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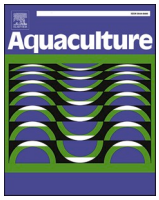

Humoral immune response in Asian seabass vaccinated with inactivated and recombinant viral nervous necrosis vaccine

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ABSTRACT

Viral nervous necrosis is a serious viral disease of marine and brackishwater finfish hatcheries. The red-spotted grouper nervous necrosis virus (RGNNV) is the most prevalent genotype among fishes in tropical and temperate regions. RGNNV was propagated in SSN-1 cell line and inactivated using BEI. The capsid protein of RGNNV was cloned and expressed in *Escherichia coli* and purified. Inactivated and recombinant VNN vaccines were produced by emulsifying the antigen with commercial adjuvant and administered to Asian seabass, *Lates calcarifer*, fingerlings and broodstock intraperitoneally. The immune response was assessed at regular intervals by indirect ELISA. The maternal antibodies in one-day post-hatch larvae obtained from the vaccinated and control broodstock were assessed by indirect ELISA. The larvae were subjected to challenge with virulent RGNNV and the relative per cent survival (RPS) was estimated. The efficacy of the vaccine was also assessed under field conditions. The recombinant vaccine produced significantly higher specific antibody levels than the inactivated vaccine in fingerlings and broodstock and the immune response was dose-dependent. Recombinant vaccine at 50 and 100 μg/fish produced significantly higher antibody levels and immune response could be observed as early as one-week post vaccination. Vaccinated broodstock produced larvae with maternal antibodies and had higher RPS when challenged at 18 days post-hatch. The vaccine produced a significant immune response even under field conditions. The vaccine can be used to vaccinate broodstock to reduce loss due to VNN in early larval stages.

1. Introduction

Viral nervous necrosis (VNN), also known as viral encephalopathy and retinopathy (VER) is a serious viral disease of many marine, brackishwater and freshwater fish species. The disease predominantly affects marine fishes, with 177 species being reported to be susceptible to the virus and epizootic outbreaks were reported in 62 of them (Bandín and Souto, 2020). VNN is caused by nervous necrosis virus (NNV), a betanodavirus belonging to the family *Nodaviridae* (Mori et al., 1992). The virus measures 25 nm in diameter with single-stranded positive sense bisegmented RNA of size 3.1 (RNA1) and 1.4 kb (RNA2) (Mori et al., 1992; Delsert et al., 1997; Nagai and Nishizawa, 1999). Like most other positive-sense RNA viruses, the nucleic acid of betanodavirus is infectious (Iwamoto et al., 2001). Four genotypes of the virus have been reported based on the T4 variable region of RNA2 viz., red-spotted grouper NNV (RGNNV), barfin flounder NNV (BFNNV), tiger puffer NNV (TPNNV) and striped jack NNV (SJNNV) (Nishizawa et al., 1997). RGNNV is the most widely prevalent genotype affecting the highest number of species in both tropical and temperate regions of Australia, Asia, Europe and the Mediterranean (Bandín and Souto, 2020). RNA1 codes for RNA-dependent RNA polymerase or protein A and a subgenomic RNA3 generated from the 3′ end of RNA1 codes for Protein B1 and B2 (Tan et al., 2001; Johansen et al., 2004; Iwamoto et al., 2005). RNA2 codes for the only structural protein of the virus, the capsid protein of size variably reported as 40–43 kDa (Mori et al., 1992; Comps et al., 1994; Chi et al., 2001; Hegde et al., 2002; Hegde et al., 2003; Gye and Nishizawa, 2016; Nallala et al., 2021). Although the deduced molecular weight of the capsid protein based on the open reading frame is 37 kDa, the protein weighs approximately 5 kDa more due to post-translational glycosylation (Chi et al., 2001). The protein also appears in trimeric form with a molecular weight of 110–120 kDa (Hegde et al., 2003; Gye and Nishizawa, 2016; Liu, 2003; Yoshimura et al., 2015) both in native virus and recombinant capsid protein expressed in bacteria and eukaryotes. The recombinant protein self-assembles into virus-like

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particles when expressed in *Escherichia coli* or in eukaryotic cells and can induce a strong immune response (Liu, 2003; Chien et al., 2018; Thie'ry et al., 2006; Moon et al., 2017; Cho et al., 2017).

Asian seabass (*Lates calcarifer*) is a carnivorous euryhaline species widely cultured in Asia Pacific region. Asian seabass is cultured in brackishwater ponds and suitable for cage culture in backwaters and open seas. VNN is an important viral disease severely hampering the hatchery production of Asian seabass seeds and also causes significant mortality in nursery and grow-out ponds. The disease is reported to be transmitted vertically and horizontally (Breuil et al., 2002; Ransangan et al., 2011). Infected broodstock shed the virus in ovarian fluids infecting the early stages of larvae (Breuil et al., 2002). Practical ways to prevent vertical transmission of the virus are to use virus-free broodstock and vaccination of brooders. Vaccination of brooders also results in the transfer of maternal antibodies to the eggs (Kai et al., 2010; Pakingking et al., 2018). The present study was undertaken to compare the immune response to recombinant and inactivated vaccines in Asian seabass, the subsequent transfer of maternal antibodies to the offspring and the protection against virulent NNV.

2. Materials and methods

2.1. Experimental animals

Asian seabass fingerlings and broodstock maintained at the fish hatchery at Muttukadu experimental station of ICAR-Central institute of brackishwater aquaculture, Chennai, India, free of VNN as tested by RT-PCR (Dalla Valle et al., 2000) were used for vaccination trials. All the animal experiments were approved by the institutional animal ethics committee (Approval No. CIBA/IAEC/2019–02). The fish were anaesthetised with 2-phenoxyethanol 500 μl L^{-1} mixed with seawater before vaccination and blood collection.

