OUTBREAK OF BETANODAVIRUS INFECTION IN CAGE-CULTURED ASIAN SEABASS (*LATES CALCARIFER* BLOCH): A CASE STUDY IN ODISHA

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ABSTRACT

Viral encephalopathy and retinopathy (VER) or viral nervous necrosis (VNN) is an infectious neuropathological disease affecting more than 120 fish species. Although VER affects mainly marine fish, the disease has also been detected in certain species reared in low-saline and freshwater environments. In the present study, a case of betanodavirus associated massive mortality was investigated in juvenile seabass Latescalcarifer, maintained in cage culture facilities in brackishwater pond in Odisha (India). The affected fish showed clinical signs typical of betanodavirus infection around 70 days post hatch (dph), resulting sudden mass mortality in all the cages affecting almost 100% of the population. Histopathology revealed massive necrosis of tubules in kidney and mild vacuolation of the nervous system, suggesting an infection by a betanodavirus. The presence of virus was detected and confirmed by nested RT-PCR assay and sequence analysis of PCR product. Sequencing of the PCR product indicated sequence homology with red-spotted grouper nervous necrosis virus (RGNNV) type. However, the reservoir of virus leading to the outbreak remains unidentified, but suspects the infected fry as a source. This is the first report of mortality associated with natural infection of betanodavirus infection in inland cage farming facilities in India.

Key Words*:* Cage culture, Asian seabass, Viral nervous necrosis, Betanodavirus, Mortality, RT-PCR.

INTRODUCTION

Viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER) is one of the major disease constraints in the aquaculture of a number of marine fish species across the world. The etiological agent is a piscine nodavirus, member of the family *Nodaviridae* and causes highly destructive disease in hatchery reared larvae and juveniles. Since its first description by Yoshikoshi and Inoue (1990) in Japanese parrotfish, *Oplegnathus fasciatus*, in Japan, the disease has been reported from over 120 fish species, mostly marine fishes in the Indo-Pacific region, Mediterranean & Scandinavia and North America (Maltese and Bovo, 2007; Bandín and Souto, 2020). In addition, a wide variety of asymptomatic fish belonging to 120 different species and 21 marine invertebrate species have been found to be susceptible to this infection and hence

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act as carriers (Costa and Thomson, 2016). The disease is characterized by neurological abnormalities (erratic swimming, spiral movements with belly-up) and a distinct vacuolization of the nervous tissue (brain, retina) (OIE 2006). Mortality associated with the disease is severe, reaching up to 100% depending on age, with younger fish being more susceptible (OIE 2015). In India, the disease has been reported in hatchery reared seabass larvae (Azad *et al*., 2005; Parameswaran *et al*., 2008) and among aquarium fishes (Jithendran *et al*., 2011a). There are very few reports of VNN in grow-out culture facilities in India. In this paper, an outbreak of VNN in juvenile Asian seabass stocked in cage culture facility in brackish water pond in Odisha has been reported with confirmatory diagnosis by molecular assays and histopathological studies.

MATERIALS AND METHODS

Case history and clinical samples

The present study was carried out at a seabass cage culture farm in a brackish water pond located at Gudugai (Balasore, Odisha) with water spread area of 1 hectare with water depth 1.5-2.0 m. The cages (dimensions: 2x2x1.5 m) were stocked (900 numbers/cage) with two batches of seabass fingerlings sourced from a hatchery in Tamil Nadu in the month of November, 2009. The fishes were provided with imported pellet feed from Indonesia. The farmers noticed abnormality in fish *viz*., gulping of air, tail and dorsal fin rot in cages in the month of January, 2010. In order to control fin rot, oxytetracycline (4g/200L) dip treatment in separate tanks thrice a day for two consecutive days was advised by local aquaculture consultant. The pond water was also treated with potassium permanganate at 1kg/ha and 120kg lime. However, sudden mass mortality of fish was observed in all cages. Mortality of fish numbering 200/day was observed and all fish died within a span of four days.The water quality parameters during the mortality were reported as: dissolved oxygen, 7.0 mg/L; pH, 8.0-8.5; salinity, 14-16 ppt and temperature, 14˚C.

