Aquaculture Research

Aquaculture Research, 2014, 1–12 doi: 10.1111/are.12578

Development, distribution and expression of a DNA vaccine against nodavirus in Asian Seabass, *Lates calcarifier* (Bloch, 1790)

S Vimal[1], M A Farook[1], N Madan[1], S Abdul Majeed[1], K S N Nambi[1], G Taju[1], N Sundar raj[1], S Venu[2], R. Subburaj[2], A. R. Thirunavukkarasu[2] & A. S. Sahul Hameed[1]

[1]OIE Reference Laboratory for WTD, PG & Research Department of Zoology, C. Abdul Hakeem College, Melvisharam, Tamilnadu, India

[2]Fish Culture Division, Central Institute of Brackishwater Aquaculture, Chennai, India

Correspondence: A S Sahul Hameed, OIE Reference Laboratory for WTD, PG & Research Department of Zoology, C. Abdul Hakeem College, Melvisharam 632 509, Vellore District, Tamilnadu, India. E-mail: cah_sahul@hotmail.com

Abstract

Fish nodavirus (betanodavirus), a viral pathogen responsible for viral nervous necrosis (VNN) was isolated from infected Asian sea bass (*Lates calcarifer*). The distribution, clearance and expression of nodavirus vaccine, on the basis of DNA vaccine (pFNCPE42 DNA-pcDNA3.1) construction, were analysed in tissues of the Asian seabass by PCR, RT-PCR, ELISA and Immunohistochemistry. Fish immunized with a single intramuscular injection of 20 μg of the pFNCPE42-DNA vaccine showed a significant increase in the serum antibody level in the 3rd week after vaccination, compared to control eukaryotic expression vector pcDNA3.1 vaccinated fish. Results from PCR studies indicated that the vaccine-containing plasmids were distributed in heart, intestine, gill, muscle and liver 10 days after vaccination. Clearance of pFNCPE42-DNA vaccine was studied at 10, 25, 50, 75 and 100 days of post vaccination (d p.v). At 100 days p.v. pFNCPE42-DNA was cleared from muscle of vaccinated sea bass. *In vitro* and *in vivo* expression of fish nodavirus capsid protein gene (FNCP) was determined by fluorescent microscopy. Asian seabass was immunized with pFNCPE42-DNA vaccine at a dose of 20 μg per fish and were challenged with betanodavirus by intramuscular injection. The vaccinated seabass was protected from nodaviral infection and 77.33% of relative percent survival (RPS) was recorded.

Keywords: betanodavirus, DNA vaccine, distribution, expression, neutralization

Introduction

Fish nodavirus (betanodavirus) is a viral pathogen and responsible for viral nervous necrosis (VNN) in teleost fish. The betanodavirus infects fish and belongs to the family Nodaviridae. This virus is a major problem in the farming of marine fish species, causing high mortality in larvae and juveniles of Asian seabass, *Lates calcarifer* worldwide. In India, this virus was isolated from infected Asian seabass (*Lates calcarifer*) larvae during the massive outbreak in sea bass hatcheries located in Chennai and Nagapattinam of Tamilnadu, India (Azad, Shekhar, Thirunavukkarasu, Poornima, Kailasam, Rajan & Ali 2005; Parameswaran, Rajesh, Ishaq Ahmed & Sahul Hameed 2008). Betanodavirus belongs to family, *Betanodaviridae* and is a small virus with particle size between 25 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, Nakai, Muroga, Arimoto, Mushiake & Furusawa 1992).

The genome of the virus consists of two single stranded RNAs (RNA1 and RNA2) (Mori *et al.* 1992; Frerichs, Rodger & Peric 1996). 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 (Mori *et al.* 1992; Nishizawa, Mori, Furuhashi, Nakai, Furusawa & Muroga 1995; Hegde, Chen, Qin, Lam & Sin 2002; Iwamoto, Okinaka, Mise, Mori, Arimoto, Okuno & Nakai 2004).

Preparation of vaccines based on the recombinant DNA technology have shown promising results in both laboratory and field trials. Vaccine is a biologically prepared antigen which helps to improve the immunity in animals against a particular disease or a group of diseases. Different types of vaccines such as heat or formalin killed whole cell vaccine, recombinant protein vaccine and DNA vaccines have been tried to protect the fish from a variety of viral and bacterial diseases (Yuasa, Koesharyani, Roza, Mori, Katata & Nakai 2002; Sommerset, Lorentzen, Lorenzen, Bleie & Nerland 2003; Sommerset, Skern, Biering, Bleie, Fiksdal & Grov 2005; Pakingking, Bautista, Ayson & Reyes 2010; Oh, Gye & Nishizawa 2013; Vimal, Abdul Majeed, Nambi, Madan, Farook, Venkatesan, Taju, Venu, Subburaj, Thirunavukkarasu & Sahul Hameed 2014; Vimal, Madan, Farook, Nambi, Abdul Majeed, Rajkumar, Venu, Thirunavukkarasu & Sahul Hameed 2014).

Current vaccine research is oriented towards replacement of conventional vaccines with newer more effective and safer approaches, such as DNA vaccines. Recently DNA vaccines have been developed for protection against fish viral diseases such as infectious hematopoietic necrosis virus (IHNV) (Corbeil, Kurath & LaPatra 2000), viral haemorrhagic septicaemia virus (VHSV) (McLauchlan, Collet, Ingerslev, Secombes, Lorenzen & Ellis 2003) and infectious pancreatic necrosis virus (IPNV) (Heras, Rodriguez Saint-Jean, Sara & Perez-Prieto 2010).

Sommerset *et al.* (2003) developed a DNA vaccine using gene encoding glycoprotein of VHSV and challenge experiments revealed that the immunity established is cross-protective against heterologous fish rhabdoviruses and also against nodavirus. Recombinant and DNA vaccines have been developed based on capsid encoding region of the Atlantic halibut nodavirus (AHNV) genome and the results revealed that immunity against nodavirus was established in the fish vaccinated with recombinant capsid protein, but not in the DNA vaccinated fish (Sommerset *et al.* 2005).

In this study, an attempt has been made to develop DNA vaccines using the capsid protein gene of Indian isolate of fish nodavirus and to determine the efficacy of the DNA vaccines to provide protective immunity in Asian seabass against nodavirus infection. Plasmid pFNCPE42-DNA may serve as a DNA vaccine against fish nodavirus.