2.2. Virus and recombinant capsid protein

RGNNV isolated earlier from Asian seabass (Nallala et al., 2021) was propagated in SSN-1 cell line (RRID:CVCL_4306) at 27 °C. The infected SSN-1 cells were freeze-thawed three times, clarified at 12,000 g at 4 °C for 20 min and the supernatant was used for virus titration and preparation of inactivated VNN vaccine. The virus was purified after inactivation as described earlier by Nallala et al., (Nallala et al., 2021). Briefly, the virus was pelleted by centrifuging at 80,000*g* for 2 h at 4 °C in a swinging bucket rotor (Beckman SW 32Ti). The pellet was resuspended in tris buffer and subjected to CsCl gradient centrifugation at densities 1.4, 1.3, 1.2 and 1.1 at 153,700*g* for 16 h at 4 °C in a swinging bucket rotor (Beckman SW 32Ti). The virus containing fractions were collected and pelleted at 200,500 g for 2 h at 4 °C in a fixed-angle rotor (Beckman 100 Ti). The capsid protein gene of RGNNV was PCR amplified, cloned and expressed in *Escherichia coli* BL21 (DE3) cells and the histidine-tagged recombinant protein was purified using nickel affinity columns (Marappan et al., 2019). The purified virus and recombinant protein were characterised by Western blot with monoclonal antibodies developed against the recombinant capsid protein of RGNNV (Makesh et al., 2022).

2.3. Preparation of inactivated and recombinant vaccine

RGNNV was titrated to estimate the 50% tissue culture infectious dose ($TCID_{50}$) in SSN-1 cells as described earlier (Nallala et al., 2021). The virus was inactivated using binary ethylenimine (BEI), prepared by dissolving 2-bromoethylamine hydrobromide (Sigma-Aldrich, USA) in 0.2 N sodium hydroxide (SRL, India), at a final concentration of 3 mM for 72 h at 25 °C. After inactivation the BEI was hydrolysed by adding sodium thiosulfate in Hanks balanced salt solution (20% *w/v*) (ThermoFisher Scientific, USA) to a final concentration of 2%. The inactivation of the virus was verified by passaging the inactivated virus three times in SSN-1 cells. The antigen content of the virus was quantified by indirect sandwich ELISA (Makesh et al., 2022). The purified recombinant protein was quantified using Pierce™ Microplate BCA protein assay kit (Thermo Fisher Scientific). The vaccines were prepared by emulsifying the inactivated virus or recombinant protein with Montanide ISA 763 A VG adjuvant (Seppic, France) in the ratio of 3:7 using a high shear mixer to get a final antigen concentration of 0.2 or 0.04 mg mL^{-1} of inactivated antigen and 1, 0.5, 0.2 or 0.04 mg mL^{-1} of recombinant protein. The vaccines were stored at 4 °C until use. The sterility of the recombinant and inactivated vaccines was checked by inoculating 0.1 mL of the vaccine into 3.0 mL of nutrient broth (Himedia, India) and fluid thioglycollate medium (Himedia) and incubating at room temperature for 7 days.

2.4. Immune response to inactivated and recombinant vaccines

Six groups of 25 Asian seabass fingerlings (body weight 21 ± 2.6 g) were immunised intraperitoneally (I/P) each with adjuvant only or 4, 20 and 100 μg of recombinant protein or 4 and 20 μg of inactivated virus in 0.1 mL of vaccine. The fish were maintained in 300 L fibre-reinforced plastic (FRP) tanks with flow-through system. The water temperature ranged from 27 to 29 °C throughout the experiment. The fish were fed with pellet feed at 5% body weight in two divided rations daily. Pre-vaccination and post-vaccination blood samples were collected every week from the 3rd week onwards from the caudal vein from six fish from each group till eight weeks post-vaccination (wpv). The serum was separated from blood samples and stored at −80 °C until use. The anti-NNV serum antibody level was assessed by indirect ELISA as described earlier (Satyanarayana et al., 2021). Briefly, polystyrene 96-well plate (Nunc MaxiSorp™) was coated with 100 μL of recombinant RGNNV capsid protein (5 μg/mL) in 100 mM carbonate buffer (pH 9.6) and incubated at room temperature for 2 h. After incubation the plate was washed three times with 300 μL of wash buffer (phosphate buffered saline with 0.05% Tween 20, PBST) per well and the wells were blocked overnight with 3% skim milk powder in PBS at 4 °C. After washing the plate three times as described earlier, two-fold serially diluted serum samples starting from 1:40 were added to each well in duplicates and incubated at room temperature for 3 h. The plate was washed three times and 100 μL of anti-seabass IgM monoclonal antibody (5 μg/mL) (produced in-house) was added to each well and incubated at room temperature for 1 h. After washing the unbound antibodies 100 μL of anti-mouse HRP conjugate (1:2000; GeNei, India) was added to each well and incubated at room temperature for 1 h. The chromogenic solution (ABTS) (Sisco Research Laboratories, India) was added to each well and incubated at room temperature for 20 min. The reaction was stopped by adding 50 μL of 1% SDS (Sisco Research Laboratories) solution and optical density (OD) was measured at 405 nm. The cut-off value for the ELISA was calculated by adding the average OD_{405} and three standard deviations of the negative serum sample at 1:40 dilution.

2.5. Broodstock vaccination

The Asian seabass broodstock tagged using pit tag were maintained in a recirculatory aquaculture system with water temperature in the range of 27–29 °C and were fed with trash fish at the rate of 5% body weight at two divided doses daily. One female (5.0 kg) and two male (2.85, 2.53 kg) broodstock were vaccinated with 1 mL of recombinant VNN vaccine containing 1 mg recombinant protein I/P. Similarly, one female (3.5 kg) and two males (1.31 and 1.21 kg) were vaccinated with 1 mL of inactivated vaccine containing 1 mg NNV capsid protein I/P. Two similar booster doses were administered at two monthly intervals. Blood samples (0.2 mL) were collected from the gill arches before and two months after each vaccination. The serum was separated from blood samples and stored at −80 °C until use. The anti-NNV serum antibody titre was determined by indirect ELISA. Rest of the unvaccinated brooders served as control.

