Non-lethal screening of Asian seabass (*Lates calcarifer*) by monoclonal antibody based indirect enzyme linked immunosorbent assay for viral nervous necrosis

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**A B S T R A C T**

Viral nervous necrosis (VNN) is a serious viral disease of several species of farmed and wild fishes. Adult fish are asymptomatic and become carriers of the virus when infected with nervous necrosis virus (VNN) and they transmit the virus to the offspring through eggs. ELISA is ideal for non-lethal screening of adult fish for VNN. Asian seabass (*Lates calcarifer*) IgM was purified using Protein A affinity column and hybridoma clones secreting monoclonal antibodies (MAB) specific to the heavy chain of IgM was developed. An Indirect ELISA using anti-sea bass IgM MAB was developed by optimizing all the reagents. The assay was used to screen adult Asian seabass from grow-out farms in comparison to RT-PCR. The assay was also used to assess the immune response in Asian seabass immunized with inactivated Red-spotted grouper NNV (RGNNV). Seabass IgM on SDS-PAGE analysis revealed three heavy chain bands of size 96, 82 and 76 kDa and a single light chain of size 25 kDa. Out of 18 positive hybridoma clones, two selected clones reacted specifically with the 76 kDa heavy chain band. Out of 28 serum samples of Asian seabass from grow-out farms 26 were positive for NNV antibodies while 22 were positive by RT-PCR. Fish immunized with inactivated RGNNV showed immune response by one week post-immunization, and the peak immune response was observed four weeks post-immunization. The assay developed can be used for non-lethal screening of adult Asian seabass for VNN and to assess the immune response after vaccination.

1. Introduction

Viral nervous necrosis (VNN) also called as viral encephalopathy and retinopathy is a serious viral disease of fish affecting more than 120 species of farmed and wild fishes [1]. The disease is caused by nervous necrosis virus (NNV) belonging to the genus *Betanodavirus* under the family *Nodaviridae*. The virus is transmitted both horizontally and vertically. Disease outbreaks are more common in larval and early juveniles before they become fully immunocompetent [1]. In adult fish the infection is usually asymptomatic and the affected fish becomes a carrier of the virus transmitting it vertically through eggs and ovarian fluids to the offspring [2]. Hence, it is important to use virus free adults for breeding. VNN can be diagnosed by the detection of nucleic acid or antigen in the predilection organs such as brain, eyes and spinal cord. Since non-lethal sampling from these organs is not possible, broodstocks can be screened by detecting NNV from ovarian biopsy, blood, gills and caudal fin clippings by reverse transcription polymerase chain reaction (RT-PCR) [3,4]. However, detection of the virus was inconsistent due to low viral load in these tissues [3,4]. Immune response to betanodavirus can be detected as early as five days post infection [5] and antibodies can be detected up to one year following natural infection [6]. Hence, antibody detection coupled with antigen detection is the ideal way to screen adult fishes for VNN.

Serum neutralization test (SNT) and enzyme linked immunosorbent assay (ELISA) are ideal methods for antibody detection and quantification of serum antibodies in fish. SNT is laborious and is often used in the absence of anti-species antibodies. ELISA is the test of choice for species for which anti-fish antibodies are available. ELISA is also an important assay for serological survey to assess the prevalence of a disease in a particular geographical location. The assay can also be used to assess the efficacy of vaccines. Non-lethal sampling method, low cost and persistence of antibodies makes this an ideal test especially for valuable broodstock [7].

There are many formats of ELISA reported for antibody detection against NNV in several species [4,5,8–17] (Table 1). In spite of this, antibody detection is not considered as a routine screening method for VNN due to insufficient research on antibody detection methods [18].