Sample collection and investigations

Affected fish samples of 10-30 g body weight were sampled on appearance of the clinical signs at 70-80 days post culture. Both sick and apparently healthy fish were sampled for laboratory investigations. Samples were collected for gross observation, bacteriology, histopathology and molecular diagnostics for viral pathogens. Wet mounts of skin and gills were prepared and examined for external parasites. Aseptically collected samples were analysed for common opportunistic bacterial pathogens. For histopathology, tissue samples from gills, liver, spleen, heart, brain, stomach and kidney were fixed in 10% neutral buffered formalin and processed by standard histology procedures.

RNA extraction, RT-PCR and sequencing

Total RNA was extracted from approximately 20 mg of preserved brain/eye tissue samples using TRIzol™ Reagent (Invitrogen, USA) following the manufacturer's protocol and screened by reverse transcription polymerase chain reaction (RT-PCR) as described earlier (Gomez *et al*., 2004). Reverse transcription followed by PCR amplification was performed with the RT-PCR kit (QIAGEN,

Table 1. Primers used in the RT-PCR reaction

Primer code	Séquences (5' to 3')	Amplicon(bp)	References
BNVUF2	THC AAG CRA CTC GYG GTG C	420 (First step)	Gomez *et al*. (2004)
BNVUR2	GCC ART AVA CRG CMC GKT CVA CRT		
BF	TCT CAC GTC ACC TGG TCG	250 (Nested)	This study
BR	CAG CTG GAA GAC TGC TCC AT		

USA). Nested PCR was performed similarly but with primary PCR product as the template. The cycling conditions include; one cycle of initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 30 s, primer annealing at 58°C for 40 s and primer extension at 72°C for 1 min and one cycle of final extension at 72°C for 10 min. A set of custom designed degenerate primers were used for the amplification of RNA2 partial sequences. For RT-PCR, a degenerate primer set designed by Gomez *et al*. (2004) and for nested PCR, a custom designed primer set (BF and BR) producing an amplicon of 250 bp was used (Table 1). Primers for nested PCR were designed based on a full length betanodavirus RNA2 reported from India (Gen Bank Accession No: GU953669) using tools available with Genscript (www.genscript.com/tools) and synthesized from Sigma (India). The PCR products were analyzed for purity and size by electrophoresis in 1.5% agarose gel after staining with 0.5% mg/mL ethidium bromide. The amplified PCR products of specific DNA bands were excised, extracted using PCR/gel extraction columns (Qiagen) and sequenced by outsourcing (M/s Xleris, Ahmedabad). The nucleotide sequences were analysed by nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), aligned with representative nucleotide sequences of four species of betanodavirus: SJNNV (NC003449), TPNNV (NC013461), BFNNV (NC013459) and RGNNV (AY324870, AB373029) retrieved from the NCBI GenBank database. Betanodavirus sequences were aligned using MEGA 4.0 (Tamura *et al*., 2007) and the neighbour joining analysis was performed using the Kimura-2 parameter model of nucleotide substitution.

Histopathology

Tissue samples from gills, liver, spleen, heart, brain, stomach and kidney fixed in 10% neutral buffered formalin were processed by standard histological method for histopathological examination. Sections of tissues (4-5 μm) were cut using Leica RM 2245 microtome and stained with routine haematoxylin and eosin (H&E) and finally mounted with DPX mountant and subjected for microscopy and microphotography.