2.6. Induced breeding of Asian seabass

After the second booster dose, the vaccinated fish and unvaccinated fish were sampled by cannulation of the oviduct using a polyethylene cannula. The maturity of the female fish was assessed by the oocyte size (> 450 μm) and males by oozing of milt when the abdomen was gently pressed. The mature male and females were administered with Luteinizing Hormone Releasing Hormone analogue (LHRHa) (Sigma-Aldrich) at the rate of 35 and 70 μg kg^{-1} body weight, respectively intramuscularly, to accelerate and synchronise spawning (Kailasam et al., 2007). The fertilised eggs obtained about 36 h post-LHRHa injection were transferred to hatching tanks and the hatched-out larvae were stocked in larval rearing tanks. The larvae were maintained following standard protocols (Kailasam et al., 2007). Larval samples from vaccinated and unvaccinated broodstock were collected from three different spawning for assessing maternal antibody and for challenge studies.

2.7. Estimation of maternal antibody in larvae

One day post-hatch larvae obtained from vaccinated and unvaccinated Asian seabass broodstock were collected in sterile tubes and stored at −20 °C until used for anti-NNV maternal antibody estimation. The larvae (50 mg) were homogenised to make a 10% suspension in PBS and centrifuged at 12,000*g* for 10 min. The supernatant was collected and an indirect ELISA was performed to assess the anti-NNV antibody level.

2.8. Challenge studies of the larvae

The larvae (18 days post hatch) ($n = 120$) obtained from vaccinated and unvaccinated Asian seabass broodstock were challenged with virulent RGNNV (RGNNVLCInd2016) (Nallala et al., 2021) by immersing the larvae in seawater containing 10^5 $TCID_{50}$ mL^{-1} NNV at 28 °C for 1 h with aeration. Subsequently, the larvae were transferred to plastic tubs of 100 L capacity and maintained with continuous aeration. The larvae were fed with artemia nauplii twice daily. The larvae were observed daily and the mortality was recorded till 15 days post challenge. The moribund and freshly dead larvae were tested by RT-PCR for RGNNV (Dalla Valle et al., 2000).

2.9. Dose optimisation of recombinant vaccine

Since the recombinant vaccine gave a better immune response, to optimise the dose required for optimum immune response, Asian seabass fingerlings (Average body weight 20 g) were divided into four groups of 80 fish each. Each group was immunised intraperitoneally with 0.1 mL of recombinant vaccine containing 100, 50 and 20 μg of recombinant capsid protein. The control group was immunised with PBS emulsified with adjuvant. The fish were maintained in 1000 L FRP tanks with a flow-through system. The fish were fed with pellet feed at 5% of body weight twice daily. The water temperature was in the range of 28 ± 1 °C throughout the experimental period. Blood (0.1 mL) was collected from the caudal vein of twelve fish from each group before vaccination and every week up to 12 wpv. The anti-NNV serum antibody titre was assessed by indirect ELISA.

2.10. Assessment of the safety of vaccine

The safety of the recombinant vaccine was assessed by administering adjuvant only, 1× (100 μg) and 3× (300 μg) doses each to 12 Asian seabass fingerlings (Average body weight 25.7 g) intraperitoneally. One group was maintained as unvaccinated control. The survival was monitored for 30 days and the changes to the internal organs such as brain, liver, kidney and spleen were assessed by gross observation and histology. The intraperitoneal lesions in the vaccinated fish were scored according to Galindo-Villegas et al. (Galindo-Villegas et al., 2019). The tissues were fixed in neutral buffered formalin and processed as per standard format. Briefly, the fixed tissues were embedded in paraffin blocks and sectioned. The cut sections (5 μm thick) were mounted on glass slides and rehydrated with descending grades of ethanol. The sections were stained with Haematoxylin and eosin and observed under a microscope.

2.11. Field trials

Field trials of the recombinant vaccine were conducted at farmer's facilities. Asian seabass fingerlings (Average body weight 40 g) were injected with recombinant VNN vaccine intraperitoneally at a dose rate of 100 μg $fish^{-1}$. Two hundred vaccinated and two hundred unvaccinated fish were stocked separately at two different places in cages in brackishwater backwaters with no history of VNN. The fish were fed with pellet feed and trash fish by the fish farmers. Blood samples were collected after two months and the survival rate was estimated at the end of the 11 months culture period.

2.12. Statistical analysis

The difference in the immune response between groups vaccinated with different vaccines and doses and the maternal transfer of antibodies were analysed by one-way ANOVA. The difference in the immune response of broodstock vaccinated with recombinant and inactivated vaccines and the immune response in field trials were analysed by the t-Student test ($P < 0.05$).

3. Results

3.1. Preparation of inactivated and recombinant vaccine

The titre of RGNNV propagated in SSN-1 cells was $10^{8.8}$ $TCID_{50}$ mL^{-1}. BEI at a concentration of 3 mM for 72 h could completely inactivate the virus as no CPE was observed even after three passages of the inactivated virus. Monoclonal antibodies developed against RGNNV reacted specifically with the virus capsid protein and recombinant capsid protein at 40 and 45 kDa, respectively (Fig. 1). The recombinant protein also exhibited a specific band at 125 kDa corresponding to the trimeric form of the capsid protein.

A. SDS-PAGE of purified Nervous necrosis virus (NNV), Lane 1- Protein marker, Lanes 2 and 3- Purified inactivated RGNNV; B. SDS-PAGE of recombinant NNV capsid protein; 1- Protein marker, 2- Purified recombinant NNV capsid protein; C. Western blot of purified NNV against anti-NNV monoclonal antibodies, 1- Purified inactivated NNV, 2-Protein marker; D. Western blot of recombinant NNV capsid protein against anti-NNV monoclonal antibodies, 1- Purified recombinant NNV capsid protein, 2. Protein marker.

3.2. Immune response to inactivated and recombinant vaccines

The recombinant vaccine produced a better immune response than the inactivated vaccine. The immune response to inactivated and recombinant vaccines was dose-dependent, with higher the antigen dose, the better the immune response. Recombinant vaccine at 100 μg gave the highest immune response, followed by 20 μg of recombinant protein. The immune response of inactivated vaccine at both the doses and recombinant vaccine at 4 μg per fish was not significantly different from the control fish. The immune response peaked between 4 and 6 wpv and declined slightly by 7 wpv before increasing by 8 wpv while the antibody level in control fish remained below the cut-off value throughout (Fig. 2).

