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Production of recombinant vaccine using capsid gene of nodavirus to protect Asian sea bass, *Lates calcarifer* (Bloch, 1790)

S. Vimal [a], N. Madan [a], M.A. Farook [a], K.S.N. Nambi [a], S. Abdul Majeed [a], T. Rajkumar [a], S. Venu [b], A.R. Thirunavukkarasu [b], A.S. Sahul Hameed [a,*]

[a] OIE Reference Laboratory for WTD, PG & Research Department of Zoology, C. Abdul Hakeem College, Melvisharam, Vellore District, Tamilnadu - 632 509, India.
[b] Fish Culture Division, Central Institute of Brackishwater Aquaculture, 75-Santhome High Road, R. A. Puram, Chennai 600 028, India.

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ABSTRACT

Fish nodavirus (betanodavirus), a viral pathogen responsible for viral nervous necrosis (VNN) was isolated from infected Asian sea bass (*Lates calcarifer*). The capsid protein gene of nodavirus was amplified and cloned in pRSET B, a prokaryotic expression vector. The construct was named as r-FNCP. The size of the capsid protein was 42 kDa and it was named as r-FNCP42. The r-FNCP42 protein was expressed as a protein with a 6-histidine tag in *Escherichia coli* BL21 with IPTG induction. Polyclonal antiserum was raised against this recombinant protein in rabbits and it recognized r-FNCP42 protein. The gene encoding capsid protein (FNCP-42) nucleotide sequence was cloned and its sequence was analysed with sequences of other isolates such as Taiwan, Malaysia, Singapore and France available in the GenBank using Bioinformatics tools such as BLASTn, clustal W analysis. Homology comparison showed 98–99% identity with other isolates. Asian sea bass was immunized with purified r-FNCP42 mixed with complete adjuvant at a dose of 50 μg per fish and was challenged with nodavirus by intramuscular injection. The vaccinated sea bass was protected from nodaviral infection and 76% of relative percent survival (RPS) was recorded.

1. Introduction

Fish nodavirus is a viral pathogen and responsible for viral nervous necrosis (VNN) in teleost fish. In India, this virus was isolated from infected Asian sea bass (*Lates calcarifer*) larvae during the massive outbreak in sea bass hatcheries located in Chennai and Nagapattinam of Tamilnadu, India (Azad et al., 2005; Parameswaran et al., 2008). Betanodavirus belongs to the family, *Betanodaviridae* and it is a small virus with particle size between 25 nm and 30 nm in diameter, non-enveloped and having icosahedral shape. The main target organ for nodavirus in infected fish is the central nervous system (CNS), including the brain, spinal cord and retina, where it causes extensive cellular vacuolation and neuronal degeneration (Mori et al., 1992).

The genome of the virus consists of two single stranded RNAs (RNA1 and RNA2) (Frerichs et al., 1996; Mori et al., 1992). The RNA1 is reported to have 3100 bases along its length and consists of a single open reading frame (ORF) which is translated into RNA dependent RNA polymerase (RdRp) or protein A (Mori et al., 1992). The RNA2 consists of 1410 bases and has a single ORF encoding the viral coat protein. The coat protein (42 kDa) is an important structural protein of betanodavirus in which the RNA segments are packaged (Hegde et al., 2002; Iwamoto et al., 2004; Mori et al., 1992; Nishizawa et al., 1995).

Vaccine preparations based on the recombinant protein technology have shown promising results in both laboratory and field trials. Nakai et al. (1995) reported that striped jack with a recombinant capsid protein from striped jack nervous necrosis virus (SJNNV) expressed in *Escherichia coli* elicited the production of virus-neutralising antibodies. Nakai (2000) and Tanaka et al. (2001) have reported encouraging survival in preliminary vaccination trials using recombinant nodavirus coat protein as the immunogen and the presence of virus-neutralising antibodies after immunization. Husgaro et al. (2001) demonstrated RPS value of 83% in juvenile turbot following i.p. (intra peritoneal) vaccination with an oil-emulsified recombinant partial capsid protein from SJNNV. Tanaka et al. (2001) demonstrated that immunization of young seven-band grouper (*Epinephelus septemfasciatus*) by intramuscular injection of recombinant coat protein produced neutralizing antibodies in high titres. Husgaro et al. (2001) used a recombinant partial capsid protein from SJNNV to immunize adult turbot (*Scophthalmus maximus*) and Atlantic halibut (*Hippoglossus hippoglossus*) by intra peritoneal injection.