Materials and methods

Nodavirus propagation

The susceptibility of fish cell lines to a betanodavirus was evaluated according to the method described by Fan, Wang, Geng, Cong, Li, Yu and Yang (2006). The fish nodavirus used in the present study was isolated from infected Asian seabass (*L. calcarifer*) larvae during a massive outbreak in seabass hatcheries located in Chennai and Nagapattinam of Tamilnadu, India (Parameswaran *et al.* 2008). The betanodavirus was propagated in the seabass kidney cell line [Epithelial cell type (SISK)] derived from Asian seabass used for infectivity studies (Sahul Hameed, Parameswaran, Ravi Shukla Bright Singh, Thirunavukkarasu & Bhonde 2006). Viral inoculum was prepared by the method described by Kang, Oh, Kim, Kawai and Jung (2003). For infection, the cells of seabass kidney cell line were inoculated in a 24-well plate and incubated for 12–24 h at 28°C to 60–70% confluence. After removing the medium, 0.1 mL of virus suspension with a titre of 10^3 $TCID_{50}$ per mL was inoculated onto the cell culture in the 24-well plate and allowed to adsorb for 1 h. Then, 0.5 mL of maintenance medium containing 5% FBS was added. The cells were incubated at 25°C and 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 × 9 *g*) at 4°C, and the viral titre was then determined in a 50% tissue culture infective dose ($TCID_{50}$) assay in 96-well tissue culture plates based on the procedure described by Reed and Muench (1938).

Collection and maintenance of experimental animals

Healthy juveniles of Asian seabass (*L. calcarifer*) fish (10–15 g in body weight) were obtained from Central Institute of Brackishwater Aquaculture,

Chennai and transported in live condition to the laboratory. In the laboratory, the animals were maintained in 500 L tanks containing UV-treated seawater (salinity 30 ppt) with continuous aeration at 24°C. The fish were fed twice a day with boiled fish meat during the acclimatization and experimental periods. After acclimatization, the fish were placed in separate tanks for experimental purpose.

Construction and preparation of plasmid (pFNCPE42) DNA vaccine

The gene encoding for ORF of the viral capsid protein, approximately 1017 bp in length was amplified using specific primer set forward primer (5′-CGC AAGCTTACCATGGTACGCAAAGGTGATAAG-3′) and reverse primer (5′-CCCGAATTCCCGGTTAGT TTTCCGAGTCAA-3′) and subsequently cloned into a eukaryotic expression vector pcDNA3.1 (Invitrogen, Carlarbad, CA, USA), behind the early cytomegalovirus promoter, yielding pcDNA3.1–FNCPE42. The pcDNA3.1–FNCP42 was verified using *Hind III* and *EcoR I* endonuclease analysis, and the recombinant plasmid was then transformed into *E. coli* DH5α cells. Recombinant clone was selected based on ampicillin resistance and confirmed by DNA sequencing. Plasmid was named as pFNCPE42. Plasmid was purified with the EndoFree Plasmid Mega purification kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions, aliquoted at 1 μg mL^{-1} in sterile endotoxin free phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4), and stored at −20°C until further use.

In vitro transfection of pFNCPE42-DNA in seabass kidney cell line (SISK) and detection through immunohistochemistry

The SISK cells (Sahul Hameed *et al.* 2006) were grown on coverslips (22 × 22 mm) for 24 h, after subconfluent monolayer cells were transfected with pFNCPE42-DNA. A concentration of 2–4 μg of pFNCPE42-DNA was added and incubated with the cells for 8 h followed by 24 h incubation in fresh complete medium (Leibovitz's L-15; Invitrogen). All transfection experiments were performed in triplicate. After 48 h, the cells were fixed with 3.7% p-formaldehyde for 10 min at 4°C, washed with PBS, permeabilized with 0.1% Triton X-100 at 4°C for 4 min, and then blocked in PBS containing 1% bovine serum albumin (BSA) for 30 min at room temperature. Polyclonal antibody against capsid protein of nodavirus (FNCP) was diluted (1:50) in PBS with 1% BSA and directly added to the fixed cells and kept for 2 h at room temperature (Sahul Hameed, Anilkumar, Stephen Raj & Kunthala 1998; Vimal, Madan *et al.* 2014; Vimal, Abdul Majeed *et al.* 2014). Then the cells were washed with wash buffer, followed by addition of the goat anti-rabbit IgG secondary antibody (IgG) conjugated with FITC at a dilution of 1:50 for 45 min at room temperature. The cells were washed and mounted with antifade 1, 4-diazobicyclo-2, 2, 2-octanex (DABCO) in mounting medium purchased from Sigma Aldrich (St. Louis, MO, USA). The slides along with cover slips were observed under a fluorescence microscope (Carl Zeiss, Jena, Germany) (Vimal, Taju, Nambi, Abdul Majeed, Sarath Babu, Ravi & Sahul Hameed 2012).

Tissue distribution and clearance of DNA vaccine

Distribution of pFNCPE42 DNA vaccine was studied by extracting DNA from the tissue samples (heart, intestine, gill, muscle and liver) at 10 days post vaccination. Clearance of DNA vaccine was studied at different days on 10th, 25th, 50th, 75th and 100th day post vaccination and confirmed by PCR (Zheng, Sun, Liu & Zhang 2006). Briefly, the tissue suspension was mixed with an appropriate amount of digestion buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 50 mM EDTA, pH 8.0, 0.5% sodium dodecyl sulphate and 0.1 mg mL^{-1} proteinase K). After incubation at 65°C for 2 h, the digests were deproteinized by successive phenol/chloroform/isoamyl alcohol extraction and DNA was recovered by ethanol precipitation and dried. The dried DNA pellets were resuspended in TE buffer (1 M Tris, pH 8.0, 0.5 M EDTA, pH 8.0) and used as a template for PCR. The extracted DNA was confirmed in the tissue samples (heart, intestine, gill, muscle and liver). Amplification was performed at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 45 s, annealing at 55°C for 45 s and extension at 72°C for 45 s and a final extension at 72°C for 10 min following the above mentioned PCR protocol described by Vimal *et al.* (2012), Vimal, Abdul Majeed, Taju, Nambi, Sundar Raj, Madan, Farook, Rajkumar, Gopinath and Sahul Hameed (2013).

