

Isolation and characterization of a nodavirus associated with mass mortality in Asian seabass (*Lates calcarifer*) from the west coast of India

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Abstract A fish nodavirus was detected in the juveniles of Asian seabass (*Lates calcarifer*) during a massive outbreak in the seabass cage culture farm located in the south west coast of India. The clinical signs of the disease included anorexia, inflated abdomen, exophthalmia, darkening of the whole body, erratic swimming and cork-screw type movement followed by death. The dead and the moribund fish were analyzed for nodavirus by reverse transcriptase-polymerase chain reaction (RT-PCR) using specific primers targeting the T4 region of RNA2 coat protein gene. This is the first report of nodavirus infection in the fresh water cage-reared seabass fish in the west coast of India. The piscine nodavirus was detected in the brain, retina and kidney of all the fishes examined. The PCR products were cloned and sequenced. The sequence analysis showed more than 90 % homology with the other coat protein gene sequence of piscine nodaviruses from other countries. The phylogenetic analysis based on the partial nucleotide sequence of RNA2 coat protein gene revealed that the virus belonged to the red-spotted grouper nervous necrosis virus, which is one of the widely distributed genotype among the other four known genotypes of piscine nodavirus.

Keywords Nodavirus · *Lates calcarifer* · Reverse transcription PCR (RT-PCR) · Viral nervous necrosis (VNN) · Coat protein gene (RNA2)

Introduction

The marine aquacultured fish production has increased rapidly during the last few years due to their high market demand and economic value. However, their rapid expansion and intensification has led to the several disease outbreaks. Among the infectious diseases, viral diseases are the most serious and cause severe losses to the sector. Many viral diseases of fish have been reported worldwide [7, 14, 21]. Piscine nodavirus causing viral nervous necrosis (VNN) has been associated with high mortalities in larvae and juveniles of fish species which are of economic importance to the aquaculture industry in Europe, Asia, Japan and Australia [20]. Viral nervous necrosis is one of the most serious viral disease problem in fish such as Mediterranean seabass (*Dicentrarchus labax*), Australian seabass (*L. calcarifer*), Turbot (*Scophthalmus maximus*), Halibut (*Hippoglossus hippoglossus*), Grouper (*Epinephelus malabaricus*), Mullet (*Mugil cephalus*), Striped jack (*Pseudocaranx dentex*), Tilapia (*Oreochromis mossambicus*) and Guppy (*Poecilia reticulata*) [1, 6, 9, 10, 18, 20, 24].

Lates calcarifer (Bloch), commonly known as Asian seabass is a euryhaline member of the family Centropomidae and is widely cultured throughout the world. Asian seabass is a potential fish candidate for farming in India, because of the fast growth rate, tolerance to a range of environmental conditions and its demand in the domestic and export markets [15]. The fish nodavirus association with mass mortality in Asian seabass (*L. calcarifer*) has

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been reported recently in India and has become a cause of concern as culture of marine fish is still in its infancy [2, 15].

Fish nodavirus, a non-enveloped, positive-strand RNA virus belongs to the genus *Betanodavirus*, family *Nodaviridae* and is the causative agent of VNN, also known as the viral encephalopathy and retinopathy (VER) and fish encephalitis. The genome of fish nodavirus is bipartite, comprising two molecules of single stranded, non-polyadenylated, positive polarity RNA1 (3.1 kb encoding the RNA-dependent RNA polymerase) and RNA2 (1.4 kb encoding the coat protein) protein precursor [20, 23]. The *Betanodavirus* has been classified based on the phylogenetic analysis of the coat protein gene sequence into four genotype clusters comprising of striped jack nervous necrosis virus (SJNNV), barfin flounder nervous necrosis virus (BFNNV), tiger puffer nervous necrosis virus (TPNNV) and red-spotted grouper nervous necrosis virus (RGNNV) [13, 22].

Several diagnostic methods have been developed for detection of piscine nodavirus which include immunohistochemistry [8], enzyme-linked immunosorbent assay (ELISA) [1], in situ hybridization [5] and capture ELISA [17]. However reverse transcriptase-polymerase chain reaction (RT-PCR) has emerged as a powerful, rapid and sensitive method for the detection of VNN [12, 16]. The T4 coat protein gene (RNA2) has been used as a target for the detection of nodavirus in SJNNV by RT-PCR [12, 15]. In 2002, fish nodavirus associated with mass mortality of Asian seabass (*L. calcarifer*) was detected by employing the RT-PCR methods [16].

In the present study, the fish nodavirus associated with mass mortality in Asian seabass (*L. calcarifer*) was detected from the west coast of India using RT-PCR. Further the phylogenetic analysis based on the partial RNA2 coat protein encoding nucleotide sequence was performed to characterize the relationship between the nodavirus which was isolated during the study and other previously characterized betanodavirus groups.