Vaccination with the recombinant capsid protein induced protection against Atlantic halibut nodavirus in juvenile turbot and humpback grouper (*Cromileptes altivelis*) (Sommerset et al., 2005; Yuasa et al., 2002). Thiery et al. (2006) vaccinated sea bass (*Dicentrarchus labrax*) intramuscularly, using virus-like particles (VLP) made from a single type of VNN coat protein spontaneously assembled in a baculovirus expression system. An oral vaccine composed of *Artemia*-encapsulated recombinant *E. coli* expressed the NNV capsid protein gene in larvae of grouper (*Epinephelus coioides*) and the results revealed that the

* Corresponding author. Tel./fax: +91 416 269487.
E-mail address: cah_sahul@hotmail.com (A.S.S. Hameed).

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vaccinated larvae showed a high degree of protection after challenge with NNV (Lin et al., 2007). Overgard et al. (2013) have studied the immune response in Atlantic halibut (*Hippoglossus hippoglossus* L.) following administration of an experimental vaccine comprising 10 μg recCP with oil adjuvant (OA), 50 μg recCP plus OA in combination with an oil adjuvant (OA).

Previous reports indicate that recombinant viral structural proteins have already been developed for other fish viral diseases, i.e. infectious hematopoietic necrosis virus (Leong and Fryer, 1993), viral haemorrhagic septicaemia virus (Lorenzen and Olesen, 1997) and infectious pancreatic necrosis virus (Knappskog et al., 1999). The aim of the present work was to clone and express the capsid protein gene of Indian nodavirus isolate and to determine the efficacy of the purified recombinant capsid protein to provide protective immunity in Asian sea bass against nodavirus infection.

2. Materials and methods

2.1. Virus isolate and virus propagation

The fish nodavirus used in the present study was isolated from infected Asian sea bass (*L. calcarifer*) larvae during a massive outbreak in sea bass hatcheries located in Chennai and Nagapattinam of Tamilnadu, India (Azad et al., 2005; Parameswaran et al., 2008). Nodavirus was propagated in kidney cell line derived from Asian sea bass and used for RNA extraction for cloning of capsid gene and infectivity assays (Sarath Babu et al., 2013). The cells were grown at 28 °C in Leibovitz's L-15 medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml Fungizone. The monolayer of cells was washed with phosphate buffer three times and then inoculated with the virus at a dose of 10^3 $TCID_{50}$. Then, L-15 medium with 5% FBS was added to the cells and incubated at 25 °C. The cells were examined daily for the appearance of a cytopathic effect (CPE) for up to 2 weeks. The virus was harvested from the infected cultures and processed by three cycles of freezing and thawing. The virus harvest was clarified by low-speed centrifugation (3000 ×*g*) at 4 °C, and the viral titre was then determined in a 50% tissue culture infective dose (TCID50) assay.

2.2. Sequence analysis and alignment

Plasmid DNA containing viral cDNAs (RNA) were purified using Qiagen plasmid minipreparation spin columns. Sequence analysis was performed on an Autoread Sequencing kit Applied Biosystems, Bangalore, India. The nucleotide sequence of nodavirus RNA2 capsid protein of Indian isolate has been deposited in GenBank (Accession No. KF146304). The sequences were aligned using Bioinformatics tools such as standard nucleotide BLAST and multiple sequence analysis clustalW (Thompson et al., 1994).

2.3. Cloning, expression and purification of FNCP gene in E. coli

The gene encoding for ORF of the major capsid protein of nodavirus, approximately 1017 kb in length was amplified by PCR using gene specific primer set (Forward Primer: FNCP-F-5′CCGGAATTCATGGTA CGCAAAGGTGAGAAG3′and Reverse Primer: FNCP-R-5′CCCAAGCTTTT AGTTTTCCGAGTCAACCCT3′). The forward primer (FNCP-F) included 10 additional bases of linker sequence with recognition site for EcoRI while the reverse primer (FNCP-R) has 9 additional bases of linker sequence with recognition site for Hind III. The amplicon of capsid protein gene was cloned into pRSET B expression vector (Invitrogen, Carlsbad, CA, USA). The cloned plasmid was transformed into *E. coli* BL21 (DE3) via the heat shock method and poured on the LB-plated ampicillin sensitive plates. Complete capsid protein gene of nodavirus was expressed as a fusion protein with a 6-histidine tagged in *E. coli* BL21 (DE3) strain under the induction of isopropyl-1, 1-thio-β-D-galactopyranoside (IPTG). The expression of recombinant capsid protein of nodavirus (r-FNCP42) was confirmed by SDS gel electrophoresis and further confirmed by Western blot using antibody raised against purified r-FNCP42 of nodavirus or whole nodavirus, and purified by affinity chromatography using a nickel–nitrilotriacetic acid (Ni–NTA) column according to the manufacturer's instructions (Qiagen, Valencia, CA, USA).