3.3. Broodstock vaccination

Vaccination of broodstocks with inactivated and recombinant vaccines resulted in significantly higher ($p < 0.05$) immune response in

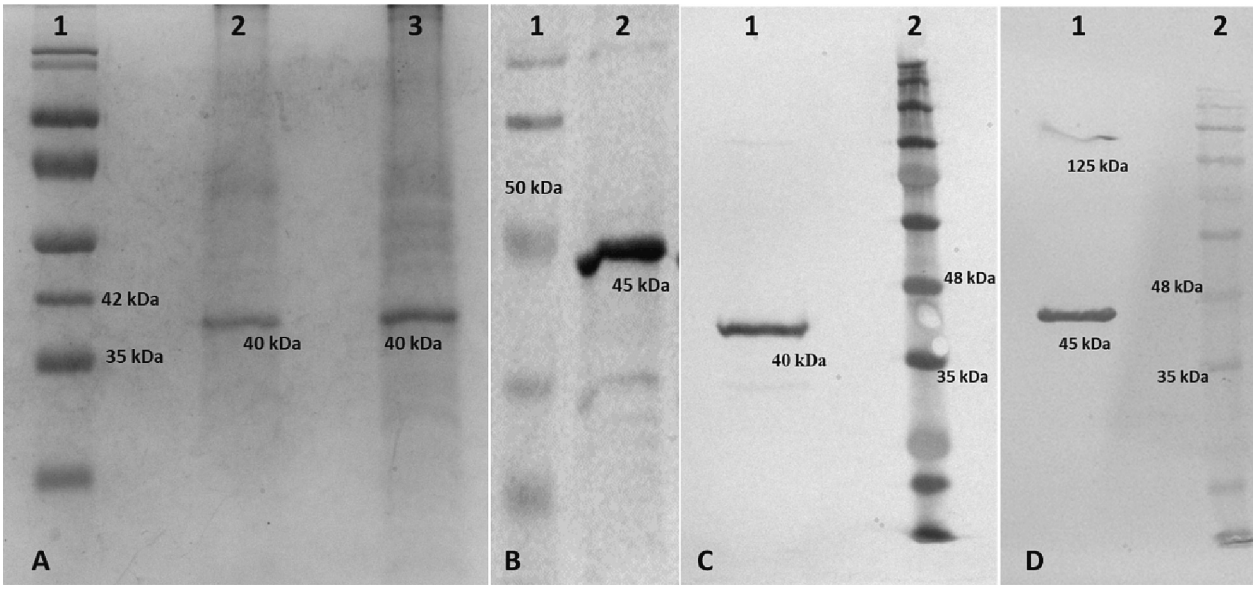

Fig. 1. SDS-PAGE and Western blot for purified NNV and recombinant capsid protein.

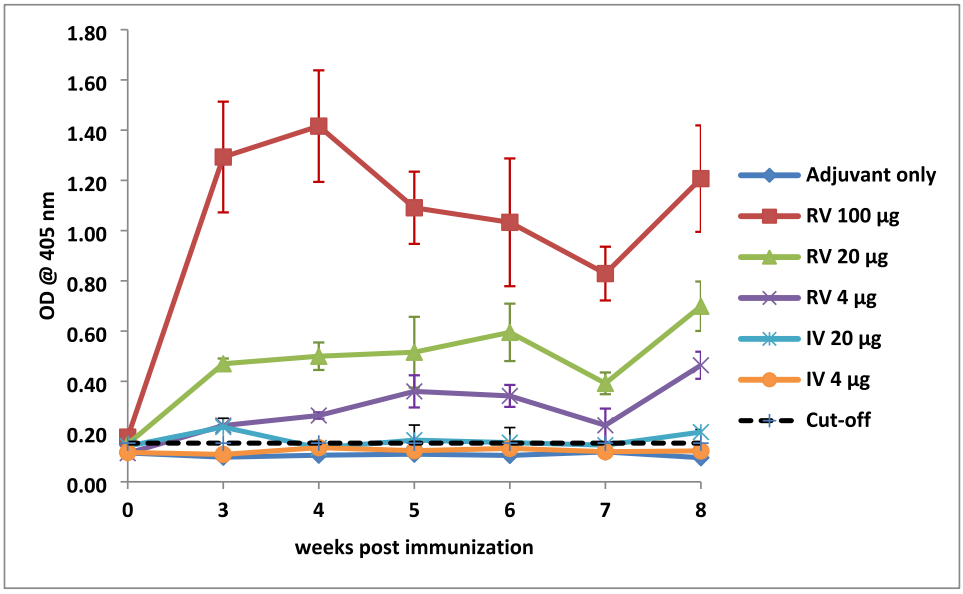

Fig. 2. Immune response of Asian seabass fingerlings vaccinated with different doses of recombinant and inactivated VNN vaccine assessed by indirect ELISA (OD_{405}). RV- Recombinant vaccine, IV- Inactivated vaccine. The graph represents the mean OD_{405} values of 6 fish in duplicate. The error bar represents the standard error for the mean of 6 samples.

recombinant protein vaccinated group compared to the inactivated virus vaccinated group (Fig. 3). Further, the pre-vaccination antibody levels influenced the immune response to vaccination. Higher pre-vaccination antibody levels resulted in a lower immune response to vaccination, while fishes with low antibody levels before vaccination produced higher antibody levels post-vaccination. The unvaccinated control broodstock did not show any significant increase in antibody levels during the study.

3.4. Estimation of maternal antibody in larvae

The maternal antibody level was significantly higher in the one day old larvae obtained from recombinant protein vaccinated brooders followed by larvae from brooders vaccinated with the inactivated vaccine (Fig. 4). However, by 3 days post hatch, no maternal antibody could be observed by indirect ELISA in the larvae obtained from vaccinated brooders.

