

Genetic diversity and latency status of betanodavirus in wild seeds of Asian seabass *Lates calcarifer* (Bloch) sampled along Indian coasts

Husne Banu¹, Sabyasachi Pattanayak², Jitendra Kumar Sundaray², & P.K. Sahoo^{2*}

¹ICAR-Central Institute of Fisheries Education, Versova, Mumbai, India

²ICAR-Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar, India

*[E-mail: pksahoo1@hotmail.com]

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Piscine nodavirus is the causative agent of viral encephalopathy and retinopathy in marine and freshwater fish worldwide. The present investigation was carried out to know the persistence and diversity of betanodavirus in wild healthy seabass seeds around east and west coasts of India from three major landing sites. The seeds obtained from two coasts were detected positive by nested PCR using RNA2 coat protein gene-specific primers. Phylogenetic analysis revealed all the three Indian coast sample sequences as close to Indian strain of betanodavirus reported earlier and those belong to red-spotted grouper nervous necrosis virus (RGNNV). These results indicate a strong latency or persistency of betanodavirus among wild healthy seeds of Asian seabass available along Indian coasts that remain as a source of viral inoculum for spreading disease in farm reared fish.

[Keywords: Nervous necrosis virus (NNV); Latent infection; *Lates calcarifer*; India; Wild seeds]

Introduction

Asian seabass (*Lates calcarifer*) is a high demand species in aquaculture due to its impressive growth rate (350 - 3000 g within six months to two years) and is consistently having high market value¹. Viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER) is a common viral disease associated with high mortality of seabass during larval rearing or intensive cage culture practices all over the world. It is caused by betanodavirus that belongs to family *Nodaviridae* and carries positive sense two segments of single strand RNA. The RNA1 (3.1 kb) and RNA2 (1.4 kb) encode for viral RNA-dependent RNA polymerase and coat protein, respectively^{2,3}. It has four genotypes, namely, striped jack nervous necrosis virus (SJNNV), tiger puffer nervous necrosis virus (TPNNV), barfin flounder nervous necrosis virus (BFNNV), and red spotted grouper nervous necrosis virus (RGNNV). Later in 2004, one more genetic group was included from turbot (TNV)⁴. However, another group isolated from Atlantic Cod (ACNNV) was included under BFNNV⁵.

The virus affects both marine and freshwater fish of over 120 farmed and wild species of 30 families⁶. Among these, sea bass, groupers, parrotfish, striped jack, tiger puffer and flatfish are the most affected species globally⁷. Hick et al.⁸ reported 100% mortality in 12 day-old cultured *L. calcarifer* batch during

cross-sectional survey of six consecutive batches (2007-2008). The source of infection was found to be horizontal transmission of virus from sub-clinically infected cohort of juvenile barramundi. After a certain age (26 days post-hatch), survivors of infection persistently carry the virus for a long period without any clinical signs of disease. Several studies also designate that apparently healthy wild species from different regions act as carrier for this virus^{9,10} and can potentially transmit the virus to other fishes.

Azad et al.¹¹ reported betanodavirus infection for the first time in India in hatchery-produced Asian sea bass larvae. Recently, Rajan et al.¹² detected betanodavirus in subclinically infected fish samples collected from farms/wild habitat of Chennai and Kakdwip area in India with a newly designed nested primer set. Apart from food fishes, this virus has also been detected in ornamental fish in India^{13,14,15,16}. Although hatchery seed production is well established for seabass, farmers are mostly dependent on wild collection of fry, fingerlings and brooders in some parts of Asia. Wild seeds are asymptomatic carriers for many diseases and sanitary status of specific Indian fish population is still obscured. The increasing viral trade between wild and hatchery/domestic fish is one of the major reasons for frequent disease outbreaks. While stocking the wild collected seeds in culture ponds, the incidence of this disease increases that leads to severe economic loss.

The present investigation was undertaken to assess the persistence of betanodavirus infection in the seeds collected from wild from three major landing centers of east (Odisha and West Bengal states) and west (Maharashtra state) coasts of India. The study further aimed at knowing any recent genotype of betanodavirus circulating along Indian coast.