2.4. SDS-PAGE

The quality of r-FNCP42 expressed in *E. coli* was analysed 12% SDS-PAGE (Laemmli, 1970). The pelleted induced, uninduced or purified sample was mixed with Laemmli sample buffer, boiled for 5 min, and electrophoresed at a constant current of 30 mA. After electrophoresis the gel was stained with Coomassie brilliant blue. Molecular weight standards were co-electrophoresed.

2.5. Preparation of polyclonal antiserum

Polyclonal antiserum against r-FNCP42 was raised in five New Zealand white rabbits (2.5–3.0 kg) using purified protein emulsified with Freund's complete adjuvant. The antiserum was collected from the immunized rabbit after completion of a standard immunization trial (Sahul Hameed et al., 1998). The immunoglobulin G (IgG) fraction was purified by Protein A-Sepharose column (Bio-Rad, Hercules, CA, USA) and stored at −80 °C (Sambrook et al., 1989). ELISA was performed to determine the titres of antiserum against r-FNCP42 and the optimal dilution of purified IgG, after serial dilutions, was found to be 1:20 000 (Schurrs and Van Weemen, 1977). Alkaline phosphatase conjugated goat anti-rabbit IgG was obtained from Sigma-Aldrich (St Louis, MO, USA) and used to detect r-FNCP42. The pre-immune rabbit serum was used as negative control.

2.6. Western blot

The Western blot was carried out to detect r-FNCP42 produced in prokaryotic system (Talbot et al., 1984). After separation on SDS-PAGE gel, the proteins were transferred onto a nitrocellulose membrane (Macherey-Nagel, Duren, Germany). After transfer, the membrane was blocked in blocking buffer at 4 °C overnight followed by incubation with primary antiserum (rabbit anti-r-FNCP42 IgG) overnight. Subsequently, the membrane was incubated in ALP-conjugated goat anti-rabbit IgG (Sigma) for 1 h and r-FNCP42 was detected with a substrate solution of 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

2.7. Vaccination and nodavirus challenge

Juveniles of Asian sea bass (10–15 g body weight) were used in challenging experiment to evaluate the efficacy of recombinant vaccine. The sea bass were divided into three groups (120 per group) for challenging experiment. In Group I, the fish were injected with PBS (Negative Control). In Group II (Non-Vaccinated Group), the fish were intramuscularly injected with Freund's complete adjuvant (Sigma). In Group III (Vaccinated Group), the fish were intramuscularly injected with purified r-FNCP42 mixed with complete adjuvant (50 μg per/fish) as followed by previous workers (Overgard et al., 2013; Sommerset et al., 2005). After 5 weeks post vaccination, fish from each group were challenged with an intramuscular injection of nodavirus at a dose of $1 \times 10^{6.5}$ $TCID_{50}$ $fish^{-1}$. After 30 days of post challenging, cumulative percentage mortalities were recorded daily and dead fish were autopsied to determine the cause of death and to detect the presence of nodavirus. Relative percent survival (RPS) was calculated using the following formula: (1% mortality of vaccinated fish / % mortality of control fish) × 100 (Amend, 1981). In another experiment, fish were divided into three groups (30 per group) as mentioned above.

After treatment as described above fish from each group were sacrificed for collection of antiserum at 5th week after post vaccination. Fish were bled from the caudal vein located at the ventral base of the spinal cord. Caudal peduncle of the sampled fish was severed with a sharp scalpel and a haematocrit capillary tube was placed at the cut end of the caudal vein to collect the blood. It was allowed to clot overnight at 48 °C and centrifuged at 2500 ×*g* for 10 min to collect serum. Sera from each group were pooled and used for analysis of antibody response and in vitro viral neutralization assay in sea bass kidney cells (SISK).