3.5. Challenge studies of the larvae

The average relative per cent survival of 3 batches of larvae obtained from seabass broodstock vaccinated with the recombinant vaccine, at the end of 15 days was 32, while that of inactivated vaccine was 11.8 when challenged at 18 dph (Fig. 5). Moribund larvae were positive for NNV by RT-PCR.

3.6. Dose optimisation of recombinant vaccine

Out of the three different doses of recombinant vaccine assessed, 50 and 100 µg vaccinated groups resulted in significantly better immune

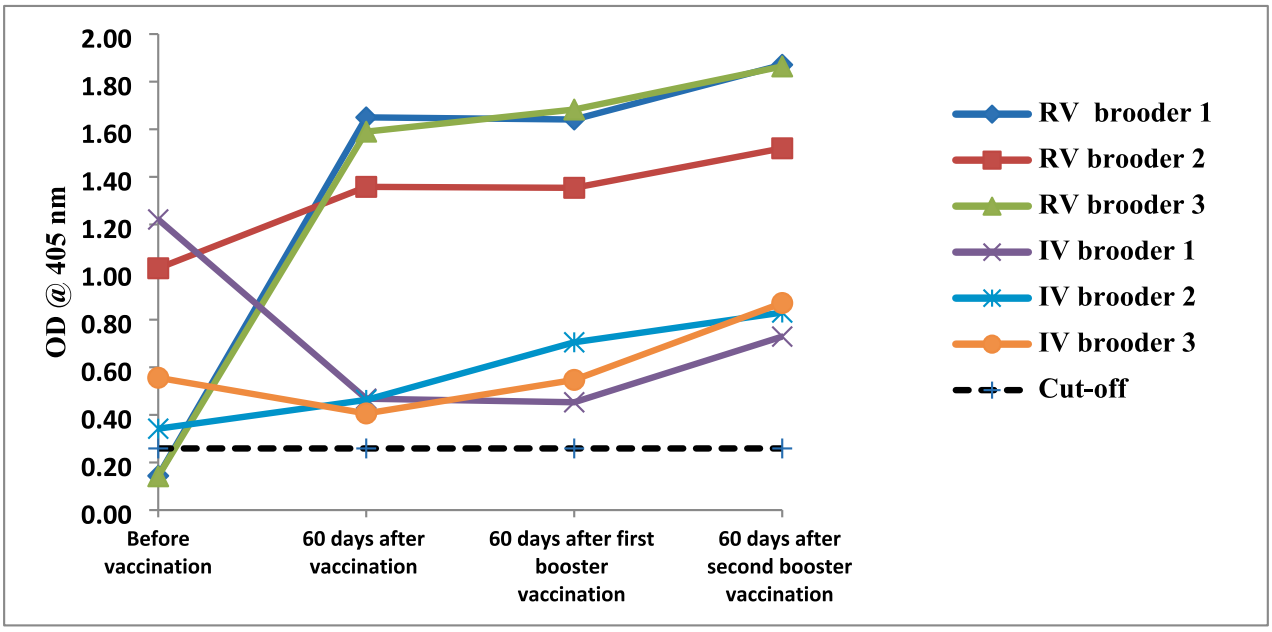

Fig. 3. Immune response of Asian seabass broodstock vaccinated with recombinant and inactivated VNN vaccine assessed by indirect ELISA (OD_{405}). RV- Recombinant vaccine, IV- Inactivated vaccine. Recombinant vaccine produced significantly (t-Student test ($P < 0.05$)) higher immune response than inactivated vaccine at all time points post-vaccination.

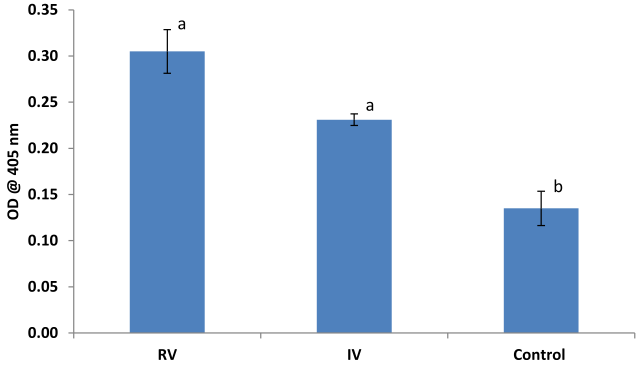

Fig. 4. Antibody level in one day post-hatch Asian seabass larvae obtained from vaccinated and control broodstock tested by indirect ELISA (OD_{405}). The bar represents the mean OD_{405} values of three samples in duplicate. The error bar represents the standard deviation for the mean of 3 samples. RV- Recombinant vaccine, IV- Inactivated vaccine. Bars with different alphabets differ significantly ($p < 0.05$)

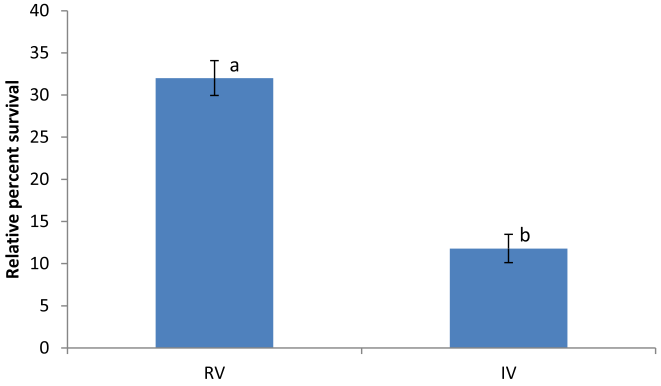

Fig. 5. Relative percent survival of Asian seabass larvae obtained from brooders vaccinated with recombinant VNN vaccine (RV) and inactivated VNN vaccine (IV). The graph represents average RPS of three different challenge experiments. The error bar represents the standard error for the mean of 3 samples. Bars with different alphabets differ significantly ($p < 0.05$).

response (Fig. 6). The immune response could be observed as early as one wpv at higher doses and the peak immune response was observed between 4 and 9 wpv. The antibody levels declined between 7 and 9 wpv before increasing again after 10 wpv while the control fish antibody level remained below the cut-off value.