Materials and Methods

Sample collection

One hundred samples (up to size ~2 cm length) of apparently healthy and live seabass fry were collected from estuarine areas of east (West Bengal and Odisha) and west (Maharashtra) coasts of India during May 2016 to June 2016 and transported to the laboratory in oxygenated polythene bags. The sampling locations are near to estuarine areas of the rivers Muri Ganga (Latitude: 21° 52' 50.1"N, Longitude: 88° 9' 53.6"E), Budhabalanga (Latitude: 21° 24' 09.5"N, Longitude 86° 55' 37.4"E) and Amba (Latitude 18° 47' 28.4"N, Longitude 72° 58' 46.7"E) of West Bengal, Odisha and Maharashtra states, respectively. The collected samples were euthanized with overdose of anaesthesia (MS222, Sigma-Aldrich Co., USA) and the head parts were removed and preserved aseptically. Half of the head portion along with half of the brain was stored in RNAlater (Sigma, Life Science, USA) and rest half was preserved in 10% neutral buffered formalin for histological study using haematoxylin and eosin stain.

RNA extraction

The head part stored in RNAlater was processed further for RNA extraction after pooling 20 fishes of each location into four pooled samples (five fish/pool). Total RNA was extracted using TRI reagent (Sigma, St. Louis, MO, USA) following the manufacturer's instructions. The resulting RNA was treated with DNase I, RNase-free (Fermentas, Thermo Fisher Scientific, Wilmington, DE, USA) followed by inactivation of DNase I according to the manufacturer's instructions. The concentration of the total RNA in the sample was quantified by measuring absorbance at 260 nm. The purity of the samples was also checked by measuring the ratio of OD260 nm and OD280 nm using NanoDrop ND1000 (Thermo Scientific) with expected obtained values between 1.8 and 2.0.

Polymerase chain reaction amplification

The primers used in this study for screening of betanodavirus were based on RNA2 sequence of four genotypes (i.e., BNV-UR1 and BNV-UF1 for reverse

transcriptase (RT)-PCR and BNV-UR2, BNVUF2 for nested PCR) following Gomez *et al.*¹⁰ (2004). The resultant amplicon sizes of 570 and 420 bp were produced in first and second step respectively. Thermo Scientific RevertAid First Strand cDNA synthesis kit (Thermo Fischer Scientific Inc., USA) was used following the manufacturer's instruction to generate complimentary DNA from one microgram of total RNA by reverse transcription. Briefly, template RNA (1 µg) was added to random hexamer primer (1 µl) and nuclease free water to make the volume to 12 µl. Subsequently, 5× reaction buffer (4 µl), Ribolock RNase inhibitor (1 µl), 10 mM dNTP mix (2 µl) and RevertAid M-MuLV RT (200 U/µl) (1 µl) were added to make the final volume of the reaction mixture to 20 µl. The mixture was incubated at 25 °C for 5 min followed by 60 min at 42 °C. The reaction was terminated by heating at 70 °C for 5 min. The reverse transcription reaction product was stored at -20 °C for further use.

The first and second step PCR were performed with 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 20 s and extension at 72 °C for 60 s. The amplification was confirmed by analyzing the PCR product in 1% agarose gel electrophoresis. The pRSET-A vector with RNA2 gene was used as positive control, and the sample without template was used as negative control for both PCR.

Gene sequencing and phylogenetic study

The amplified products were sequenced commercially in triplicate per sample of each sampling site. Single nucleotide-nucleotide alignment was performed using the Basic Local Alignment Search Tool (BLAST) of NCBI (<http://www.ncbi.nlm.nih.gov/blast>) to find out the homology. The sequences from three location samples were deposited in the NCBI GenBank database. Multiple alignment was performed with MEGA5¹⁷ by using ClustalW algorithm¹⁸. Phylogenetic analysis of RNA2 sequences was performed through neighbour-joining method available in MEGA5 with Kimura 2-parameter distance model and reliability of groupings was evaluated by using the bootstrap test with 1000 replications.

Results and Discussion

Polymerase chain reaction

Most of the *L. calcarifer* tissue pools from each sampling location were detected positive for betanodavirus. Out of four pools of 20 wild fish

samples, collected from Kolkata Muriganga Estuary area, two pools were found to be positive in both first step and nested PCR (Figs 1a and 1b). In case of the samples obtained from Odisha and Maharashtra coasts, all pools were found to be positive only in nested PCR (Figs 1c and 1d).

Histopathology

The histoarchitecture of wild seabass seed samples was found to be normal (Fig. 2). There was no vacuolative degeneration in retina of eye or in the brain. An increase in melanomacrophage centres was only noticed in head kidney tissues.