2.8. Analysis of antibody response

After 5 week post vaccination, fish from each treatment group were assayed for antibody response against FNCP42 by sandwich enzyme-linked immunosorbent assay (ELISA) following the protocol described previously (Rajesh Kumar et al., 2007). The wells of flat-bottomed ELISA plate were coated with sera from vaccinated fish in PBS overnight at 4 °C. The plates were then washed thoroughly with PBS and blocked with 2% BSA in PBS for 1 h at 37 °C. Subsequently, the plates were washed thoroughly with PBS/T and incubated with primary antiserum

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India       ATGGTACGCAAAGGTGAGAAGAAATTGGCAAAACCCGCGACCACCAAGGCCGCGAATCCG 60
Taiwan      ATGGTACGCAAAGGTGAGAAGAAATTGGCAAAACCCGCGACCACCAAGGCCGCGAATCCG 60
France      ATGGTACGCAAAGGTGATAAGAAATTGGCAAAACCCGCGACCACCAAGGCCGCCAATCCG 60
Singapore   ATGGTACGCAAAGGTGAGAAGAAATTGGCAAAACCCGCGACCACCAAGGCCGCGAATCCG 60
Malaysia    ATGGTACGCAAAGGTGAGAAGAAATTGGCAAAACCCGCGACCACCAAGGCCGCCAATCCG 60
            ********************************************************** ******

India       CAACCCCGCCGACGTGCTAACAATCGTCGGCGTAGTGATCGCACTGACGCACCTGTGTCT 120
Taiwan      CAACCCCGCCGACGTGCTAACAATCGTCGGCGTAGTAATCGCACTGACGCACCTGTGTCT 120
France      CAACCCCGCCGACGTGCTAACAATCGTCGGCGTAGTAATCGCACTGACGCACCTGTGTCT 120
Singapore   CAACCCCGCCGACGTGCTAACAATCGTCGGCGTAGTAATCGCACTGACGCACCTGTGTCT 120
Malaysia    CAACCCCGCCGACGTGCTAACAATCGTCGGCGTAGTAACCGCACTGACGCACCTGTGTCT 120
            ************************************ ************************

India       AAGGCCTCGACTGTAACTGGATTTGGACGTGGGACCAATGACGTCCATCTCTCAGATATG 180
Taiwan      AAGGCCTCGACTGTAACTGGATTTGGACGTGGGACCAATGACGTCCATCTCTCAGGTATG 180
France      AAGGCCTCGACTGTAACTGGATTTGGACGTGGGACCAATGACGTCCATCTCTCAGGCATG 180
Singapore   AAGGCCTCGACTGTAACTGGATTCGGACGTGGGACCAATGACGTCCATCTCTCAGGTATG 180
Malaysia    AAGGCCTCGACTGTGACTGGATTTGGACGTGGGACCAATGACGTCCATCTCTCAGGTATG 180
            **************.******** ******************************** ***

India       TCGAGAATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGAACAGACGGATACGTTGTTGTT 240
Taiwan      TCGAGAATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGAACAGACGGATACGTTGTTGTT 240
France      TCGAGAATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGAACAGACGGATACGTTGTTGTT 240
Singapore   TCGAGAATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGAACAGACGGATACGTTGTTGTT 240
Malaysia    TCGAGAATCTCCCAGGCCGTCCTCCCAGCCGGGACAGGAACAGACGGATACGTTGTTGTT 240
            ************************************************************

India       GACGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAG 300
Taiwan      GACGCAACCATCGTTCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAG 300
France      GACGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAG 300
Singapore   GACGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAG 300
Malaysia    GACGCAACCATCGTCCCCGACCTCCTGCCACGACTGGGACACGCTGCTAGAATCTTCCAG 300
            ************** ********************************************

India       CGATACGCTGTTGAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGC 360
Taiwan      CGATACGCTGTTGAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGC 360
France      CGATACGCTGTTGAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGC 360
Singapore   CGATACGCTGTTGAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGC 360
Malaysia    CGATACGCTGTTGAAACACTGGAGTTTGAAATTCAGCCAATGTGCCCCGCAAACACGGGC 360
            ************************************************************

India       GGTGGTTACGTTGCTGGCTTCCTGCCTGATCCAACTGACAACGATCACACCTTCGACGCG 420
Taiwan      GGTGGTTACGTTGCTGGCTTCCTGCCTGATCCAACTGACAACGACCACACCTTCGACGCG 420
France      GGTGGTTACGTTGCTGGCTTCCTGCCTGATCCAACTGACAACGATCACACCTTCGACGCG 420
Singapore   GGTGGTTACGTTGCTGGCTTCCTGCCTGATTCAACTGACAACGATCACACCTTCGACGCG 420
Malaysia    GGTGGTTACGTTGCTGGCTTCCTGCCTGATCCAACTGACAACGATCACACCTTCGACGCG 420
            ******************************** ************* ***************