Materials and methods

Sample collection

Twelve moribund Asian seabass juvenile samples (6–10 cm size) were collected randomly from four different cage cultures in freshwater farms at Neeleshwaram, Kasargode district of Kerala in the west coast of India, where the outbreak occurred. The moribund juveniles were preserved in RNA later (Ambion) and were transported to the laboratory for immediate processing and extraction of RNA.

Total RNA extraction

RNA extraction was done from the brain, eye and kidney of individual juveniles using TRIzol[®] LS reagent (Invitrogen, Life technologies, UK) according to the protocol provided by the manufacturers. Briefly, 750 µl of TRIzol reagent was added to the individual tissue samples of the brain, kidney, retina and then homogenized with a micro pestle. Following incubation for 5 min at room temperature, they were centrifuged at 12,000×g for 15 min at 4 °C. RNA was precipitated from the aqueous phase with isopropanol, washed with 75 % ethanol and dissolved in 50 µl of diethylpyrocarbonate (DEPC) treated water. Extracts were subsequently treated with DNase I (Fermentas International Inc, Burlington, Canada), according to the supplier guidelines to remove any of the remaining DNA contaminant. The DNase treated RNA was then checked for its purity. The concentration and purity of the RNA was measured using NanoDrop[®] spectrophotometer (ND-1000, V3.3.0, Thermo Fisher Scientific, USA) and the purity was confirmed by electrophoresis. The samples were stored at –80 °C for subsequent use.

Reverse transcription

The reverse transcription was carried out according to the instructions provided by manufacturer (Fermentas International Inc, Burlington, Canada). About 938 ng µl⁻¹ RNA was used in each reaction for cDNA synthesis. The presence of fish nodavirus in seabass was analysed by PCR targeting LC and T4 region of RNA 2 coat protein gene as described by Nishizawa et al. 1994. LCNV primer: (F) 5'-GTTCCCTGTACAACGATTCC-3', (R) 5'-GGATT TGACGGGGCTGCTCA-3' and T4NV primer: (F) 5'CGT GTCAGTCATGTGTCGCT-3', (R) 5'-CGAGTCAACAC GGGTGAAGA-3' were used for the study [12, 15].

PCR was carried out in 30 µl reaction mixture containing 10× buffer (100 mM of Tris-HCl, pH 8.3, 20 mM of MgCl₂, 500 mM of KCl, 0.1 % gelatin), 200 mM of dNTPs (dATP, dTTP, dGTP, dCTP), 10 pmol each of forward and reverse primer and 1U of *Taq* DNA polymerase (Bangalore Genei, India). Amplification was carried out in a thermocycler (MJ Research, USA) and the programme included an initial delay at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C (for T4NV primers)/50 °C (for LCNV primers) for 30 s, extension at 72 °C for 30 s and a final delay at 72 °C for 5 min. LC and T4 gene amplified and cloned into pDrive cloning vector and were used as positive control. The PCR products were resolved on agarose gel (1.5 %) stained with ethidium bromide (0.5 µg ml⁻¹), photographed and analyzed using gel documentation system (Herolab, Weisloch, Germany).

Cloning of coat protein gene and DNA sequencing

The PCR products of both the coat protein genes were purified by PCR purification kit (Qiagen, Germany) according to the protocol mentioned in the manual provided with the kit. Purified PCR products were ligated into pDrive Cloning Vector (PCR cloning kit, Qiagen, Germany). Three clones of LCNV and four clones of T4NV were sequenced (M/s Chromous Biotech Ltd, Bangalore). The sequences obtained were analyzed by BLAST programme of the national center for biotechnology information (NCBI) (<http://blast.ncbi.nlm.nih.gov/>).

Phylogenetic analysis

The RNA2 coat protein (partial gene sequence) region was aligned with other betanodavirus coat protein gene sequences available in the GenBank of NCBI and phylogenetic tree was constructed using the MegAlign program (Windows version 5.05, DNASTAR, USA). Sequences of fish nodavirus were obtained from the GenBank database: India *L. calcarifer* (HM017076), India *L. calcarifer* (HM017077), Malaysia *L. calcarifer* (EU380202), Malaysia *L. calcarifer* (FJ617262), Malaysia *L. calcarifer* (FJ617265), Taiwan *E. lanceolatus* (AF245004), Australia *L. calcarifer* (EF591371), Australia *L. calcarifer* (EF591372), Japan *E. akaara* (D38636), China *E. akaara* (EF558369), Singapore *Poecilia reticulata* (AF499774), Malaysian *L. calcarifer* (GQ120525), Thailand *E. malabaricus* (AF175518), Singapore *L. calcarifer* (AF1755), Singapore *E. tauvina* (AF318942), Japan *Takifugu rupripes* (D38637), Japan *T. rupripes* (EU236149), Japan *Verasper moseri* (D38635), Japan *V. moseri* (EU236147), Japan *V. moseri* (EU826138), Japan *P. dentex* (AF175519), Japan *P. dentex* (D30814), Japan *P. dentex* (AB056572).