Sequence analysis

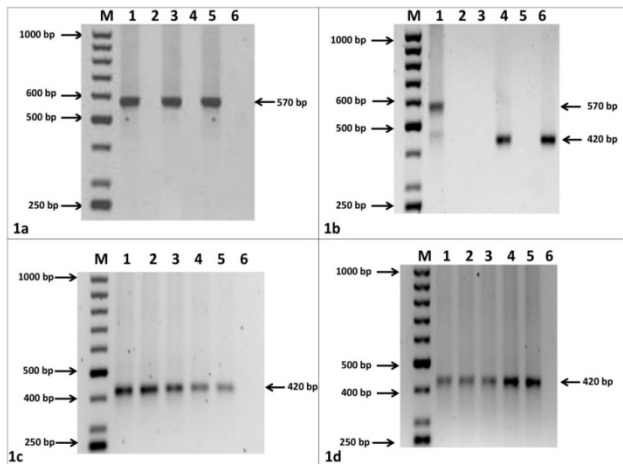
The partial RNA2 gene sequences of three NNV strains were deposited under the Accession No. MG575046, MG575047 and MG575048 for Odisha, West Bengal and Maharashtra samples, respectively in the NCBI GenBank. The nucleotide sequence similarity by National Centre for Biotechnology Information (NCBI) BLAST program (<http://www.ncbi.nlm.nih.gov/>) revealed that Kolkata, Odisha and Maharashtra strains have high similarity with RGNNV coat protein gene sequences having accession numbers AY744705.1 and KC696562.1. The multiple alignments with available sequences in the GenBank of

NCBI representing four betanodavirus genotypes revealed that all three sequences in this study were homologous with RGNNV genotype.

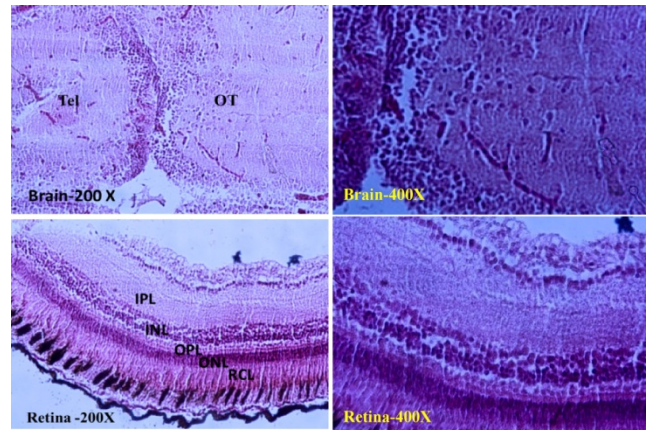
The sequence similarity between different genotypes is shown in Figure 3. All three sequences obtained in this study from three different locations of the Indian coast were 100% similar with each other.

However, the percentage similarity of Kolkata, Maharashtra and Odisha strains with Indian *L. calcarifer* NNV (FR669249.1, GU953669.1, GU826692.1, JF412273.1, JF412272.1) strains detected earlier was higher (98.2-99.4%) in comparison to TPNNV (74.5%), BFNNV (78.6%) and SJNNV (74.5%).

The deduced amino acid sequences from 167 to 246 of RNA2 gene were aligned. There was a marked variation at position corresponding to aa 245 and 208 among the current three derived sequences and few other reported Indian RGNNV strains. At 245 position, methionine (M) was replaced with leucine (L) and at 208 tyrosine (Y) was replaced with cysteine (C) in all three sequences obtained in this study. Apart from



Figs 1a-1d — [Fig. 1a- Agarose gel electrophoresis of RNA2 first step PCR amplification for West Bengal sample, Lane 1; Positive control, Lane 2; Negative control, Lanes 3-6; West Bengal pooled sample, Lane M; Marker-50 bp]; [Fig. 1b-RNA2 nested PCR amplification for West Bengal sample, Lane 1; Positive control, Lane 2; Negative control, Lanes 3-6; West Bengal pooled sample, Lane M; Marker-50 bp]; [Fig. 1c- RNA2 nested PCR amplification for Maharashtra. Lanes 1-4; pooled samples, Lane 5; Positive control, Lane 6; Negative control; Lane M; Marker-50 bp]; [Fig. 1d- RNA2 nested PCR amplification for Odisha samples. Lanes 1-4; pooled samples, Lane 5; Positive control, Lane 6; Negative control; Lane M; Marker-50 bp].