India       CTTCACGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAAAGCAGAACAGTCCGACCT 480
Taiwan      CTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAAAGCAGAACAGTCCGACCT 480
France      CTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAAAGCAGAACAGTCCGACCT 480
Singapore   CTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAAAGCAGAACAGTCCGACCT 480
Malaysia    CTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAAAGCAGAACAGTCCGACCC 480
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Fig. 1. Clustal W multiple sequence alignment of nodavirus RNA2 FNCP42 gene sequence of Indian isolate of fish nodavirus with sequences of nodavirus isolates of Taiwan, France, Singapore and Malaysia. Asterisk symbol indicates nucleotides that are identical to those of India at a given position. The numbers at the end of the last line designate the position of the nucleotide residues.

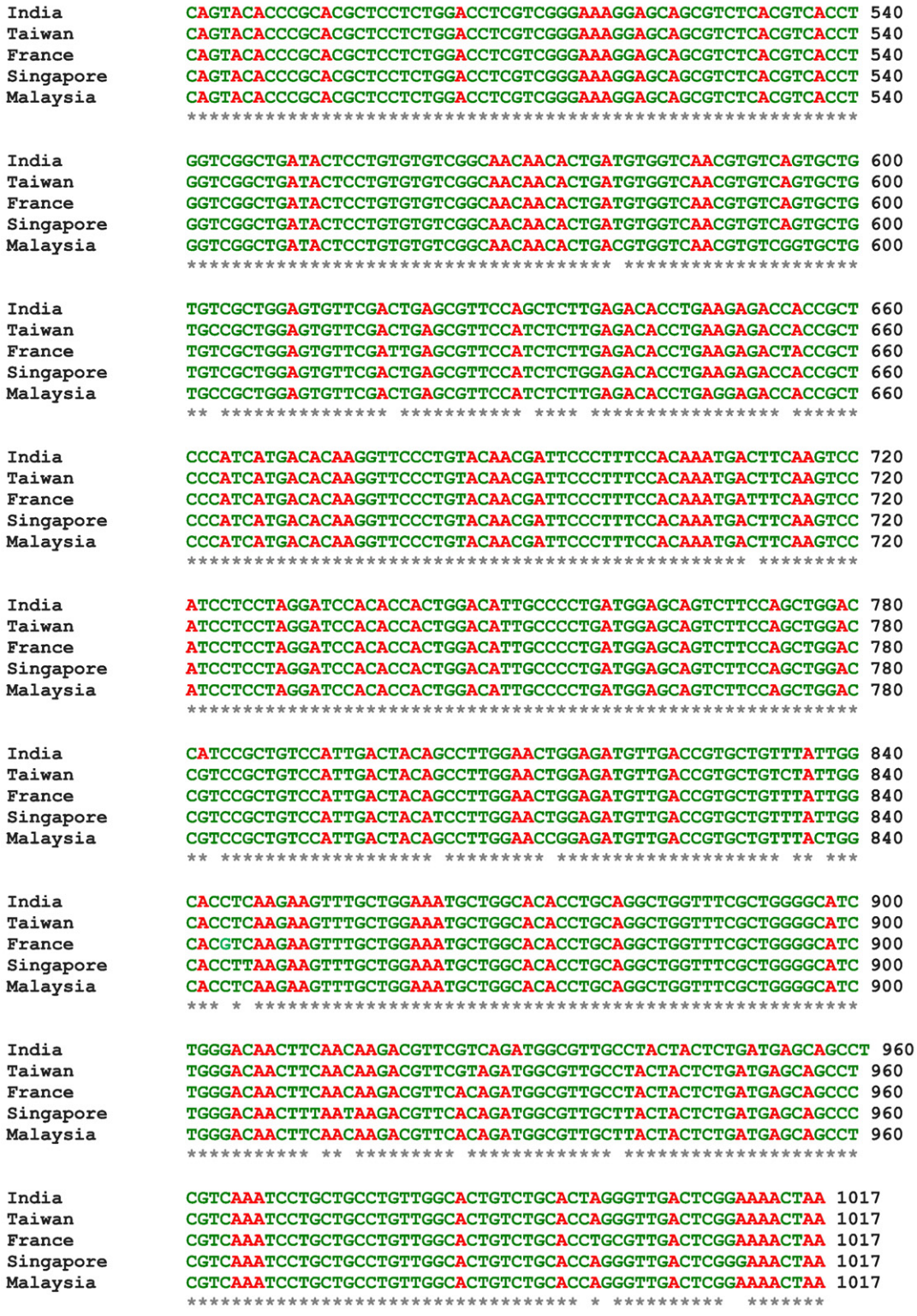

Fig. 1. (*continued*).