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Concentration of each analyte such as antigen, test serum dilution, conjugate and substrate used in ELISA needs to be optimized for better reproducibility of the assay [19]. Most of the ELISA reported for antibody detection against NNV did not specify the concentration of antigen or the secondary antibody. ELISA for fish antibodies has the inherent problem of non-specific binding of the antibodies to blocking reagents and antigen [8,20]. Use of anti-species polyclonal antibodies adds to the non-specific binding. Use of monoclonal antibody (MAB) reduces non-specific bindings to a great extent. However, anti-species MABs are commercially available only for a few species. The commercially available Asian seabass (Lates calcarifer) IgM MAB (Aquatic Diagnostic Ltd, Scotland, UK) at different dilutions did not give optical density (OD) higher than the background OD of the plate [17]. Hence, the study was aimed to develop hybridoma clones secreting MABs against heavy chain of Asian seabass IgM and optimize all the reagents of ELISA to reduce the background OD. The ELISA developed can be used for non-lethal screening of VNN and to assess the immune response to VNN vaccines in Asian seabass.

### 2. Materials and methods

#### 2.1. Virus propagation, inactivation and production of recombinant capsid protein

Red-spotted grouper nervous necrosis virus (RGNNV) (RGNNV-Lclnd2016) isolated in this laboratory, was propagated in SSN-1 cells (RRID: CVCL_4306). The 50% tissue culture infective dose (TCID\(_{50}\)) was calculated using SSN-1 cells in 96 well tissue culture plate [21]. The virus was inactivated using 3 mM binary ethylenimine (BEI) by constant slow stirring at 25 °C for 48 h [22]. Complete inactivation of the virus was confirmed by inoculating the inactivated virus onto SSN-1 cells and observing no cytotoxic effect even after 3 passages. The capsid protein gene of RGNNV was RT-PCR amplified, cloned into pET-28b(+) vector and the recombinant protein was expressed in *Escherichia coli* BL21 (DE3) cells as described earlier [23]. The purified recombinant capsid protein was used to coat ELISA plates.
2.2. Purification and characterisation of Asian seabass IgM

Three adult Asian seabass (weighing 3.4 ± 0.45 Kg) maintained at fish hatchery of Muttukadu experimental station of ICAR-Central Institute of Brackishwater Aquaculture, Chennai, India was used for blood collection. All invasive procedures involving fish were carried out with the approval of the institute animal ethics committee of Central Institute of Brackishwater Aquaculture, Chennai (Approval number: C IRA/IAEC/2019-02). The fish were anaesthetized by adding 2-phenoxyethanol at a dose of 200 μl 1−1 of seawater to the holding tank and blood was collected from the gill arches. The blood was allowed to clot and the serum was separated. The serum was clarified at 5000 × g for 10 min and the supernatant was used to purify IgM using HitrapTM Protein A HP column (GE Healthcare, Sweden) following the manufacturer’s protocol. The purified IgM was quantified using PierceTM BCA Protein Assay Kit (Thermo Fisher Scientific, USA). The molecular weight of the heavy chain and light chain of IgM was estimated by SDS-PAGE analysis [24]. Briefly, the IgM was boiled in 4X sample loading buffer (VWR Life sciences, India) for 5 min and electrophoresed on a polyacrylamide gel consisting of 5% stacking gel and 12% separating gel. The gel was stained with Coomassie brilliant blue R-250 and destained. The gel was observed under Molecular imager® Gel DocTM XR+ system (Bio-Rad, India) and the molecular weight of the heavy chain and light chain was estimated using Image Lab Software 5.1 (Bio-Rad, India).