RESULTS AND DISCUSSION

Mariculture and brackishwater finfish culture in India is dominated by seabass culture either in cages in coastal areas or land based culture facilities and typically involves the stocking of 10–20 g fish sourced from nurseries into cages or ponds. Fish culture in cages has been identified as one of the eco-friendly and at the same time intensive culture practices for increasing fish production. The present study carried out at a seabass cage culture facility in

a brackishwater pond exhibited mass mortality of juvenile fish within a month after stocking. The affected fish showed clinical signs typical of betanodavirus infection, including spiral movement on the surface of the water, erratic and uncoordinated swimming, and lethargy at later stages of the disease and swimming with the belly up usually on the surface of the water, darkening of the skin and swim bladder over-inflation. The disease was first observed in one of the cages around 70 dph, subsequently sudden mass mortality in all the cages affecting almost 100% of the population. The fish samples received for investigation did not reveal any parasitic or systemic bacterial infection. However, the representative pooled samples subjected for RT- PCR analysis showed strong positive results in first step and nested RT-PCR reactions with the amplification of 420 and 250 bp PCR products (Fig. 1). On histology, the mild vacuolationin the brain tissue was observed (Fig. 2a). The histology of kidney samples revealed intra-nuclear inclusions in tubular epithelial cells, fatty changes in liver and myonecrosis with mononuclear infiltrations in muscles (Fig. 2 b-d). In the present study, the characteristic clinical symptoms with massive mortality, mild vacuolations seen in the brain and a gene specific RT-PCR confirmed the aetiological agent as betanodavirus.

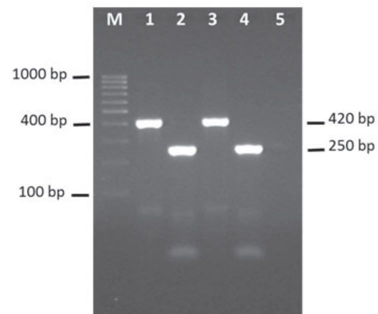

Fig 1. Agarose gel electrophoresis of RT-PCR products for VNN in Asian seabass fingerlings

(Lanes: M- 100 bp ladder,; 1- Fish sample by first step PCR, 2- Nested PCR; 3- Positive control on first step PCR; 4- Positive control on nested PCR; 5- Negative control)

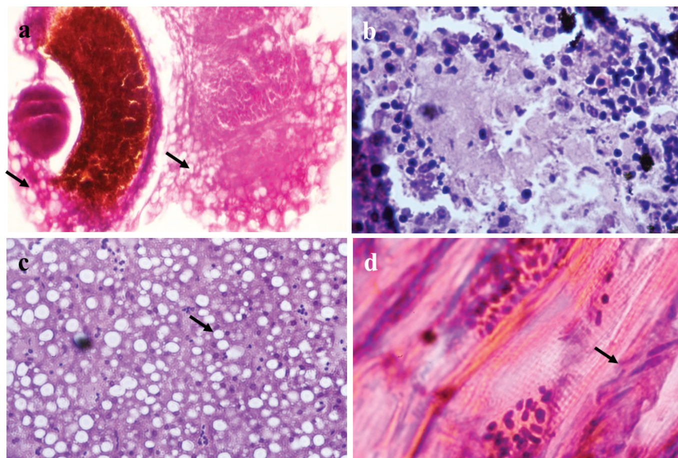

Fig 2. Histopathology of Asian seabass juvenile fish

(a) Histological section of eye and brain showing vacuolation (400x); (b) Kidney showing massive necrosis of tubules (250x); (c) Fatty changes in liver (100x); and, (d) Myonecrosis with mononuclear infiltraion (100x). H & E stain