raised against r-FNCP at 37 °C for 2 h. The plates were washed with PBS/T and PBS three times each for 2 min and further incubated with 100 μl of anti-rabbit IgG conjugated with alkaline phosphatase for 1 h. The plates were finally washed with PBS/T and PBS three times each for 2 min and developed with the substrate p-nitrophenyl phosphate in substrate buffer. The optical density was measured at 405 nm using an automated ELISA reader (Labsystems, USA).

2.9. In vitro neutralization assay

The neutralization test (constant virus plus antibody dilutions) was applied to test the neutralization ability of the antibody raised against r-FNCP42 in rabbit and vaccinated fish. In the test, 50 μl of 10^2 $TCID_{50}$ nodavirus solution was mixed with an equal volume of serial 2-fold dilutions of cultured medium containing polyclonal anti-r-FNCP42

raised in rabbit or fish and the mock medium (negative control). The mixtures were incubated at 37 °C for 1 h, and then each mixture was added into 4 wells (0.2 ml well^{-1}) of a 96-well microtitration plate with 60% confluent of SISK cells. Evidence of cytopathic effect (CPE) was checked after 6 days incubation at 25 °C, and the 50% neutralization dose (ND_{50}) was determined according to the method described previously (Nakai et al., 1995). The ND_{50} is defined as the inverse of the highest dilution factor of antibody that results in half of the infected cells showing CPE.

2.10. Statistical analysis

Data are expressed as mean ± SD. A statistical analysis was performed using one-way ANOVA for antibody response, and a log rank test using the Kaplan Meier method was used for survival analysis. $p < 0.05$ was taken to indicate statistical significance. All experiments were carried out in triplicates.

3. Results

3.1. Sequence analysis of RNA2

RNA2 capsid protein nucleotide sequence of Indian nodavirus isolate from Asian sea bass (*L. calcarifer*) was cloned and sequenced using standard procedures. Alignment of the multiple sequences showed that it was 98–99% identical at both the nucleotide and the amino acid levels with other isolates of nodavirus reported in Taiwan, Malaysia, Singapore and France and the results are shown in Fig. 1.

3.2. Cloning of RNA2capsid protein

The gene encoding capsid protein of nodavirus was cloned into pRSET B, a prokaryotic expression vector and sequenced. Sequencing results revealed that the capsid protein gene was correctly cloned into expression vector. The recombinant plasmid was obtained and designated as r-pFNCP42.

3.3. Expression and purification of recombinant capsid protein in E. coli

The gene encoding capsid protein of nodavirus was expressed as a recombinant protein with a 6-histidine tag in *E. coli* BL21. It was designated as r-FNCP42. The induced and uninduced r-FNCP42-BL21 (DE3) were analysed by SDS PAGE and the results showed a prominent band corresponding to 42 kDa molecular weight in the induced r-FNCP42-BL21. No protein was found at the same position in uninduced r-FNCP42-BL21 or in induced and uninduced BL21 harbouring pRSET B vector (Fig. 2). The expression of the r-FNCP42 was confirmed by Western blot analysis using antiserum raised against whole nodavirus and it revealed a prominent band corresponding to 42 kDa. The results are shown in Fig. 3.

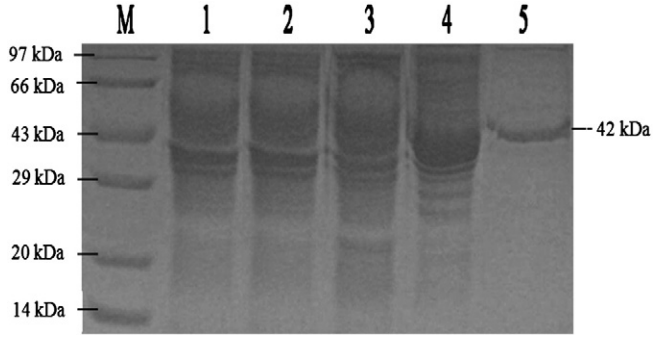

Fig. 2. SDS-PAGE analysis showed the expression of r-FNCP42 in *Escherichia coli* BL21 (DE3). Lane M — 97 kDa Protein marker, Lane 1 — non induced pRSET B-BL21 (DE3); Lane 2 — induced pRSET B-BL21 (DE3) (without insert); Lane 3 — non-induced r-FNCP42-BL21 (DE3); Lane 4 — induced r-FNCP42-BL21 (DE3), and Lane 5 — purified r-FNCP42 (Ni–NTA purification system).