Tel- telencephalon; OT-optic tectum; IPL-inner plexiform layer; INL-inner nucleated layer; OPL-outer plexiform layer; ONL-outer nucleated layer; RCL-layers of rods and cones

Fig. 2 — H & E stained section of seabass whole head portion. The tissue was preserved in 10% neutral buffer formalin and 5 µm thick sections were stained with hematoxylin and eosin.

MG575047	MG575046	MG575048	FR669249	GU953669	GU826692	JF412273	JF412272	D38636.1	D38637.1	EU826138	D30814.1
MG575047	100	100	99.4	98.5	99.1	98.2	98.5	98.8	74.5	78.6	74.5
MG575048		100	99.4	98.5	99.1	98.2	98.5	98.8	74.5	78.6	74.5
MG575046			99.4	98.5	99.1	98.2	98.5	98.8	74.5	78.6	74.5
FR669249 :RJNNV INDIA				99.1	99.7	98.8	99.1	99.4	74.5	79.2	74.5
GU953669 :RJNNV INDIA					98.8	99.7	100	98.5	74.8	78.9	74.5
GU826692 :RJNNV INDIA						98.5	98.8	99.1	74.8	78.9	74.8
JF412273 :RJNNV INDIA							99.7	98.2	74.5	78.6	74.2
JF412272 :RJNNV INDIA								98.5	74.8	78.9	74.5
D38636.1 RJNNV									73.9	78.6	74.5
D38637.1 TPNNV										77.1	80.6
EU826138. BFNNV											73
D30814.1 SJNNV											

Fig. 3 — Identity matrix obtained by using Jotun Hein Method of MegaAlign package. The digits in upper right angle depict nucleotide percentage similarity between RNA2 sequences of NNV strains and except Kolkata, Maharashtra and Odisha all other sequences are retrieved from NCBI.

these changes, at positions 224 and 226, amino acids varied among Indian strains from lysine (K) to arginine (R) and from proline (P) to glutamine (Q), respectively (Fig. 4).

The Figure 5 includes RNA2 sequences from different betanodavirus genotypes reported worldwide and further Indian strains of betanodavirus formed a single clade along with other RGNNV strains. The black beetle virus RNA2 was included as an outlier.

The first report of piscine nodavirus in India was from Chennai where 15-21 day post-hatchlings of *L. calcarifer* in hatchery suffered from heavy mortality (60-90%)¹¹. Further, several reports of betanodavirus outbreak from farms as well as wild environment along the coastline of southern India were reported^{12,19,20,21,22}. In 2014, Banerjee and his co-workers reported a massive outbreak of betanodavirus

in seabass juveniles in cage culture from south-west India (Kerala state)²³. In most of the culture practices, brood stocks or larvae are being collected from wild population and hence, the chances of virus entry into culture system become easy. There was no earlier report on presence of this virus in wild seeds collected from Odisha, Kolkata and Maharashtra coasts which are the major collection centres of wild seeds for culture. Hence, the present study was undertaken to know the presence of betanodavirus contamination along east and west coasts of India in wild *L. calcarifer* larvae; and also the sequences obtained were analyzed to know the evolved variations in the present circulated strains/genotypes.

Gomez *et al.*¹⁰ (2004) used nested PCR primers for detection of piscine nodavirus in 131 apparently healthy fish. The same method was also used to screen wild apparently healthy marine fish and invertebrates collected in southern Korea^{24,25}. These studies indicated that betanodavirus could persist in wide range of marine fishes and invertebrates which can act as asymptomatic carriers of disease. The present study could detect persistence of virus in maximum tissue pools of apparently healthy *L. calcarifer* seeds and possibly those could act as a source of inoculum for horizontally spreading the virus in culture systems. There are number of studies concerning vertical transmission of betanodavirus through infected ovarian fluid, sperm and fertilized eggs⁶. Valero *et al.*²⁶ (2015) demonstrated presence and replication of NNV in testis of both European seabass and Gilthead seabream. In the present study, wild juveniles of maximum two months old were detected positive for virus, which signifies that vertical pathway possibly is the major route for viral transmission in wild.

