-CP-RNA-2 pVAX1 DNA vaccine and challenged with *Mr*NV after 20 and 40 dpv. The values are significantly ($P \leq 0.001$) differed by column ($F = 11.73$) and non-significantly ($P > 0.05$) differed by row ($F = 0.960$).

Table 2
Lysozyme activity carried out in the haemolymph of *M. rosenbergii* treated with *Mr*NV-CP-RNA-2 pVAX1 DNA vaccine and challenged with *Mr*NV after 20 and 40 dpv.

Dilutions	Zone of inhibition (mm)			
	Control		*Mr*NV-pVAX1	
	20 dpv	40 dpv	20 dpv	40 dpv
Blank Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
10^{-1} (mm)	2.18^{a} ± 0.45	3.76^{b} ± 0.32	8.82^{c} ± 0.76	11.36^{d} ± 1.05
10^{-2} (mm)	1.54^{a} ± 0.11	2.89^{b} ± 0.02	6.23^{c} ± 0.15	9.87^{d} ± 0.33
10^{-3} (mm)	1.13^{a} ± 0.03	1.89^{b} ± 0.04	5.45^{c} ± 0.65	7.76^{d} ± 0.56

Means with different lowercase letters are statistically different from each other (one-way ANOVA, $P < 0.05$ and subsequent post-hoc multiple comparison with SNK test).

influence haemocyte abundance [32]. In this study, the haemolymph took 167.64 and 174.05 s to coagulate in the control groups of 20 and 40 dpv, respectively. Surprisingly, the coagulation time was drastically decreased to 117 and 103.59 s in the *Mr*NV-CP-RNA2-pVAX1 DNA vaccine treated *M. rosenbergii* of 20 and 40 dpv groups, respectively. The reduction of coagulation times is due to the influence of immune enhancement by the vaccine which led to the reduction of viral load. Generally, if microbial load is higher in haemolymph, it will take more time to coagulate. In this experiment, the vaccinated prawns had less coagulation time, which was also observed in another study when the herbal immunostimulant treated cultivable shrimp species were challenged with WSSV [27,33,34] and *Vibrio harveyi* egg yolk antibody (IgY) treated *Fenneropenaeus indicus* were challenged with *V. harveyi* [35]. The same trend was also observed in the THCs level. There was 37.84×10^5 cells ml^{-1} in the blank control prawn, which was drastically reduced to 19 to 21×10^5 cells ml^{-1} in the control groups which were not vaccinated. The vaccinated groups helped to improve the THCs level of 32–36 $\times 10^5$ cells ml^{-1} due to immune enhancement. *M. rosenbergii* treated with higher concentration of recombinant RNA-dependent RNA polymerase protein (RdRp) of *Mr*NV showed significantly higher total haemocyte count at different period post injection [30]. Inactivated WSSV vaccine with herbal immunostimulants treated *Penaeus mondon* had higher level of THCs when compared with non-vaccinated groups [36].

ProPO activating system stimulates several cellular defense reactions, including phagocytosis, nodule formation, encapsulation, and haemocyte locomotion [37]. Activated haemocytes also produce extra bactericidal substances, such as H_2O_2 and superoxide anion (O_2^-) that may increase disease resistance [19]. In the present study, the proPO value was drastically down when the *Mr*NV infection happened. Further, the value was gradually increased at stable levels in the vaccinated groups. The *Mr*NV-pVAX1 treated prawn had improved proPO values compared with the control group due to the immunostimulation by the vaccines. When foreign material is recognized, different effector mechanisms are activated, such as the prophenoloxidase (proPO) system, phagocytosis, and encapsulation [21]. Cellular reactions involve phagocytosis, nodule formation and encapsulation, while humoral reactions involve the prophenoloxidase activating cascade and immune related proteins such as lysozymes, lectins and antimicrobial peptides [38]. The *Mr*NV-CP-RNA2-pVAX1 DNA may interact with the pattern recognition receptors (PRPs) and stimulate to activate the proPO system, leading to the secretion of proPO enzyme. WSSV-VP28 gene encoding DNA vaccinated shrimp showed significantly high level of prophenoloxidase and superoxide dismutase (SOD) when compared to the control groups [39]. Shelby and Popham [40] have reported that the phenoloxidase is having virucidal activity by inactivating the insect virus *Helicoverpa zea* single nucleopolyhedro virus (HzSNPV) *in vitro* in *Heliothis virescens*. In the present experiment, increasing O_2^- activity was observed in *Mr*NV-CP-RNA2-pVAX1 DNA vaccine treated prawns, which seems to act as a promoter of prawn immune system against the *Mr*NV infection. Since O_2^- is the first product to be released from the respiratory burst, the measurement of O_2^- has been accepted as a direct and accurate way of measuring respiratory burst activity [41,42]. The level of intracellular superoxide anion production also observed the same way like proPO improvement in the *Mr*NV-CP-RNA2-pVAX1 DNA vaccine treated prawns. The oral administration of ß-1,3 glucan effectively enhanced PO and SOD activities and the survival of shrimp also increased when challenged with WSSV [42]. WSSV-VP28 encoded in pVAX1 also highly influenced to improve the SOD levels after 14th day injection in immunized shrimps [43]. Total lysozyme is a measurable humoral component of the non-specific defense mechanism after activation of the immune system with immunomodulants [44]. The present study revealed that intra agar lysozyme activity was higher in the vaccinated group than the control group, suggesting that the *Mr*NV-pVAX1 helped to improve the immunity by producing more lysozymes to kill or degrade the pathogens. Lysozyme is one of the enzymes that can hydrolyse components of the cell walls of certain bacteria [45]. The anti-*Vibrio harveyi* IgY, an edible antibody, coated diet fed *F. indicus* achieved increased lysozyme activity against pathogenic Vibrio infection [35]. The herbal immune adjuvant *Asparagus racemosus* enhanced lysozyme activity in tilapia fed low (0.1%) and medium (0.5%) doses [46] and higher immune responses in *P. monodon* treated with anti-WSSV IgY, which was produced from *A. racemosus* extracts [47].

The oral administration of *Mr*NV-CP-RNA2-pVAX1 DNA vaccine through bacterial cells are found to be effective in preventing to reduce the infection and improve immunity. The prolonged feeding or delivery of vaccines with safety protocols helps to enhance the immunity in a controlled manner of antigen presentation. Vaccination trial results show significant resistance in the shrimp injected twice with rVP292 (RPS: 52%) against WSSV by the 30th day post-initial vaccination. This suggests that the generation of a more specific immune response (long-term protection) provided by injection of virus-specific proteins like VP292 is possible to prevent the entry of the virus [48]. Our studies clearly indicate that oral *Mr*NV-CP-RNA2-pVAX1 DNA vaccine delivery helped to increase the survival of 75%; reduce the *Mr*NV load of 79%; improved the THC count of 47%; activation of prophenoloxidase system at 95% level and 66% improvement of lysozyme activity at a very significant levels in the prolonged delivery at 40 dpv from control group after *Mr*NV challenge. However, more intensive studies like *Mr*NV load quantification by quantitative PCR and specific immune gene expression at quantitative and semi quantitative PCR levels are needed for the successful application *Mr*NV-CP-RNA2-pVAX1 DNA vaccine as a tool to control the *Mr*NV infection in freshwater prawn aquaculture. In conclusion, the study is offering a platform for further research on recombinant vaccine development and its commercial application in freshwater prawn aquaculture in a big way against viral infection.

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