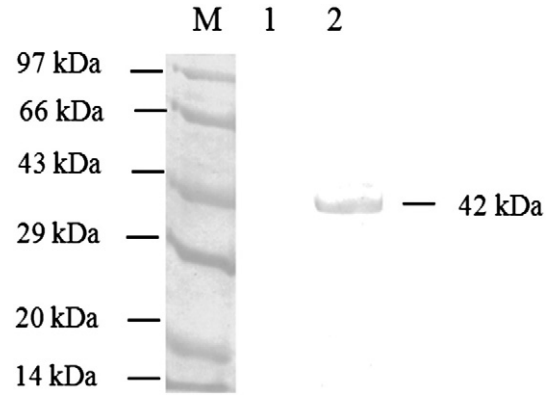

Fig. 3. Western blot analysis of recombinant capsid protein of nodavirus. Lane M — 97 kDa Protein marker, Lane 1 — Un-induced r-FNCP42-BL21 (DE3), and Lane 2 — purified recombinant capsid protein (r-FNCP42).

3.4. Antibody response

The humoral immune response of Asian sea bass to immunization with the r-FNCP42 vaccine was assessed by sandwich ELISA on 5th week after post vaccination. Antibody levels against nodavirus were found to be statistically significant at the serum dilution of 1:2000 in vaccinated fish when compared to negative controls of PBS and FCA. The results are shown in Fig. 4.

3.5. Vaccination and challenge experiment

The Asian sea bass was vaccinated intramuscularly using recombinant r-FNCP42 and the vaccinated sea bass was challenged by intramuscular injection of nodavirus. The vaccinated sea bass was protected from nodaviral infection and 76% of relative percent survival (RPS) was observed. Cumulative percentage mortality rate was recorded and the results are shown in Fig. 5.

3.6. In vitro neutralization assay

The results of in vitro neutralization test revealed that antibody raised against r-FNCP42 neutralized the infectivity of the virus at the dose of 50 μl, 10^2 $TCID_{50}$ up to 1:128 dilution and no CPE was observed in SISK cell lines whereas serum from non-vaccinated fish did not neutralize the virus and showed prominent CPE in SISK cell line. The

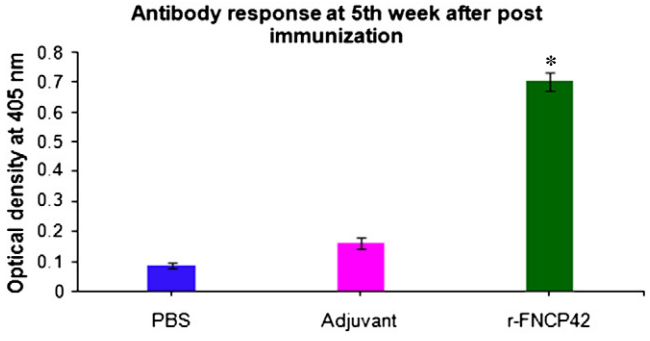

Fig. 4. ELISA detection of anti-FNCP antibody in serum from fish immunized with purified recombinant vaccine (r-FNCP42) and control fish injected with PBS and Adjuvant control. Each column represents the mean optical density (OD) among replica groups measuring data at a serum dilution of 1:2000. A statistical analysis was performed using one-way ANOVA for antibody response between treatment and control groups. *Denotes significant differences of r-FNCP42 ($p < 0.05$).