Total of 10 tissue pools were detected positive in nested PCR, whereas Kolkata samples were also found to be positive even in first step RT-PCR. The low level of virus titer in asymptomatic wild seeds was very difficult to be detected in nPCR unless the template concentration increased. Even the isolated virus from carrier fish was difficult to confirm by cell culture method²⁷. Further, the histoarchitecture of seabass seeds was almost normal. As per the previous reports of NNV infection in seabass, the brain and retinae show numerous vacuolative degeneration and necrosis¹¹. However, there were no such changes in neuron cells as well as in the kidney of wild collected samples. It indicated the latency or subclinical status

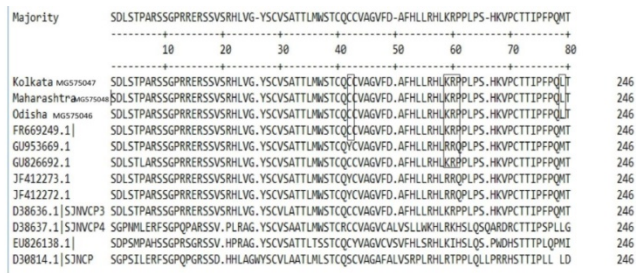


Fig. 4 — Amino acid alignment of RNA2 sequences (a.a 167-246) of all Indian NNV strains including Kolkata, Maharashtra and Odisha. The aa variations are outlined. The alignment result is obtained from Mega5 software.

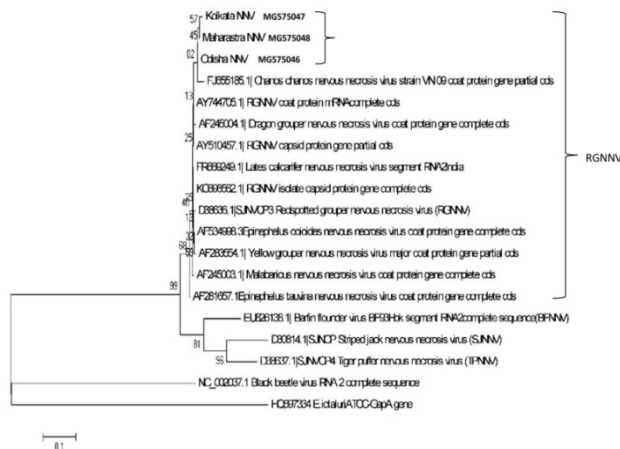


Fig. 5 — Phylogenetic analysis of RNA2 coat protein gene sequences of four NNV genotypes including three new sequences. It is constructed through Kimura 2 Parameter distance model and neighbour-joining method available in Mega 5. The reliability of phylogenetic groupings was evaluated using the bootstrap test with 1000 bootstrap replications.

of virus in seeds and probably the disease flares up when other predisposing factors aggravate the situation in culture systems. It had also been previously speculated that betanodavirus may 'hide' in a subclinical state in broodstock until some mechanism activates its expression during maturation and spawning²⁸.

The detected virus RNA2 gene sequences (499-838) which partly includes highly variable region i.e. 666-842 bp was compared with other strains of NNV. The multiple alignment showed an absence of six bases in previously detected sequences of RGNNV and BFNNV²⁹. The identity matrix revealed that all sequences have more than 90% similarity with previously reported Indian betanodavirus which are from south India region. The amino acid alignment revealed minor substitutions within Indian NNV sequences at 245, 226, 224 and 208 positions. Only the position 245 comes under 232–255 amino acid region (nt 695–765), which is one of the major putative host-specificity determinants³⁰.

According to earlier report of Panzarin et al.³¹ (2012), maximum amino acid substitution of RGNNV group falls within the C-terminal region (aa 238–340). Again the amino acid residues at 217–256 and aa 257–341 determine major antigenic diversity in RGNNV and SJNNV, respectively³². However, beyond 246 positions there was no marked change in amino acid sequences of viral RNA2 gene from India. Panzarin et al.³¹ (2012) reported that the virus tends to form strong clusters according to geographical regions and to their farming status (wild or farmed). Our phylogeny based analysis corroborated the previous report and three new sequences formed a single cluster with Indian strains of betanodavirus. According to John et al.³³ (2014), an Indian strain of virus (LCNNV-In01, FR669249) belongs to RGNNV genotype and similar findings were obtained in this study.

Conclusion

This study suggested persistence of RGNNV virus in wild *L. calcarifer* seeds along the east and west Indian coasts. The absence of closeness of culture farm to the sampling area indicated possibility of vertical transmission of virus along the coastline and suggested the importance of seed quarantine before putting into culture systems.

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