Transcriptional analysis of pFNCPE42 gene by RT-PCR

Total RNA was extracted from the pFNCPE42 DNA vaccinated tissues samples from heart, intestine, gill, muscle and liver tissues and pFNCPE42 DNA transfected SISK cell line. For transcriptional analysis, the samples were subjected to RT-PCR. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA), as per the manufacturer's instructions. The extracted RNA was dissolved in nuclease-free water. The contaminating DNA was removed by treatment with DNase I (Merck, Bangalore, India) at 37°C for 30 min and then re-extracted with phenol-chloroform. The DNase treated total RNA was denatured by heating at 85°C for 10 min in 10 mL DEPC-water containing 100-pmol oligo-dT primer (Bangalore Genei). The first strand cDNA was synthesized by the addition of 3 μL 5 × M-MuLV buffer,1 μL 100 mM DTT, 1 μL 10 mM dNTPs and 10 U RNasin (Bangalore Genei) making a total volume of 10 μL including 100 U M-MuLV reverse transcriptase (New England Bio-labs, Ipswich, MA, USA). The mixture was kept at 37°C for 1 h for cDNA synthesis. The cDNA was subjected to PCR with a primer set specific to FNCP gene. The RT-PCR products were then analysed by electrophoresis on a 1% agarose gel stained with ethidium bromide and visualized by ultraviolet transillumination (Vimal *et al.* 2012).

In vivo expression of pFNCPE42-DNA by immunohistochemistry

The expression of FNCP gene in different tissues of pFNCPE42-DNA vaccinated sea bass was analysed by immunohistochemistry. The tissue samples of heart, intestine, gill, muscle and liver were collected from the fish and fixed with 10% formaldehyde (made in 0.1 M PBS, pH 7.4). After 48 h, the tissue samples were processed and embedded in paraffin wax, and then cut into sections of 5 μm thickness using microtome. The paraffin sections were deparaffinised in xylene, and hydrated through descending graded levels of alcohol to distilled water. The sections were then treated with trypsin (0.1% w/v in PBS, biochemical grade, Hi Media, Mumbai, India) for 10 min and washed twice with PBS-Tween 20 (T-20, Hi Media, Mumbai, India; 0.01% v/v with PBS). Non-specific antibody binding sites were blocked for 30 min using 3% bovine serum albumin (in PBS, pH 7.4) and washed with PBS/T. The sections were treated with the primary antibody rabbit antiserum specifically raised against recombinant capsid protein of nodavirus at a dilution of 1:50 (sterile PBS, pH 7.4) for 1 h in a humid chamber. Sections were washed with PBS-T and treated with goat anti-rabbit (IgG) FITC conjugate at a dilution of 1:50 for 30 min at room temperature. After washing, it was mounted using glycerol following the protocol of (Vimal *et al.* 2012). The slides were observed under a fluorescent microscope (Carl Zeiss).

Enzyme-linked immunosorbent assay (ELISA)

The expression of FNCP gene in different tissues of vaccinated seabass was confirmed by ELISA using the antiserum raised against recombinant capsid protein (r-FNCP42) of nodavirus. The wells of flat-bottomed ELISA plate were coated with the suspension of tissue samples (heart, intestine, gill, muscle and liver) of fish injected with pFNCPE42-DNA 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 antiserum raised against 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 goat anti-rabbit IgG conjugated with alkaline phosphatase for 1 h. The plates were again 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 (Thermo Labsystems, Beverly, MA, USA).

Experimental nodavirus challenge

Juveniles of Asian seabass were used in challenging experiments to evaluate the efficacy of DNA vaccine. The seabass were divided into three groups (120 per group) for challenging experiment. In Group I (Negative Control), the fish were injected with PBS. In Group II (Non-vaccinated group), the fish were intramuscularly injected with pcDNA3.1. In Group III (Vaccinated group), the fish were intramuscularly injected with one dose of 20 μg of pFNCPE42-DNA. After 21 days post vaccination, the fish in all the groups were challenged with an intramuscular injection of

betanodavirus at a dose of $1 \times 10^{6.5}$ $TCID_{50}$ per fish. After 30 days of post challenging, cumulative percentage mortalities were recorded daily. Dead fish were autopsied to determine the cause of death and to detect the presence of virus. 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 (90 per group) as mentioned above. After vaccination as described above in different groups, different tissue samples namely heart, intestine, gill, muscle and liver were collected from 20 fish at 10 days after post vaccination for PCR, RT-PCR, ELISA and immunohistochemistry analyses. After 21 days of post vaccination, blood sample was collected from 20 fish to determine the analysis of antibody response in vaccinated fish by ELISA as described by Rajesh Kumar, Parameswaran, Ishaq Ahmed, Syed Musthaq and Sahul Hameed (2007). *In vitro* viral neutralization assay was also studied in Seabass kidney cell lines. Clearance of DNA vaccine was studied in remaining fish on 10, 25, 50, 75, and 100 days of post vaccination and confirmed by PCR. The experiment was carried out in triplicates.

Analysis of antibody response

After 3rd 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).

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 pFNCPE42-DNA vaccinated fish. In the test 50 µl of 10^2 $TCID_{50}$ nodavirus solution was mixed with an equal volume of serial twofold dilutions of cultured medium containing polyclonal anti-r-FNCP42 raised in rabbit or pFNCPE42-DNA vaccinated 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 per well) 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, Mori, Arimoto & Muroga 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.

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.

Results

In vitro and *in vivo* expression of FNCP gene

The ability of the constructed DNA vaccine to express FNCP gene in eukaryotic (SISK cell line) cells transfected with pFNCPE42-DNA, and the negative control pcDNA3.1 were studied. After 48 h of incubation after transfection, only pFNCPE42-DNA transfected cells expressed the protein FNCP, which was detected by fluorescent microscope (Fig. 1a). *In vivo* expression of FNCP protein could also be detected in the muscle, heart, intestine, gill and liver in fish intramuscularly injected with pFNCPE42-DNA by fluorescent microscopy (Fig. 1b) and ELISA analysis (Table.1).

Distribution and clearance of pFNCPE42-DNA

The distribution of pFNCPE42-DNA in different tissues of vaccinated fish injected with pFNCPE42-DNA was determined by PCR and the results revealed the presence of pFNCPE42-DNA in the muscle, heart, intestine, gill and liver after 10 days of post vaccination. The PCR product was observed at the corresponding size of 1017 bp specific to the FNCP gene of nodavirus and the results are shown in Fig. 2a. Clearance of pFNCPE42-DNA from different organs of injected seabass was studied at different time intervals by PCR and the results revealed that pFNCPE42-DNA was cleared from intestine, gill and liver on 75 days p.v but not from the muscle. At 100 days p.v. pFNCPE42-DNA was cleared from muscle of vaccinated seabass. The results are shown in Fig. 2b.