The first described PCR protocol for betanodavirus targeting the capsid protein sequences of NNV is considered as the gold standard for its confirmatory diagnosis (Nishizawa *et al*., 1994). VNN as a disease entity has been recognized as a potential threat to fin fish aquaculture in India, and the disease has been reported in hatchery reared seabass larvae (Azad *et al*., 2005; Parameswaran *et al*., 2008). However, the finfish culture is yet to take off in a big way to exhibit the disease manifestations in wider areas. Reports of sub-clinical infection in both cultured and wild fish exist (Jithendran *et al*., 2011b). Severe vacuolation in the brain, spinal cord and retinal tissues of the eye are considered the most important and reliable diagnostic features of VNN in fishes (Johansen *et al*., 2004; Maltese and Bovo, 2007), though sub-clinical cases of infection with very low viral load is reported to be difficult to diagnose by first step PCR. However, these clinical manifestations may vary depending on the stress conditions and age of the fish. In this study, the onset of disease was observed about a month after transport of fish from hatchery to the grow-out culture facilities. The fish at that time were at the early juvenile stage, which is critical period characterized by high losses in susceptible fish species. Hence the pre-existing sub-clinical infection accompanied with transportation stress could have elicited the infection as an outbreak detectable by first step PCR itself. Pathogeni city of VNN in fingerlings of 80 days post-hatch (dph) seabass has been found lower than that to the 14 dph larval fish (Wu *et al*., 2013). The study suggests the need for a proper surveillance protocol for fish seeds meant for aquaculture and transportation considering the high risk of introduction of the virus to otherwise naïve areas.

Studies on genetic identity of Indian isolates of betanodavirus in seabass indicated that RGNNV is the only genotype reported so far (Binesh and Jithendran, 2013; John *et al*., 2014). Natural infections of nodaviruses in marine fish occur within a wide range of water temperatures and salinity. RGNNV is the largest group with high infectivity and broad host range belonging to warm water and a potential threat to wild fish population. Majority of the suspected mortality and clinical signs exhibited during the ascending phase of temperature during the change of season in India. However, so far no data is available on distribution of various genotypes, viral transmission between wild and farmed fish, and the direction of flow of pathogen. A detailed monitoring plan in wild and farmed fish is required to decipher the risk of pathogen transfer between farmed and wild fish populations. Use of pathogen free fingerlings and brood stock for aquaculture and monitoring programme for virus in hatcheries, and aquaculture farms may help in understanding the significance and loss due to VNN. The spread of VNN in the marine aquaculture system could be attributed to either vertical or horizontal transmission (Breuil *et al*., 2002; Azad *et al*., 2006). Nodaviruses can spread both horizontally between fish and vertically from brood stock to off spring (Munday *et al*., 2002). In this study, the disease occurred in areas where there was no previous history of such outbreak due to VNN. Hence, the possibility of pre-existed virus in fish seed transported to the locality or in pond water cannot be excluded. The reservoir of

virus and the origin of the outbreak remain unidentified as the fishes were in cages in a brackish water pond with possible interaction with wild fish as well. This is the first report of mortality associated with natural infection of betanodavirus infection in inland seabass cage culture facilities in India. The RT-PCR and the sequence of the target product (420 bp) on NCBI BLAST analysis showed 100% sequence homology to RGNNV genotype. Further studies on experimental infection, genetic characterization of isolated viral strains accompanied with the understanding of epidemiological dispersion of the virus in farmed and wild fish are required for effective control of betanodavirus in culture systems.

ACKNOWLEDGEMENTS

The authors are grateful to the Director, Central Institute of Brackish water Aquaculture (Chennai) and Director, Central Institute of Freshwater Aquaculture (Bhubaneswar) for providing necessary facilities during this study.

REFERENCES

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

Azad, I.S., Jithendran, K.P., Shekhar, M.S., Thirunavukkarasu, A.R. and de la Pena, L.D. (2006). Immunolocalisation of nervous necrosis virus indicates vertical transmission in hatchery produced Asian seabass (*Lates calcarifer* Bloch) - A case study. *Aquaculture*, **255**: 39-47.

Bandín, I. and Souto, S. (2020). Betanodavirus and VER Disease: A 30-year Research Review. *Pathogens*, **9**: 106; doi: 10.3390/pathogens9020106.

Binesh, C.P. and Jithendran, K.P. (2013). Genetic characterization of betanodavirus isolates from Asian seabass *Lates calcarifer* (Bloch) in India. *Archives of Virology,* **158**: 1543-1547.