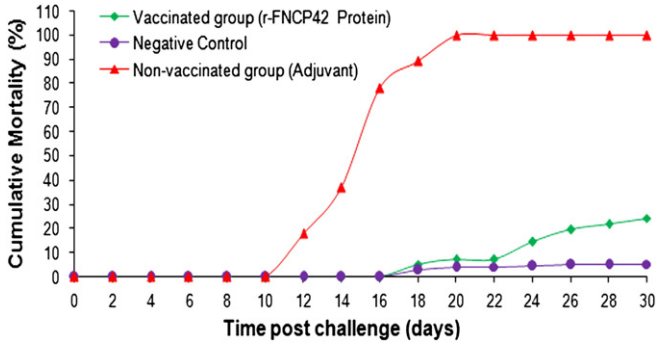

Fig. 5. Cumulative percent mortality of vaccinated and non-vaccinated Asian sea bass at different times after challenging with fish nodavirus.

results indicate that nodavirus can be neutralized by the antiserum in a dose-dependent manner.

4. Discussion

Genome sequence analysis of RNA2 capsid protein nucleotide sequence of Indian isolate of fish nodavirus has confirmed that this isolate has more than 98–99% similarity with other fish nodavirus isolates namely red spotted grouper nervous necrosis virus, *Dicentrarchus labrax* encephalitis virus, Asian sea bass nervous necrosis virus, and *Epinephelus tauvina* nervous necrosis virus (ETNV). The results of present study showed that nucleotide sequence of RNA2 capsid protein gene of Indian nodavirus isolate closely resembles the Taiwan, Malaysia, Singapore and French isolates (Lee et al., 2002; Ransangan and Manin, 2012; Tan et al., 2001; Thiery et al., 2004). Previous studies have reported that phylogenetic analysis based on the nucleotide sequence similarity of capsid protein gene of 25 strains of nervous necrosis virus (NNV) from various parts of the world revealed that fish nodaviruses are closely related to one another (Nishizawa et al., 1997). This high conservation at the genome organization level is supported by the antigenic similarity between fish nodavirus as demonstrated by cross-reactivity (Delsert et al., 1997; Thiery et al., 1999).

A previous report revealed that immunization of striped jack using the recombinant protein of striped jack nervous necrosis virus (SJNNV) led to the development of neutralizing antibodies (Nakai et al., 1995). An increase in specific antibody response following immunization of adult turbot with oil-emulsified recombinant partial capsid protein of SJNNV has been reported (Husgaro et al., 2001). Young seven band grouper vaccinated twice with intramuscular injection of recombinant capsid protein exhibited high protection (RPS: 69–88%) against the experimental challenge at the dose of $10^{3.4}$ TCID50/ml of the betanodavirus (Tanaka et al., 2001). Nodavirus capsid protein of striped jack nervous necrosis virus (SJNNV) was expressed in *E. coli* and vaccination trails using the expressed recombinant protein were conducted in turbot and the immune response of the vaccine against SJNNV evaluated (Husgaro et al., 2001). Protection of juvenile turbot against Atlantic halibut nodavirus (AHNV) demonstrated that vaccination with recombinant capsid protein vaccine could induce the production of neutralizing antibodies and provide protection against experimental challenge with the nodavirus at 33 days of post-vaccination challenge (Sommerset et al., 2005). An oral NNV vaccine composed of Artemia-encapsulated recombinant *E. coli* expressing the NNV capsid protein gene was used to vaccinate grouper larvae (Lin et al., 2007). The vaccinated larvae showed a certain degree of protection after challenge with NNV achieving a Relative Percentage Survival of 64.2% and 69.5%. Our results revealed that recombinant capsid protein (r-FNCP42) vaccinated fish were protected from nodavirus challenge with 76% of RPS which was significant with survival analysis. Rabbit antiserum against capsid protein of *E. tauvina* nervous necrosis virus neutralized the nodavirus infected sea bass cells (Hegde et al., 2002). Our results showed that the rabbit antiserum against the purified recombinant protein of nodavirus neutralized the infectivity of nodavirus at the concentration of 50 μl of 10^2 $TCID_{50}$ in sea bass kidney cells (SISK).

This study demonstrates the cloning capsid protein gene of nodavirus in pRSET B, sequenced, and expressed recombinant capsid protein successfully. The expression of r-FNCP42 was confirmed by Western blot analysis. In vitro neutralization assay performed using antiserum raised against the r-FNCP42 in rabbit and fish vaccinated with r-FNCP42 was able to neutralize the nodavirus in sea bass kidney cells. The humoral immune response of Asian sea bass to immunization with the purified r-FNCP42 protein produced moderate levels of anti-FNCP antibody.

In conclusion, the purified recombinant capsid protein (r-FNCP42) emulsified with complete adjuvant at a dose of 50 μg/fish could induce better humoral immunity and cellular immunity as well as protect Asian sea bass against nodavirus infection.

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