3.7. Assessment of safety of the vaccines

The recombinant vaccine was safe at 1× and 3× doses as no mortality were observed up to 30 days post-vaccination. Post-mortem examination of the vaccinated fingerlings revealed mild adhesions and remnants of vaccine in the abdominal cavity at the site of injection (Fig. 7). A score of 1 was given to the intraperitoneal lesions in the vaccinated fish (Galindo-Villegas et al., 2019). However, no histopathological changes were observed in 1× and 3× groups (Fig. 8).

3.8. Field trials

The water temperature in the field trial at both the locations ranged from 26 to 28 °C. The water salinity at trial 1 ranged from 17 to 28 ppt and at trial 2 the salinity ranged from 26 to 30 ppt. Field trial of recombinant protein vaccine resulted in significant immune response at 2 months post-vaccination at both the trials compared to the control group although the magnitude of immune response was lower than in laboratory trials (Fig. 9). No VNN outbreak was reported during the culture period. The survival of vaccinated fish in trial 1 was 76% and 78% for the vaccinated and unvaccinated stocks, respectively while in trial 2 the survival was 81% for both the stocks. The vaccinated fish did not show any vaccine residues or adhesions in the peritoneal cavity at the end of the culture period.

4. Discussion

Inactivated VNN vaccine can be prepared by inactivating NNV using

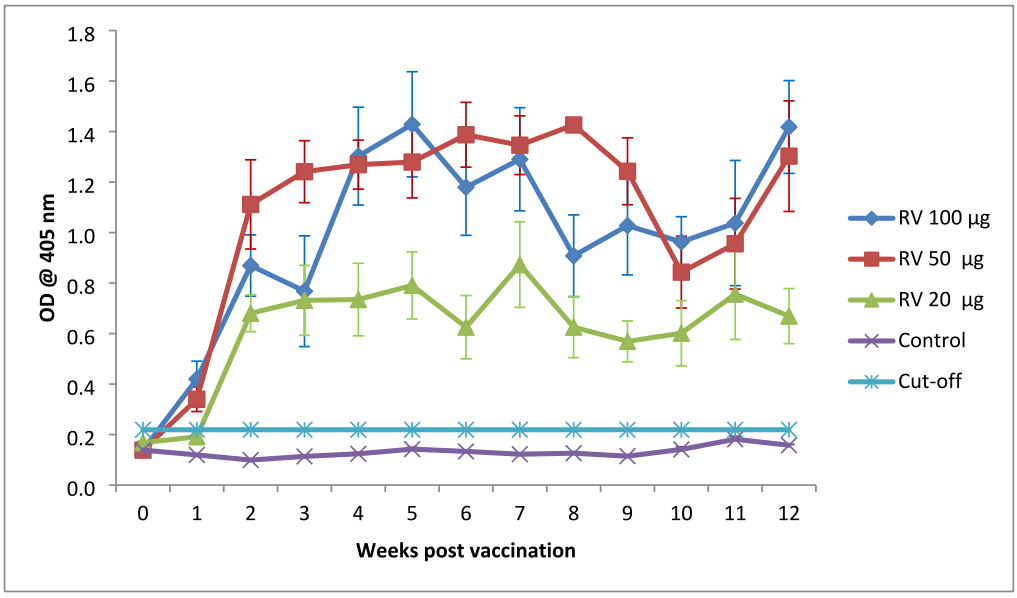

Fig. 6. Serum antibody level of Asian seabass fingerlings vaccinated with recombinant VNN vaccine at three different doses assessed by indirect ELISA (OD_{405}). The graph represents the mean OD_{405} values of 12 fish in duplicate. The error bar represents the standard error for the mean of 12 samples. RV- Recombinant vaccine.

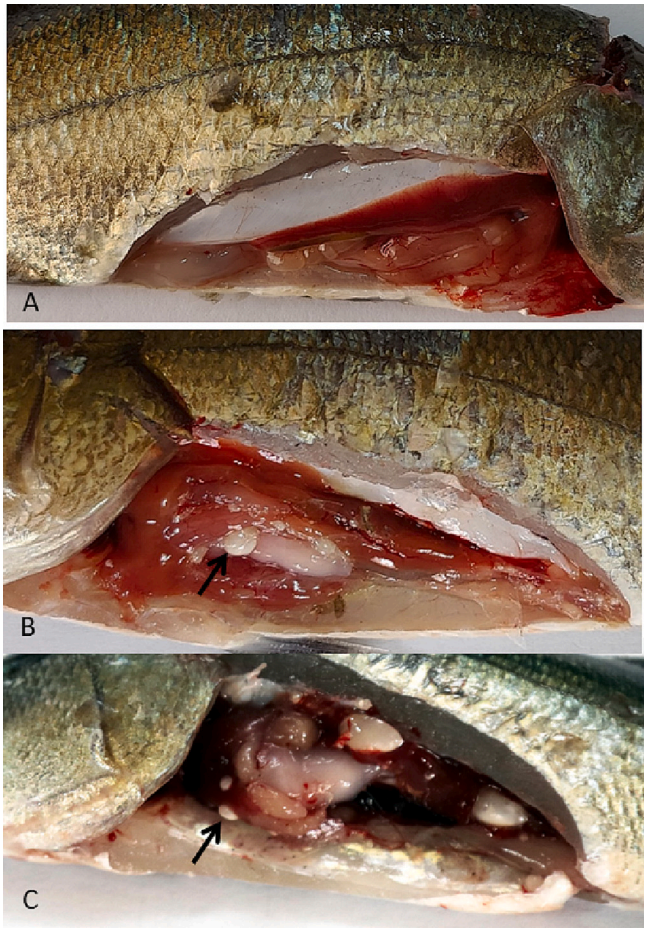

Fig. 7. Post mortem examination of the Asian seabass fingerlings vaccinated with recombinant VNN vaccine revealed remnants of vaccine (arrow) and mild adhesions at the site of injection. A: Unvaccinated control fish; B: 4 weeks post vaccination; C: 8 weeks post vaccination.