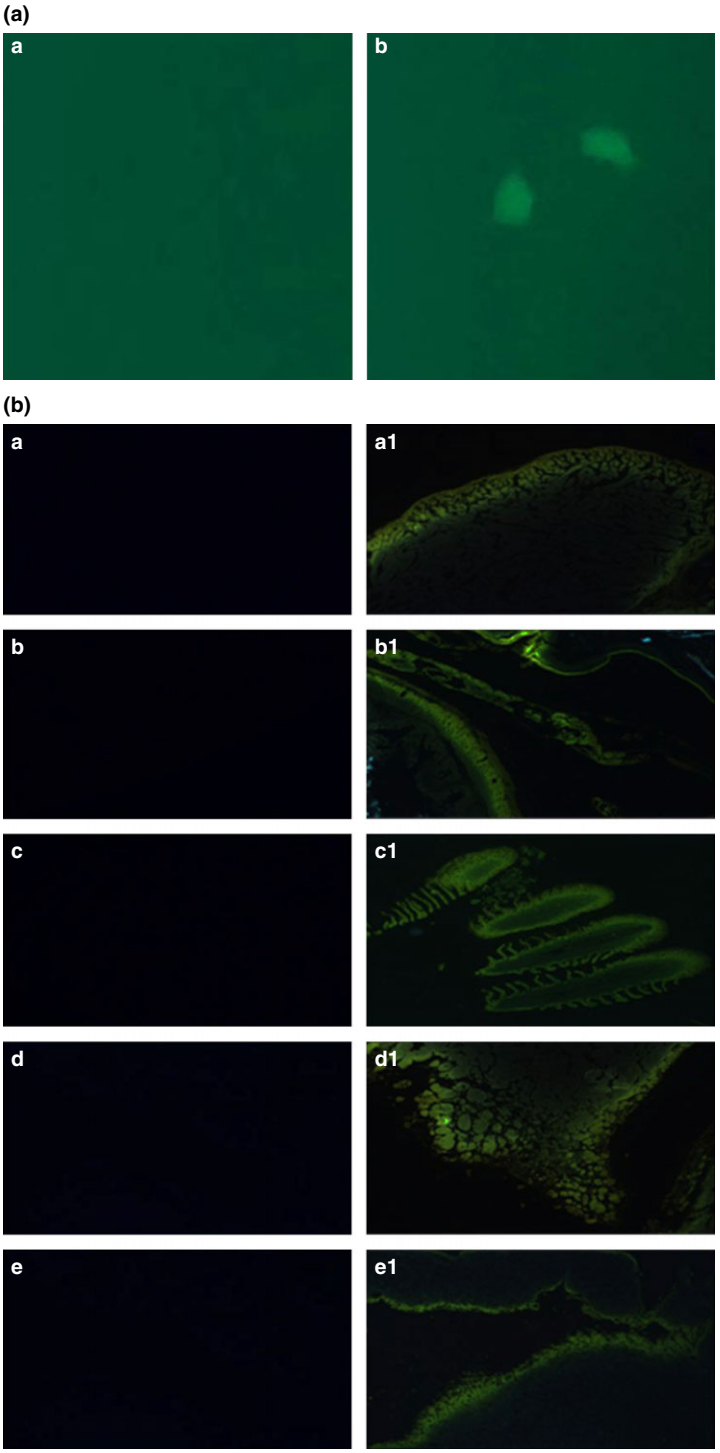

Figure 1 (a) *In vitro* gene expression of pFNCPE42-DNA vaccine in sea bass kidney cell line by immunofluorescence detection. (a) pcDNA 3.1 transfected sea bass kidney cell line and (b) pFNCPE42-DNA vaccine transfected sea bass kidney cell line (200× magnification). (b) *In vivo* gene expression of fish nodavirus capsid protein gene in different organs of fish intramuscular injection with pFNCPE42-DNA by immunofluorescence detection. a to e – Asian sea bass vaccinated with pcDNA3.1 (a) Heart tissue, (b) Intestine tissue, (c) Gill tissue, (d) Muscle tissue, (e) Liver tissue. a1 to e1 – Asian sea bass vaccinated with pFNCPE42-DNA (a1) Heart tissue, (b1) Intestine tissue, (c1) Gill tissue, (d1) Muscle tissue, (e1) Liver tissue.

In vitro and *in vivo* transcription analyses of pFNCPE42-DNA

Transcription analyses of the FNCP gene was performed by RT-PCR reaction in pFNCPE42-DNA transfected SISK cell line and different tissues (muscle, heart, intestine, gill and liver) of fish intramuscularly injected with pFNCPE42-DNA. RT-PCR product was observed at the corresponding size of 1017 bp specific to the FNCP gene of

Table 1 *In vivo* gene expression of fish nodavirus capsid protein gene in different organs of fish intramuscular injection with pFNCPE42-DNA by ELISA

	Mean ELISA OD (405) ± SE	
Organ analysed	**Fish injected with Plasmid pcDNA3.1**	**Fish injected with Plasmid FNCPE42-DNA**
Heart	0.0916 ± 0.006	0.9313 ± 0.06909
Intestine	0.0465 ± 0.002	0.4977 ± 0.02192
Gill	0.0572 ± 0.003	0.5122 ± 0.02447
Muscle	0.0832 ± 0.005	0.8725 ± 0.05618
Liver	0.0711 ± 0.005	0.6036 ± 0.06010

nodavirus and the results are shown in Fig. 3a *(in vitro)* and Fig. 3b *(in vivo)*. No amplification was observed in cells transfected with pcDNA3.1 and PBS injected fish.