Breuil, G., Pepin, J.F.P., Boscher, S. and Thiery, R. (2002). Experimental vertical transmission of nodavirus from broodfish to eggs and larvae of the sea bass, *Dicentrarchus labrax* (L.). *Journal of Fish Diseases*, **25**: 697-702.

Costa, J.Z. and Thompson, K.D. (2016). Understanding the interaction between Betanodavirus and its host for the development of prophylactic measures for viral encephalopathy and retinopathy. *Fish Shellfish Immunology,* **53**: 35-49.

Gomez, D.K., Sato, J., Mushiake, K., Isshiki, T., Okinaka, Y. and Nakai, T. (2004). PCR-based detection of betanodaviruses from cultured and wild marine fish with no clinical signs. *Journal of Fish Diseases*, **27**: 603-608.

Jithendran, K.P., Shekhar, M.S., Kannappan, S. and Azad, I.S. (2011a). Nodavirus infection in freshwater ornamental fishes in India - diagnostic

histopathology and nested RT-PCR. *Asian Fisheries Science*, **24**: 12-19.

Jithendran, K.P., Binesh, C.P., Sujeet Kumar, Ananda Raja, R. and Ambasankar, K. (2011b). Epidemiology of viral nervous necrosis in marine fishes using molecular based diagnostics. Proceedings of the Asian-Pacific Aquaculture 2011, Kochi, January 17-20, 2011.

Johansen, R., Grove, S., Svendsen, A.K., Modahl, I. and Dannevig, V. (2004). A sequential study of pathological findings in Atlantic halibut, *Hippoglossus hippoglossus* (L.) through out one year after an acute outbreak of viral encephalopathy and retinopathy. *Journal of Fish Diseases*, **27**: 327-341.

John, K.R., Rosalind George, M., Jeyatha, B., Saravanakumar, R., Sundar, P., Jithendran, K.P. and Koppang, E.O. (2014). Isolation and characterization of Indian betanodavirus strain from infected farm-reared Asian seabass *Latescalcarifer* (Bloch, 1790) juveniles. *Aquaculture Research*, **45**: 1481-1488.

Maltese, C. and Bovo, G. (2007). Viral encephalopathy and retinopathy. *Ittiopatologia*, **4**:93-146.

Munday, B.L., Kwang, J. and Moody, N. (2002). Betanodavirus infections in teleost fish: a review. *Journal of Fish Diseases*, **25**: 127-142.

Nishizawa, T., Mori, K., Nakai, T., Furusawa, I. and Muroga, K. (1994). Polymerase chain reaction (PCR) amplification of RNA of stripped jack nervous necrosis virus (SJNNV). *Diseases of Aquatic Organisms*, **18**: 103-107.

OIE, (2006). *Manual of Diagnostic Tests for Aquatic Animals.*2006, OIE, Paris, France.

OIE, (2015). Viral encephalopathy and retinopathy In: OIE (Ed.) *Manual of Diagnostic Tests for Aquatic Animals 2015,* Chapter 2.3.11 pp. 1-19. Office International des Epizooties, Paris, France. Accessed online http://www.oie.int/international-standard-setting/aquatic-manual/access-online/ on 20th January, 2016.

Parameswaran, V., Rajesh Kumar, S., Ishaq Ahmed, V.P. and Sahul Hameed, A.S. (2008). A fish nodavirus associated with mass mortality in hatchery-reared Asian seabass, *Latescalcarifer*. *Aquaculture*, **275**: 366-369.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007). MEGA 4.0: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, **24**: 596-1599.

Wu, Y.C., Kai, Y.H. and Chi, S.C. (2013). Persistently betanodavirus-infected barramundi (*Lates calcarifer*) exhibit resistances to red sea bream iridovirus infection. *Development and Comparative Immunology,* **41**:666-674.

Yoshikoshi, K. and Inoue, K. (1990). Viral nervous necrosis in hatchery-reared larvae and juveniles of Japanese parrotfish, *Oplegnathus fasciatus* (Temminck & Schlegel). *Journal of Fish Diseases*, **13**:69-77.