UV (Valero et al., 2018), formalin (Kai and Chi, 2008; Pakingking Jr et al., 2009; Pakingking et al., 2010) and BEI (Kai et al., 2010; Kai and Chi, 2008). BEI-inactivated vaccine is reported to induce better protection (79–95 RPS) than formalin-inactivated vaccine (39–43 RPS) (Kai and Chi, 2008). BEI inactivation is a linear reaction throughout the loss of nearly all measurable infectivity with little loss of antigenicity and immunogenicity (Cunliffe, 1973). Hence, BEI was used for inactivation of NNV for vaccine production. The bacterial-expressed recombinant protein formed trimers during self-assembly into virus-like particles. Thus, the recombinant vaccine consisted of purified virus-like particles devoid of infectious nucleic acid.

The antigen dose range selected for optimisation was based on previous reports for recombinant protein vaccines where 50 µg/fish could provide 76% RPS (Vimal et al., 2014). An inactivated vaccine containing 100 µg of antigen was not prepared since this concentration could not be obtained even when undiluted inactivated cell culture supernatant was used for vaccine preparation. The immune response of recombinant vaccine was significantly higher than the inactivated vaccine in Asian seabass fingerlings and broodstock, although the same quantity of antigen was administered. This may be because the inactivated vaccine contained 10% fetal bovine serum and cellular proteins in addition to the viral antigen. These proteins might have competed with the viral antigen for immune response resulting in antigen competition. Reduced immune response to multivalent vaccine due to antigen competition (Thu Lan et al., 2021) or antigen inhibition (Nikoskelainen et al., 2007) has been reported in fish. Hence, the immune response to the viral antigen in the inactivated vaccine was comparatively less than the recombinant vaccine where purified antigen was administered. The use of purified inactivated virus for vaccine production might have resulted in an equivalent immune response. However, it is laborious and expensive to propagate NNV in cell culture system and purify them. Therefore, the recombinant vaccine, which is relatively easier to produce and purify, is ideal for vaccine production. Further, the recombinant protein self-assembled into virus like particles, thus mimicking purified viral particles devoid of infectious nucleic acid resulting in a better immune response. In addition, the risk of improper inactivation is avoided when recombinant protein is used for vaccine preparation. The specific antibody levels in some of the broodstock were higher even before vaccination probably due to the previous exposure to the virus. High natural antibodies prior to immunization results in reduced or no immune

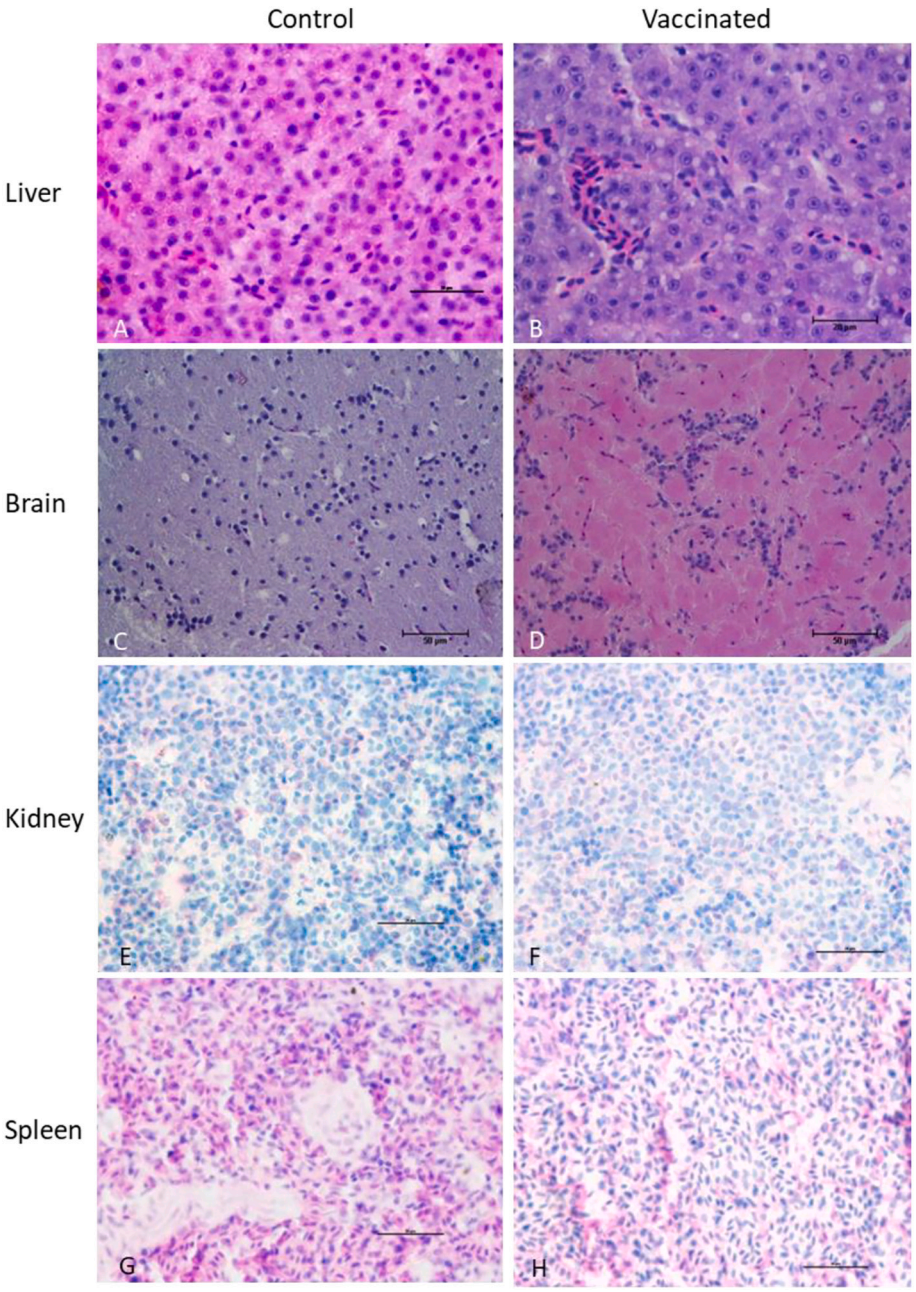

Fig. 8. Histological sections of liver, brain, kidney and spleen of control and recombinant VNN vaccinated Asian seabass fingerlings. A, C, E, G- liver, brain, kidney and spleen sections of control fish respectively; B, D, F, H- liver, brain, kidney and spleen sections of fish vaccinated with 3× recombinant vaccine respectively.