Results

Clinical signs, case history and gross pathology

The Asian seabass larvae were reared in freshwater demonstration cages (18 m³) at a stocking density of 5,000 numbers. At the time of stocking, all the larvae were active by swimming and had normal body coloration. Gradually a few of the juveniles developed slightly darker body coloration about 10 days after stocking of fish in the cages. Severe mortality was observed after 15 days of stocking and continued up to 50 days. Moderate mortality occurred even after 50 days particularly during the water exchange and size grading of the fish. The smaller sized fishes of about 6–10 cm in length were more prone to the disease symptoms than the bigger ones. During the study, VNN

affected fish showed clinical signs such as regression in feeding, floating on the surface of the cages, pale gills, inflated abdomen, exophthalmia, darkening of the whole body, cork-screw type whirling movement, erratic swimming and finally death. The outbreak of this disease resulted in severe mortality of up to 70–80 % within 2–3 weeks of stocking period.

Reverse transcriptase PCR

All the twelve fish samples from which the brain, retina and kidney were tested, showed positive for nodavirus by RT-PCR using both sets of primers. The RT-PCR amplification of the T4 region of the coat protein gene (T4NV-F2/R3 primers) gave an amplicon product size of 426 bp (Fig. 1A) while that with LCNV-F/R primers yielded a 294 bp amplified product (Fig. 1B).

Sequence analysis

The sequences that were obtained during the study were analyzed using the BLAST programme of NCBI and submitted to GenBank, with the accession numbers obtained as HM017076 (Betanodavirus LCNV coat protein gene, partial cds) and HM017077 (Betanodavirus T4NV coat protein gene, partial cds). The RNA2 coat protein gene sequence of nodavirus from the seabass obtained in the present study showed more than 90 % homology to the nucleotide sequences of piscine nodavirus isolated from the other regions.

Phylogenetic tree analysis

The DNA sequence (HM017076 and HM017077) of the nodavirus coat protein gene sequence was compared with 21 other coat protein gene sequences of piscine nodavirus available in the GenBank. At least two to three sequences were taken to represent every genotype of Betanodaviruses. The phylogenetic tree constructed based on betanodavirus coat protein gene sequence revealed that the current nodavirus isolates of the seabass belonged to the RGNNV genotype (Fig. 2).

Discussion

The disease outbreak resulting in the mass mortality in cage cultured Asian seabass was due to the nervous necrosis virus infection as confirmed by the clinical signs, RT-PCR and DNA sequencing. The clinical signs like exophthalmia, darkening of the whole body, whirling movement, erratic swimming and cork-screw type movement are the typical signs of seabass nervous necrosis virus

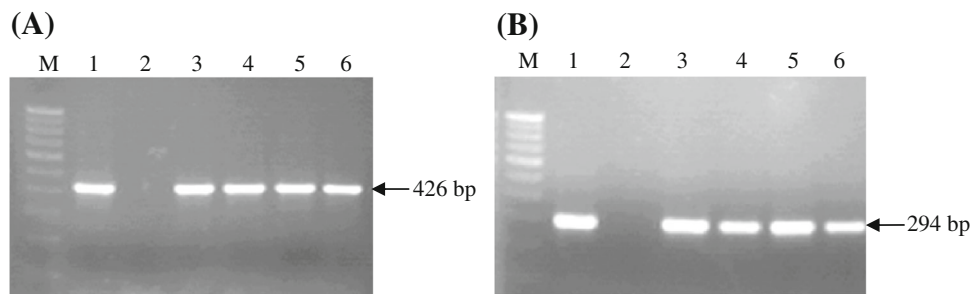
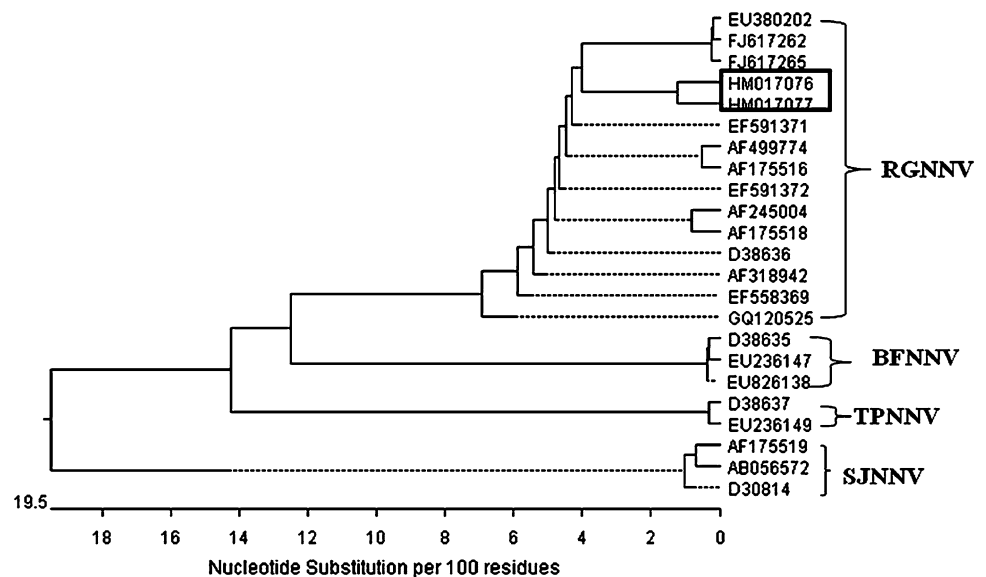