Protection of seabass from nodavirus challenge

Asian seabass immunized with the pFNCPE42-DNA vaccinated group encoding for FNCP was challenged with betanodavirus by intramuscular challenge. Mortalities following exposure to the virus were lower in fish vaccinated with pFNCPE42-DNA compared to those of the negative

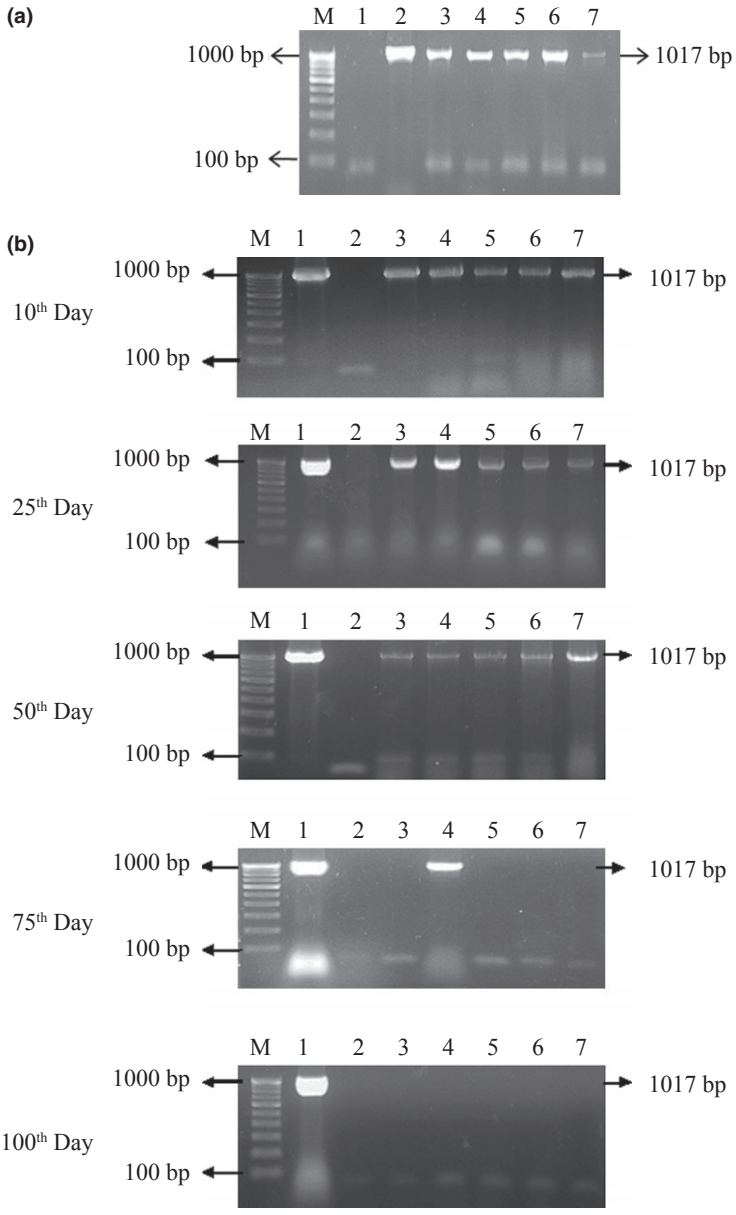

Figure 2 (a) Agarose gel showing distribution of pFNCPE42-DNA vaccine in fish tissues on 10 days of post vaccination (d p.v) and confirmed by PCR. Lane M – Marker, Lane 1 – Negative, Lane 2 – Positive; Lane 3 – Gills, Lane 4 – Muscle, Lane 5 – Intestine, Lane 6 – Heart, Lane 7 – Liver. (b) Agarose gel showing the clearance of pFNCPE42-DNA vaccine in fish tissues on 10, 25, 50, 75 and 100 days of post vaccination (d p.v) and confirmed by PCR. Lane M – Marker, Lane 1 – Positive, Lane 2 – Negative; Lane 3 – Gills, Lane 4 – Muscle, Lane 5 – Intestine, Lane 6 – Heart, Lane 7 – Liver.

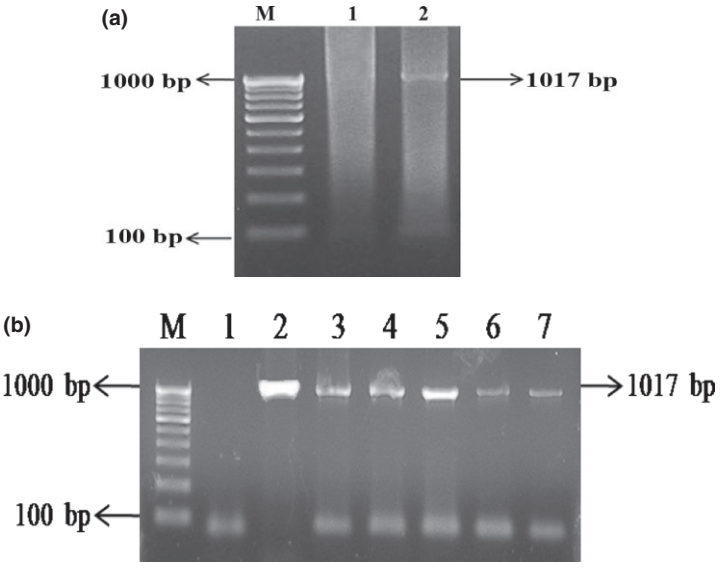

Figure 3 (a) Agarose gel showing transcription analysis of pFNCPE42-DNA in SISK cell line (*in vitro*) by RT-PCR. Lane M – Marker, Lane 1 – pcDNA3.1 transfected sea bass kidney cells, Lane 2 – pFNCPE42-DNA vaccine transfected SISK cell line PCR. (b) Agarose gel showing transcriptional analyses of pFNCPE42 DNA vaccine in fish tissues on 10 days of post vaccination (d p.v) and confirmed by RT-PCR. Lane M – Marker, Lane 1 – Negative, Lane 2 – Positive; Lane 3 – Gills, Lane 4 – Muscle, Lane 5 – Intestine, Lane 6 – Heart, Lane 7 – Liver.

control groups injected with PBS alone and non-vaccinated injected with virus. Cumulative percentage mortality rate was recorded and the results are shown in Fig. 4. The pFNCPE42-DNA vaccine protected fish from the nodavirus with 77.33% of RPS which was significant with survival analysis.

Analysis of antibody response

The humoral immune response of Asian seabass to immunization with the pFNCPE42-DNA vaccine was assessed by sandwich ELISA on 3rd week after post vaccination. Antibody levels against nodavirus were found to be statistically significant at the serum dilution of 1:1000 in vaccinated fish, when compared to negative controls of PBS buffer and pcDNA3.1. The results are shown in Fig. 5.

In vitro neutralization assay

The results of *in vitro* neutralization test revealed that antibody raised against pFNCPE42-DNA vaccinated fish sera neutralized the infectivity of the virus at the dose of 50 µL, 10^2 $TCID_{50}$ up to 1:128 dilution. 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 results indicate that nodavirus can be neutralized by the antiserum in a dose-dependent manner.