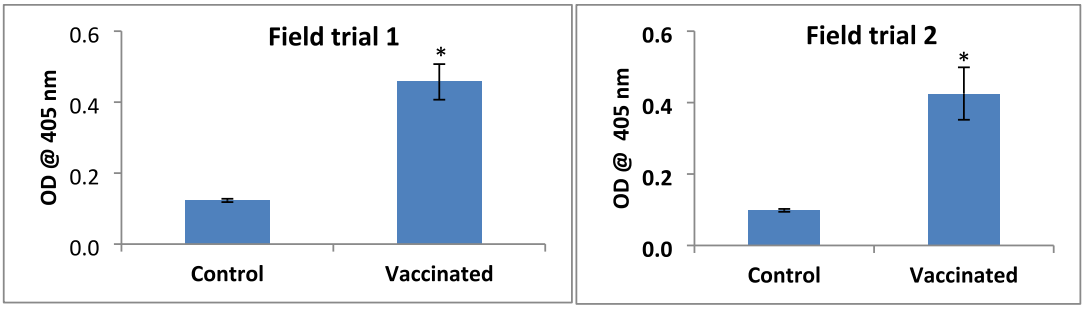

Fig. 9. Serum antibody levels of control and vaccinated Asian seabass fingerlings assessed by indirect ELISA (OD_{405}). The bar represents the mean OD_{405} values of 6 samples in duplicate. The error bar represents the standard error for the mean of 6 samples. Asterisks denote statistically significant differences between control and vaccinated groups according to the t-Student test ($P < 0.05$).

response (Sinyakov et al., 2002).

The specific antibody level started to decline after 5 weeks and increased after 7 weeks, especially in groups vaccinated with 20 μg or more antigen although this increase was not observed in control fish thus, ruling out any possible natural infection with NNV. This was also observed in the recombinant protein dose optimisation experiment, where the antibody levels declined after 5–8 weeks and increased after 10 weeks, while there was no increase in antibody levels in the control group. This phenomenon could not be explained and has to be studied further. Challenge experiment involving vaccinated and control fish at 8 wpv with virulent RGNNV by intramuscular injection did not produce mortality in any of the groups (data not shown) since L. *calcarifer* is resistant to RGNNV above 5 weeks of age (Jaramillo et al., 2017).

The presence of NNV-specific antibodies in one dph larvae revealed the maternal transfer of antibodies. Since only one set of brooders were used for each vaccine, more trials needs to be carried out to study the effect of individual differences on the transfer of maternal antibodies to the larvae. The antibodies developed against the RGNNV isolate have been reported earlier to have virus neutralising properties (Nallala et al., 2021). Larvae obtained from broodstock vaccinated with recombinant vaccine had higher antibody levels since broodstock vaccinated with recombinant vaccine had higher serum antibody titre. The maternal antibodies could not be detected by 3 dph by indirect ELISA. However, the larvae obtained from vaccinated broodstocks had higher RPS when challenged at 18 dph, indicating that anti-NNV antibodies may still be present, although at undetectable levels, offering partial protection. Vaccination of brooders also prevents vertical transmission of the virus as the eggs obtained from vaccinated grouper (*Epinephelus tukula*) were reported to be free of NNV, while the eggs from unvaccinated brooders were positive for NNV by nested RT-PCR (Kai et al., 2010).

Dose optimisation experiment was done to study whether antigen levels between 20 and 100 μg could provide a better immune response. The study indicated that recombinant protein at 50 μg could give an immune response equivalent to 100 μg; hence, 50 μg dose is sufficient for the rationalisation of antigen used. Liu et al., (Liu et al., 2006) reported that 100 and 250 μg of NNV VLPs produced 13% higher antibody levels than 10 μg in grouper *Epinephelus lanceolatus*. However, 50 μg dose was not studied. Thie'ry et al., (Thie'ry et al., 2006) reported dose-dependent immune response to betanodavirus in European seabass with specific antibody levels increasing with increasing doses from 0.1 μg to 20 μg. However, in the present study, it is observed that the immune response peaks at a dose of 50 μg per fish, beyond which there is no significant increase in antibody level. The recombinant vaccine produced a significant immune response even at field conditions, although the magnitude of immune response was lower than in experimental conditions probably because the fish might have been exposed to other antigens/pathogens, which might have resulted in antigen competition and reduced immune response.

In conclusion, purified recombinant protein vaccine could produce a significant immune response in Asian seabass broodstock and fingerlings. A 50 μg/fish dose is optimum for inducing a significant immune response. Vaccination of broodstock with recombinant VNN vaccine results in the transfer of maternal antibodies to the larvae and offers partial protection during the early larval stages.

CRediT authorship contribution statement

Following are the contributions made by authors of the manuscript submitted.

Makesh M: Experimental design, RGNNV propagation, Vaccine development, Immunization, Blood collection, ELISA, Manuscript preparation.

Venkata Satyanarayana Nallala: Bench work of vaccine production, Immunization, blood collection, ELISA, Challenge studies, Manuscript editing.

K. Muddukrishnaiah: Immunization, Blood collection, Fish maintenance.

Sujeet Kumar: Safety testing of vaccine, Histology.

G. Thiagarajan: Broodstock and fingerling maintenance, larval rearing, Blood collection.

Ashok Kumar Jangam: Statistical analysis, Manuscript editing.

R. Subburaj: Induced breeding, Broodstock and fingerling maintenance, larval rearing.

M. Kailasam: Guidance, Broodstock maintenance, sample collection, Manuscript editing.

Vijayan K.K.: Supervision, Funding acquisition, Manuscript editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2023.739384.

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