2.3. Development and characterization of MAb against Asian seabass IgM

All animal procedures for hybridoma development in this study were approved by Institutional Animal Ethics Committee (IAEC) of Vellore Institute of Technology, Vellore, India (VIT/IAEC/14/Nov/5/29). Four weeks old BALB/c mice (n=2) were immunized subcutaneously with approximately 50 μg of purified Asian seabass IgM per mouse, emulsified in Freund’s complete adjuvant (Sigma-Aldrich, USA). Two booster doses emulsified in Freund’s incomplete adjuvant (Sigma-Aldrich, USA) were administered similarly at three weeks interval. Blood collected from orbital plexus seven days after the second booster dose was used to determine anti-Asian seabass IgM antibody titer by indirect ELISA [25]. The mice with highest serum antibody titer was given a final booster dose (100 μg) intraperitoneally. Four days later, the spleen cells were isolated and fused with Sp2/0-Ag14 myeloma cells (ATCC CRL-1581) and plated on 96 well tissue culture plate containing feeder cells in DMEM supplemented with 1X HAT (Thermo Fisher Scientific, USA). Hybridoma clones were screened for secretion of anti-Asian seabass IgM antibodies by indirect ELISA [25] and positive hybridoma cells showing high OD405 values were cloned twice by limiting dilution and then cryopreserved. MAbs were purified from hybridoma culture supernatants using MAb trap kit (GE Healthcare, Sweden) following the manufacturer’s protocol. The isotype of MAb was determined by IsoStrip Mouse Monoclonal Antibody Isotyping Kit (Sigma-Aldrich, USA). The specificity of the MAb was tested by Western blot against purified Asian seabass IgM. Mabs were also tested against serum from milkfish (Chanos chanos), tilapia (Oreochromis mossambicus), pearlsnout (Euphus suratensis) and Mangrove red snapper (Lutjanus argentimaculatus) by Western blot for possible cross-reaction. Sera of these fish were diluted 1:150 in PBS and electrophoresed on 12% SDS-PAGE gel, transferred to PVDF membrane and blotted against two selected Mabs. MAb (1E12) showing high specific reactivity with Asian seabass IgM heavy chain was selected for optimizing ELISA for anti-NNV antibody detection.

2.4. Collection of samples

Seventy eight serum samples collected from the caudal vein of Asian seabass fingerlings (36±3.6 g) raised in fish hatchery (28±1 °C), free of VNN as tested by RT-PCR, were used as negative serum samples. Positive serum samples were obtained from ten adult Asian seabass (3.4 ± 0.45 kg) immunized intraperitoneally each with one ml of inactivated RGNV (108 TCID50 ml−1) emulsified in Montanide™ ISA 763 A VG (Seppic, France). Blood samples were collected from the gill arches one month after the immunization. Test serum samples (n=28) from adult Asian seabass (weighing 4.5 ± 0.85 kg) from two grow out farms located at coastal areas near Chennai, were collected from the gill arches for screening for antibodies against NNV. Ovarian biopsy samples were also collected from the corresponding fish using a cut down cannula in RNAlater (Sigma-Aldrich, USA) for RT-PCR for NNV. Test serum samples were also collected from Asian seabass fingerlings immunized with inactivated RGNV. Asian seabass fingerlings (36±3.6 g; n=30) raised in fish hatchery (28±1 °C) free of VNN as tested by RT-PCR, were immunized intraperitoneally each with 0.1 ml of inactivated RGNV (108 TCID50 ml−1), emulsified with Montanide™ ISA 763 A VG (Seppic, France). Control fish (n=30) were immunized with adjuvant only. Blood samples (0.1 ml) (n=8) were collected from the immunized and control groups before and every week post immunization up to 8 weeks.

2.5. Indirect ELISA

The ideal concentration of antigen (recombinant NNV capsid protein), secondary antibody (anti-Asian seabass IgM mouse MAb) and conjugate (anti-mouse HRP conjugate) (Genei laboratories Pvt Ltd, India) required for indirect ELISA were determined by checker-board titration. Recombinant capsid protein of RGNV (5 μg ml−1) suspended in 100 mM carbonate buffer (pH 9.6) was used to coat 96-well polystyrene plates (Nunc MaxiSorp™) at 100 μl well−1 and incubated for 2 h at room temperature (RT). The plates were washed three times with 300 μl well−1 of wash buffer (Phosphate buffered saline containing 0.05% v/v Tween 20, PBST). The wells were blocked with 3% (w/v) skim milk powder in PBS (SM-PBS) overnight at 4 °C. After washing three times with wash buffer, serum samples diluted in PBS were added in two-fold serial dilutions starting from 1:20 in each well in duplicate and incubated at RT for 3 h. The plates were washed three times and 100 μl of purified anti-Asian seabass IgM MAb (5 μg ml−1) in 0.3% SM-PBS were added to each well and incubated at RT for 1 h. After the subsequent wash step, 100 μl of anti-mouse HRP conjugate diluted 1:2000 in 0.3% SM-PBS was added to each well and incubated at RT for 1 h. After the final wash, 100 μl of thechromogenic solution containing ABTS (15 mg ml−1) and 10 μl of 30% (w/w) Hydrogen peroxide in citrate buffer, pH 4.2 was added to each well and incubated at RT for 20 min. The reaction was stopped with 50 μl of 1% SDS solution well−1 and OD was measured at 405 nm in a microplate reader (Tecan, Switzerland). Blank, positive control and negative control were included in all the assay plates. The cut-off value of the indirect ELISA was determined as the average OD405 at 1:40 dilution + 3 standard deviations (SD) of the negative samples. Test samples showing OD405 values above cut-off at 1:40 dilution were considered positive for NNV antibodies.