Discussion

In this study, investigation was carried out to test the efficacy of DNA vaccine against fish nodavirus using the gene encoding for major capsid protein

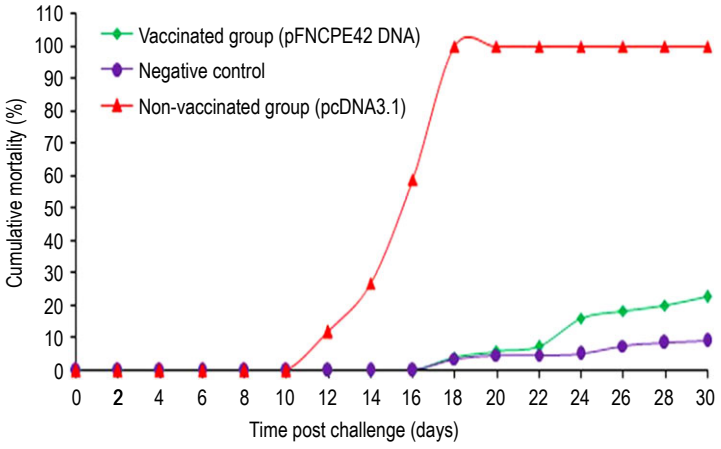

Figure 4 Development of cumulated mortality in pFNCPE42-DNA vaccinated and control sea bass after challenge with nodavirus.

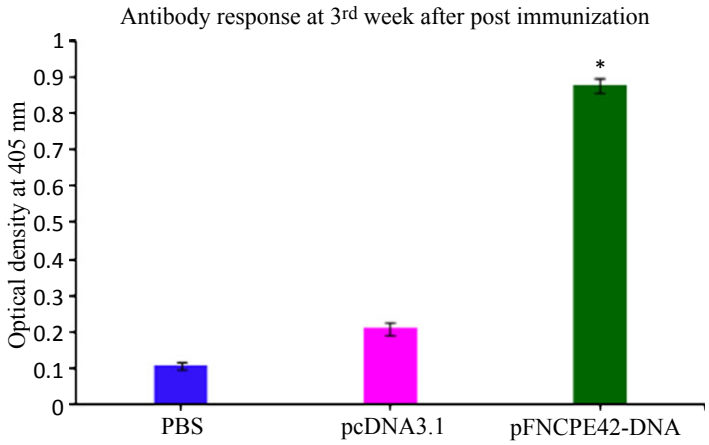

Figure 5 Enzyme-linked immunosorbent assay (ELISA) detection of anti-FNCP antibody in serum from fish immunized with pFNCPE42-DNA and control fish injected with PBS and plasmid control (pcDNA3.1). Each column represents the mean optical density (OD) among replica groups measuring data at a serum dilution of 1:1000. A Statistical analysis was performed using one-way ANOVA for antibody response between treatment and control groups. *Denotes significant differences in pFNCPE42-DNA ($P < 0.05$).

(FNCPE42) from nodavirus. The results showed that the immunization of Asian seabass with DNA vaccine induced moderate protection against experimental challenge with nodavirus. The DNA vaccinated seabass produced moderate level of antibody response, which might be due to low level of antigen expression in the host tissue.

Vaccination with major capsid protein (MCP) has been shown to be effective against nodavirus infection in fish (Lin, Lin, Chen & Yang 2007). However antigens, which are purified and structurally mature forms are important for effective immunization. DNA vaccines offer several advantages over classical antigen vaccines (i.e. live vaccine, inactivated vaccine and recombinant vaccines) (Hegde, Lam & Sin 2005; Kai & Chi 2008; Nishizawa, Gye, Takami & Oh 2012). DNA vaccine using capsid gene has already been applied in fish and the results showed partial protection to fish against experimental challenge with IHNV (Leong & Fryer 1993), VHSV (Lorenzen & Olesen 1997) and IPNV (Cuesta, Chaves-Pozo, de las Heras, Saint-Jean, Perez-Prieto & Tafalla 2010).

Tissue distribution and clearance of DNA vaccine in vaccinated fish was studied. The results revealed the distribution of pFNCPE42-DNA in different tissues of fish injected with pFNCPE42-DNA in the muscle, heart, intestine, gill and liver after 10 day post vaccination by PCR. Tissue distribution of DNA vaccine in different organs of vaccinated fish was also demonstrated by different workers (Kanellos, Sylvester, Ambali, Howard & Russell 1999; Kanellos, Sylvester, Howard & Russell 1999; Sommerset *et al.* 2003; Zheng *et al.* 2006). Clearance of pFNCPE42-DNA from different organs of injected seabass was studied at different time intervals by PCR and the results revealed that pFNCPE42-DNA vaccine was found to be stable up to 75 days in the muscle tissue of vaccinated Asian seabass. Our results agree with the results of previous works (Kanellos, Sylvester, Ambali *et al.* 1999; Kanellos, Sylvester, Howard *et al.* 1999; Sommerset *et al.* 2003; Zheng *et al.* 2006). In Japanese flounder, the DNA vaccine was detected in injected muscle till 90 days p.i (Zheng *et al.* 2006). The aim of the present study was to develop a DNA vaccine for nodavirus and to carry out a complete study on its distribution in tissues and clearance. *In vitro* and *in vivo* transcription analyses of pFNCPE42-DNA vaccinated fish and transfected seabass kidney cells were confirmed by RT-PCR. The RT-PCR results revealed that the expression of DNA vaccines steadily increased at 10 days p.v. RT-PCR product was observed at the corresponding size of 1017 bp specific to the FNCP gene of nodavirus. *In vitro* and *in vivo* gene expression of pFNCPE42-DNA in injected fish and transfected seabass kidney cell could express FNCP gene and this was confirmed by immunoflurescence detection and ELISA analyses. The serum collected from the fish vaccinated with pFNCPE42-DNA

neutralized the infectivity of the virus and no CPE was observed in SISK cell line. This indicates host immune response to DNA vaccine against nodavirus.

The usefulness of vaccinating fish challenged with nodavirus after 21 days post vaccination by i.m. injection of naked pFNCPE42-DNA encoding capsid protein of nodavirus was studied and the results are documented. Previous studies reported that pVHSV-G vaccinated turbot challenged with nodavirus induced protection at 35 days p.v (Sommerset *et al.* 2005), whereas in pAHNV-C DNA vaccinated fish challenged with AHNV at 9–33 days p.v, no mortality was observed. However, Sommerset *et al.* (2003) constructed a DNA vaccine encoding the VHSV glycoprotein (G) and observed protection of fish from nodaviral infection with RPS of 100% at 8 days p.v and 63% at 35 days p.v. In the present study, 77.3% of RPS was observed in Asian sea bass vaccinated with DNA vaccine after nodaviral challenge.