Fig. 1 Detection of fish nodavirus by reverse transcriptase PCR. **A** T4NV-F2/R primer; M: 100 bp DNA ladder, Genei, Bangalore, Lane 1: nodavirus positive control, Lane 2: negative control, Lanes

3–6: nodavirus positive samples. **B** LCNV-F/R primer; M: 100 bp DNA ladder, Lane 1: nodavirus positive control, Lane 2: negative control, Lanes 3–6: nodavirus positive samples

Fig. 2 Phylogenetic tree deduced from the variable region of coat protein sequences of 23 betanodaviruses. HM017076 and HM017077 are the T4 coat protein partial gene sequences generated in this study



infection [2, 15, 25]. Viral nervous necrosis associated mass mortalities in cage-reared seabass (*D. labrax*) have been recorded earlier [8]. In India nodavirus infection was demonstrated in freshwater ornamental fish, Guppy (*P. reticulata*) by experimental infection [6]. In this study the VNN infection in *L. calcarifer* reared in freshwater cage culture system is being reported. It was observed that seabass juveniles were more susceptible to VNN from the day 10 onwards with high mortalities of 80 % being recorded during 15–30 days of stocking. Further, it was also noted that the smaller sized fishes were more prone to VNN infection than the bigger sized fishes in the same cage. This could be attributed to various stress factors like overcrowding in the cages, cannibalistic nature of seabass, size grading procedure applied in cage culture system etc. Azad and his colleagues [2] also have reported that the larger larvae of Asian seabass are less affected than their smaller siblings in the hatchery.

Reverse transcriptase-polymerase chain reaction has been a rapid molecular diagnostic technique which has

wide application in the diagnosis of piscine nodavirus. The PCR based methods have been developed to detect RNA2 coat protein gene for the identification of betanodavirus by targeting T1 to T5 regions [12]. The T4 region of the VNN viral coat protein gene (RNA2) has also been used for diagnostic purposes [2, 12, 14]. The Office International des Epizooties (OIE) has recommended the use of the primer specific for conserved T4 region of coat protein gene, since it can be used for the piscine nodavirus detection from different geographical locations [2]. During the study all the 12 samples were positive for nodavirus by both the sets of primers i.e. for T4NV and LCNV, targeting different T4 regions of the RNA2 coat protein of the virus by RT-PCR.

In the present study T4 region of the coat protein gene was sequenced and the analysis showed that the sequence exhibited more than 90 % similarity with strains of nodavirus reported from other Asian countries. The phylogenetic comparison of this sequence obtained in the study with other available sequences showed that the viral strain

obtained in the study belonged to the RGNNV genotype (Fig. 2). According to Nishizawa et al. [13] RGNNV is one of the widely distributed nodavirus genotype in piscine nodavirus group.

To control the nodavirus infections in the aquaculture system it is important to understand the epidemiology and pathogenic mechanisms of the virus. Generally, the virus can be transmitted horizontally in aquaculture system through the carrier animals, inlet water feed, vertically through the brood stock. The Piscine nodavirus is known to infect both marine and freshwater fishes as demonstrated by several experimental studies [6, 18, 19]. The vertical transmission of VNN from the brood fish including Asian seabass to their offspring has been demonstrated [3, 4]. The horizontal transmission of nodavirus in Atlantic halibut (*H. hippoglossus*) larvae has also been reported. Further it has been observed that some of the VNN infected larval fishes can survive the infection and act as a carrier for the next generation [11]. Similarly in the present study, though some fishes were observed to survive even after 50 days of culture period, they can act as carriers later and cause infection to the next generation by both vertical and horizontal routes. Sensitive screening procedures for the nodavirus such as PCR and ELISA, along with the better management practices for avoiding the stress are considered to be the most practical strategy for controlling VNN infection in the seabass aquaculture system.

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