2.6. RT-PCR

Total RNA was extracted from the ovarian biopsy samples collected in RNAlater using TRIzol reagent (Termo Fisher Scientific, USA) following the manufacturer’s protocol. One microgram of RNA was reverse transcribed into cDNA using iScript™ cDNA synthesis kit (Bio-Rad, India). Nested RT-PCR for NNV was carried out using gene-specific primers [3] as described earlier [23]. The PCR products were electrophoresed on 1.5% (w/v) agarose gel and stained with ethidium bromide. The gel was analyzed in a gel documentation system (Bio-Rad, India).

2.7. Statistical analysis

ELISA OD405 values were given as the average of the duplicate samples. The OD405 values between the control and immunized groups was analyzed by one way ANOVA and p < 0.05 was considered statistically significant.
3. Results

3.1. Purification and characterization of Asian seabass IgM

Purification of immunoglobulin from adult Asian seabass serum using protein A column yielded 0.9 mg of IgM ml\(^{-1}\) of serum. On SDS-PAGE analysis the immunoglobulin yielded three heavy chain bands of molecular weight of approximately 96, 82 and 76 kDa and a single light chain of molecular weight 25 kDa respectively (Fig. 1).

3.2. Development and characterization of MAbs against Asian seabass IgM

The average indirect ELISA OD\(_{405}\) values of immunized mice were 2.023 and 1.916 at 1:100 dilutions while the control mouse serum OD\(_{405}\) value was 0.069. A total of 18 positive hybridoma clones were obtained upon screening the cell culture supernatant of hybridoma clones by ELISA. Two clones (2B6 and 1E12) which produced high OD\(_{405}\) in indirect ELISA were selected and subcloned twice to ensure monoclonality. Both the clones reacted with the 76 kDa heavy chain of Asian seabass IgM (Fig. 2). The heavy chain isotype of the MAbs were 2B6: IgG\(_1\) and 1E12: IgG\(_{2a}\) and the light chain isotype of both the MAbs were kappa. The MAbs did not react with IgM of any of the other fish species tested.

3.3. Indirect ELISA and RT-PCR

The negative samples gave an average OD\(_{405}\) of 0.132 ± 0.02819 at 1:40 dilution. The cut-off value of the ELISA was determined as average OD\(_{405} + 3\, SD = 0.217\). The positive serum samples obtained from immunized adult produced an OD\(_{405}\) of 1.191 ± 0.143 at 1:40 dilution by indirect ELISA. Twenty six out of 28 test serum samples collected from grow-out farms tested positive for NNV antibodies by indirect ELISA. Out of the 28 samples six samples tested negative and the rest of the samples tested positive for NNV by nested RT-PCR (Fig. 3; Table 2). Test serum samples from Asian seabass fingerlings immunized with inactivated RGNNV showed immune response from one week post-immunization although significant immune response (\(p < 0.05\)) could be observed only by two weeks post-immunization. The immune response peaked at four weeks and decreased thereafter. The control serum showed OD\(_{405}\) below the cut-off value throughout the experimental period (Fig. 4).