This study demonstrates that DNA vaccination against nodavirus in Asian seabass using pFNCPE42-DNA is very effective although both transcription and translation of the DNA-encoded antigen were demonstrated in *in vitro* and *in vivo* studies. Previous studies reported that fish DNA-vaccination systems work exceptionally well with IHNV and VHSV in rainbow trout (Anderson, Mourich, Fahrenkrug, LaPatra, Shepherd & Leong 1996; Anderson, Mourich & Leong 1996). *In vitro* neutralization assay performed using antiserum collected from pFNCPE42-DNA vaccinated fish was able to neutralize the nodavirus in sea bass kidney cells. The humoral immune response of Asian seabass to immunization with the pFNCPE42-DNA produced moderate levels of anti-FNCP antibody.

In conclusion, the results revealed that DNA vaccination in seabass, with the major capsid encoding gene from nodavirus, induces a significant humoral immune response and moderate protection against nodavirus experimental infection. Further vaccine designs and vaccination trials should focus on improving the levels of expression and antigenicity of this DNA vaccine. The results suggest that the Asian seabass adequately responds to a DNA intramuscular injection, providing an antigen and producing a specific immune response. The plasmid (pFNCPE42-DNA) is therefore a promising candidate for a DNA vaccine against the nodavirus infection.

Acknowledgments

The authors thank the management of C. Abdul Hakeem College for providing the facilities to carry out this study. This study was funded by Department of Biotechnology, Government of India, New Delhi, India, under the Indo-Norway programme on vaccine development.

References

Amend D.F. (1981) Potency testing of fish vaccines. In: *Developments in Biological Standardization*, Vol. **49** (ed. by D.P. Anderson & W. Hennessen), pp. 447–454., Fish Biologics: serodiagnostics and vaccines. S. Karger, Basel.

Anderson E.D., Mourich D.V., Fahrenkrug S.C., LaPatra S., Shepherd J. & Leong J.C. (1996) Genetic immunization of rainbow trout (*Oncorhynchus mykiss*) against infectious hematopoietic necrosis virus. *Molecular Marine Biology and Biotechnology* **5**, 114–122.

Anderson E.D., Mourich D.V. & Leong J.C. (1996) Gene expression in rainbow trout (*Oncorhynchus mykiss*) following intramuscular injection of DNA. *Molecular Marine Biology and Biotechnology* **5**, 105–113.

Azad I.S., Shekhar M.S., Thirunavukkarasu A.R., Poornima M., Kailasam M., Rajan J.J.S. & Ali S.A. (2005) Nodavirus infection causes mortalities in hatchery-produced larvae of *Lates calcarifer*: first report from India. *Diseases of Aquatic Organism* **63**, 113–118.

Corbeil S., Kurath G. & LaPatra S. (2000) Fish DNA vaccine against infectious hematopoietic necrosis virus: efficacy of various routes of immunisation. *Fish and Shellfish Immunology* **10**, 711–723.

Cuesta A., Chaves-Pozo E. & de Las Heras A.I., Saint-Jean S.R., Perez-Prieto S. & Tafalla C. (2010) An active DNA vaccine against infectious pancreatic necrosis virus (IPNV) with a different mode of action than fish rhabdovirus DNA vaccines. *Vaccine* **28**, 3291–3300.

Fan T.J., Wang L.Y., Geng X.F., Cong R.S., Li M.Y., Yu Q.T. & Yang X.X. (2006) Studies of the propagation of turbot reddish body iridovirus in vitro. *Journal of Ocean University of China* **36**, 767–774.

Frerichs G.N., Rodger H.D. & Peric Z. (1996) Cell culture isolation of piscine neuropathy nodavirus from juvenile sea bass *Dicentrarchus labrax*. *Journal of General Virology* **77**, 2067–2071.

Hegde A., Chen C.L., Qin Q.W., Lam T.J. & Sin Y.M. (2002) Characterization, pathogenicity and neutralization studies of a nervous necrosis virus isolated from grouper, *Epinephelus tauvina*, in Singapore. *Aquaculture* **213**, 55–72.

Hegde A., Lam T.J. & Sin Y.M. (2005) Immune response of freshwater fish, guppy, *Poicelia reticulate* and gouramy, *Trichogaster trichopterus* to recombinant coat protein of *Epinephelus tauvina* nervous necrosis virus. *Aquaculture* **249**, 77–84.

Heras A.I.D.L., Rodriguez Saint-Jean S., Sara I. & Perez-Prieto S.I. (2010) Immunogenic and protective effects of an oral DNA vaccine against infectious pancreatic necrosis virus in fish. *Fish and Shellfish Immunology* **28**, 562–570.

Iwamoto T., Okinaka Y., Mise K., Mori K., Arimoto M., Okuno T. & Nakai T. (2004) Identification of host-specificity determinants in betanodaviruses by using reassortants between Striped jack nervous necrosisvirus and Sevenband grouper nervous necrosis virus. *Journal of Virology* **3**, 1256–1262.

Kai Y.H. & Chi S.C. (2008) Efficacies of inactivated vaccines against betanodavirus in grouper larvae (*Epinephelus coioides*) by bath immunization. *Vaccine* **26**, 1450–1455.

Kanellos T., Sylvester I.D., Ambali A.G., Howard C.R. & Russell P.H. (1999) The safety and longevity of DNA vaccines for fish. *Immunology* **96**, 307–313.

Kanellos T., Sylvester I.D., Howard C.R. & Russell P.H. (1999) DNA is as effective as protein at inducing antibody in fish. *Vaccine* **17**(7–8), 965–972.

Kang M.S., Oh M.J., Kim Y.J., Kawai K. & Jung S.J. (2003) Establishment and characterization of two cell lines derived from flounder, *Paralichthys olivaceus* (Temminck and Schlegel). *Journal of Fish Diseases* **26**, 657–665.

Leong J.C. & Fryer J.L. (1993) Viral vaccines for aquaculture. *Annual Review of Fish Diseases* **3**, 225–240.

Lin C.C., Lin J.H.Y., Chen M.S. & Yang H.L. (2007) An oral nervous necrosis virus vaccine that induces protective immunity in larvae of grouper (*Epinephelus coioides*). *Aquaculture* **268**, 265–273.