4. Discussion

IgM is the predominant immunoglobulin isotype present in serum of teleosts and is expressed as a tetramer [26]. Each monomer consists of
Table 2
Comparison of anti-NNV antibody detection by indirect ELISA and NNV detection by RT-PCR.

| Sample | Indirect ELISA (OD) | NNV-specific RT-PCR | NNV-specific nested RT-PCR |
|--------|---------------------|---------------------|----------------------------|
| 1      | + (0.557 ± 0.021)   | −                   | −                          |
| 2      | + (0.613 ± 0.018)   | −                   | −                          |
| 3      | + (0.504 ± 0.028)   | −                   | −                          |
| 4      | + (0.502 ± 0.012)   | −                   | −                          |
| 5      | + (0.370 ± 0.025)   | +                   | +                          |
| 6      | + (0.683 ± 0.011)   | +                   | +                          |
| 7      | + (0.304 ± 0.013)   | +                   | +                          |
| 8      | + (0.699 ± 0.016)   | −                   | −                          |
| 9      | + (0.602 ± 0.019)   | −                   | −                          |
| 10     | + (0.469 ± 0.011)   | +                   | +                          |
| 11     | + (0.552 ± 0.014)   | −                   | −                          |
| 12     | + (0.403 ± 0.010)   | +                   | +                          |
| 13     | + (0.634 ± 0.013)   | +                   | +                          |
| 14     | + (0.741 ± 0.018)   | −                   | −                          |
| 15     | + (0.679 ± 0.015)   | +                   | +                          |
| 16     | + (0.905 ± 0.021)   | −                   | −                          |
| 17     | + (0.653 ± 0.019)   | −                   | −                          |
| 18     | + (0.699 ± 0.018)   | −                   | −                          |
| 19     | + (0.901 ± 0.028)   | +                   | +                          |
| 20     | + (0.656 ± 0.034)   | −                   | −                          |
| 21     | + (0.828 ± 0.020)   | +                   | +                          |
| 22     | + (0.660 ± 0.025)   | +                   | +                          |
| 23     | − (0.144 ± 0.018)   | −                   | −                          |
| 24     | + (0.439 ± 0.028)   | +                   | +                          |
| 25     | + (0.342 ± 0.030)   | +                   | +                          |
| 26     | + (0.556 ± 0.025)   | −                   | −                          |
| 27     | + (0.307 ± 0.027)   | +                   | +                          |
| 28     | − (0.142 ± 0.012)   | −                   | −                          |

+ = Positive; − = Negative; Average indirect ELISA OD_{405} ± SD values of Asian seabass serum at 1:40 dilution are given in bracket. OD values above 0.217 (Average negative sample OD + 3 SD) were considered as ELISA positive.

Fig. 4. Anti-NNV serum antibody levels (OD_{405} at 1:40 dilution of serum) in control and immunized Asian seabass fingerlings (n=8) at different time points. The bar represents mean OD values of 8 samples in duplicate. Error bar represents standard deviation for the mean of 8 samples.

Two heavy chains and two light chains and the size of the heavy and light chains vary with species. The size of heavy chain and light chain of Asian seabass was previously reported as 86 and 24 kDa by Crosbie and Nowak [27] and as 83 and 27 kDa by Choudhury and Pani Prasad [28] respectively. However, in the present study, three types of heavy chain measuring approximately 96, 82 and 76 kDa were observed in the IgM purified using protein A affinity column. The SDS-PAGE image of the purified IgM obtained was similar to that reported earlier for Asian seabass by Crosbie and Nowak [27]. The MAbs developed against purified IgM reacted with the 76 kDa band only. The band was confirmed as IgM heavy chain by LC-MS/MS analysis. Although reports of more than one IgM isotype in teleosts are rare, two types of IgM differing in net charge which can be separated by anion exchange chromatography was reported in Atlantic salmon (Salmo salar) [29–32]. The other two bands (96 and 82 kDa) obtained in the present study needs to be characterized further to ascertain whether more than one isotype of IgM exists in Asian seabass. IgM concentration in serum of teleost fish is reported to be between 800 and 9000 μg ml⁻¹ [33]. The serum IgM concentration of salmon, halibut, haddock and cod were ≤ 1, 4, 7 and 11.5 mg ml⁻¹ of serum of adult fish [34]. The serum IgM concentration of adult Asian seabass measured was within this range (0.9 mg ml⁻¹). The serum IgM concentration depends on the age, size, sex, season, and environment in addition to the infection and vaccination status [31]. The serum collected for IgM purification was from naive fish with no history of vaccination.

Development of MAbs against IgM of several fish species has been reported and MAbs against a few species are available commercially. Although the MAb against Asian seabass is available commercially, it did not work in ELISA format for quantifying the serum antibody levels in Asian seabass [17]. We had a similar experience when using the commercially available Asian seabass IgM MAb. In the present work hybridoma clones secreting MAbs against heavy chain of IgM of Asian seabass were developed which could be used to quantify specific antibodies in serum.

Coating of ELISA plate with recombinant capsid protein of NNV is a better option since the use of NNV infected cell culture supernatant will inhibit binding of NNV to the substrate [16] and purification of NNV from cell culture supernatants is laborious [11]. Use of carbonate buffer (pH 9.6) to coat NNV antigen to plates was reported to lower the OD values significantly due to the possible denaturation of the surface projection of NNV [35]. However, in our study, there was no reduction in the OD_{405} values when carbonate buffer (pH 9.6) was used instead of PBS to coat recombinant coat protein of NNV. Although different antigen concentration and dilutions were reported for coating ELISA plate, checker board titrations revealed that 500 ng well⁻¹ to be optimum in terms of sensitivity and reagent economy. Blocking the plate with 3%
skim milk overnight resulted in low background OD405 compared to 1 h blocking at RT. Further, 1:40 dilution of test serum was found optimum as it provided a good balance between sensitivity and low background OD. The duration and temperature of each incubation step was also optimized by incubating the plates at different temperature and duration. Earlier reports on ELISA for anti-NNV antibody detection used different dilutions of secondary antibody. Unless the secondary antibody is commercially available, it is always better to quantify the concentration of the antibody used for better reproducibility and to avoid batch to batch variations. In our study, a concentration of 500 ng well-1 of anti-Atlantic seabass IgM MAb was found to be optimum. Use of MAb's as secondary antibodies reduces non-specific binding of secondary antibodies with other analytes. Further, dilution of secondary antibodies and conjugate in PBS containing 0.3% skim milk reduced non-specific binding and reduced background OD. The study describes an indirect ELISA with all the reagent concentrations and steps optimized for low background OD and better sensitivity.

The assay can be used to screen adult Asian seabass for the presence of antibodies against NNV. The assay correlated with virus detection by nested RT-PCR. A good correlation between ELISA and PCR was reported earlier [17]. All the fish except two tested positive for NNV antibodies while six samples tested negative by nested RT-PCR indicating that PCR alone would be insufficient to screen fish for NNV probably due to low viral load in the sample. Brain, retina and spinal cord being the predilection organs [36,37], samples from these organs are ideal for screening. Since non-lethal sample collection from these organs is not possible, samples like ovarian biopsy, blood, gills and caudal fin clips are usually used for screening [4]. However, these samples are not ideal for screening since the viral load in these organs are very low [3,4] and hence it can be coupled with antibody screening to assess the status of exposure of fish to NNV.

The assay can also be used to quantify the immune response of Asian seabass to vaccination against NNV. The antibody response can be detected as early as one week post immunization and reached a peak by four weeks post immunization. Similar results were also reported in European seabass (Dicentrarchus labrax) [5] where the immune response to betanodavirus was detected as early as five days post-infection and a peak immune response was reported in Australian bass (Macquaria novaculaeata) [17] at four weeks post-immunization.

Declaration of Competing Interest

None.

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Supplementary materials

Supplementary material associated with this article can be found in the online version, at doi:10.1016/j.fisrep.2021.100011.

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