Lorenzen N. & Olesen N.J., (1997). Immunization with viral haemorrhagic septicaemia antigens. In: *Fish Vaccinology*, Vol. **90** (ed. by R. Gudding, A. Lillehaug, P.J. Midtlying & F. Brown), pp. 201–209. *Developments in Biological Standardization*. Karger, Basel, New York.

McLauchlan P.E., Collet B., Ingerslev E., Secombes C.J., Lorenzen N. & Ellis A.E. (2003) DNA vaccination against viral haemorrhagic septicaemia (VHS) in rainbow trout: size, dose, route of injection and duration of protection-early protection correlates with Mx expression. *Fish and Shellfish Immunology* **15**, 39–50.

Mori K., Nakai T., Muroga K., Arimoto M., Mushiake K. & Furusawa I. (1992) Properties of a new virus belonging to Nodaviridae found in larval striped jack (*Pseudocaranx dentex*) with nervous necrosis. *Virology* **187**, 368–371.

Nakai T., Mori K., Arimoto M. & Muroga K. (1995) Neutralizing antibody production in striped jack immunized with a recombinant coat protein of striped jack nervous necrosis virus (SJNNV). *Proceedings of The European Association of Fish Pathologists, Seventh International Conference, Diseases of Fish and Shell Fish Palma de Mallorca*, pp. 10–15. Sept. Asian Fisheries Society, Manila, Philippines.

Nishizawa T., Mori K., Furuhashi M., Nakai T. & Furusawa I. & Muroga K. (1995) Comparison of the coat protein genes of five fish nodaviruses, the causative agents of viral nervous necrosis in marine fish. *Journal of General Virology* **76**, 1563–1569.

Nishizawa T., Gye H.J., Takami I. & Oh M.J. (2012) Potentiality of a live vaccine with nervous necrosis virus (NNV) for sevenband grouper *Epinephelus septemfasciatus* at a low rearing temperature. *Vaccine* **30**, 1056–1063.

Oh M.J., Gye H.J. & Nishizawa T. (2013) Assessment of the sevenband grouper Epinephelus septemfasciatus with a live nervous necrosis virus (NNV) vaccine at natural seawater temperature. *Vaccine* **31**, 2025–2027.

Pakingking J.R.R., Bautista N.B., Ayson E.G. & Reyes O. (2010) Protective immunity against viral nervous necrosis (VNN) in brown-marbled grouper (*Epinephelus fuscogutattus*) following vaccination with inactivated betanodavirus. *Fish and Shellfish Immunology* **28**, 525–533.

Parameswaran V., Rajesh Kumar S., Ishaq Ahmed V.P. & Sahul Hameed A.S. (2008) A fish nodavirus associated with mass mortality in hatchery-reared Asian Seabass, Lates calcarifer. *Aquaculture* **275**, 366–369.

Rajesh Kumar S., Parameswaran V., Ishaq Ahmed V.P., Syed Musthaq S. & Sahul Hameed A.S. (2007) Protective efficiency of DNA vaccination in Asian Seabass (*Lates calcarifer*) against *Vibrio anguillarum*. *Fish and Shellfish Immunology* **23**, 316–326.

Reed L.J. & Muench H. (1938) A simple method of estimating fifty percent end points. *American Journal in Hygiene* **27**, 493–497.

Sahul Hameed A.S., Anilkumar M., Stephen Raj M.L. & Kunthala J. (1998) Studies on the pathogencity of systemic ectodermal and mesodermal baculovirus and its detection in shrimp by immunological methods. *Aquaculture* **160**, 31–45.

Sahul Hameed A.S., Parameswaran V., Ravi Shukla Bright Singh I.S. & Thirunavukkarasu A.R. & Bhonde R.R. (2006) Establishment and characterization of India's first marine fish cell line (SISK) from the kidney of sea bass (*Lates calcarifer*). *Aquaculture* **257**, 92–103.

Sommerset I., Lorentzen E., Lorenzen N., Bleie H. & Nerland A.H.A. (2003) DNA vaccine directed against a rainbow trout rhabdovirus induces early protection against a nodavirus in challenge in turbot. *Vaccine* **21**, 4661–4667.

Sommerset I., Skern R., Biering E., Bleie H., Fiksdal I.U. & Grov S. (2005) Protection against Atlantic halibut nodavirus in turbot is induced by recombinant capsid protein vaccination but not following DNA vaccination. *Fish and Shellfish Immunology* **18**, 13–29.

Vimal S., Taju G., Nambi K.S.N., Abdul Majeed S., Sarath Babu V., Ravi M. & Sahul Hameed A.S. (2012) Synthesis and characterization of CS/TPP nanoparticles for

oral delivery of gene in fish. *Aquaculture* **358–359**, 14–22.

Vimal S., Abdul Majeed S., Taju G., Nambi K.S.N., Sundar Raj N., Madan N., Farook M.A., Rajkumar T., Gopinath D. & Sahul Hameed A.S. (2013) Chitosan tripolyphosphate (CS/TPP) nanoparticles: preparation, characterization and application for gene delivery in shrimp. *Acta Tropica* **128**(3), 486–493.

Vimal S., Madan N., Farook M.A., Nambi K.S.N., Abdul Majeed S., Rajkumar T., Venu S., Thirunavukkarasu A.R. & Sahul Hameed A.S. (2014) Production of recombinant vaccine using capsid gene of nodavirus to protect Asian sea bass, *Lates calcarifier* (Bloch, 1790). *Aquaculture* **418–419**, 148–154.

Vimal S., Abdul Majeed S., Nambi K.S.N., Madan N., Farook M.A., Venkatesan C., Taju G., Venu S., Subburaj R., Thirunavukkarasu A.R. & Sahul Hameed A.S. (2014) Delivery of DNA vaccine using Chitosan-Tripolyphosphate (CS/TPP) nanoparticles in Asian sea bass, *Lates calcarifer* (Bloch, 1790) for protection against nodavirus infection. *Aquaculture* **420–421**, 240–246.

Yuasa K., Koesharyani I., Roza D., Mori K., Katata M. & Nakai T. (2002) Immune response of humpback grouper, *Cromileptes altivelis* (Valenciennes) injected with the recombinant coat protein of betanodavirus. *Journal of Fish Diseases* **25**(1), 53–66.

Zheng F.R., Sun Q.X., Liu H.Z. & Zhang J.X. (2006) Study on the distribution and expression of a DNA vaccine against lymphocystis disease virus in Japanese flounder (*Paralichthys olivaceus*). *Aquaculture* **261**, 1128–1134.

