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Viral Nervous Necrosis: A Challenge to Finfish Aquaculture

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Introduction

Aquaculture is the fastest growing food-producing sector in the world. The growth of this sector is phenomenal, contributing significantly to food security, export earnings, and employment generation. At present, India produces 4.6 million tons which make 8.7% of world aquaculture production and stands second after the Republic of China [1]. In India, the coastal aquaculture has seen its prominence since the early eighties and historically remained synonymous with shrimp culture through rising production to the highest level of 4.345 lakh MT during 2014-15 and export of 3.575 lakh MT worth of Rs. 22,468.12 crores ($ 3709.76 million) [2].

However, during the last two decades, shrimp industry experienced a series of viral disease outbreak across the world recently due to many emerging diseases. This emphasized the need for diversification and sustainable development of the sector. In India, apart from shrimp, the culture of finfish like Asian seabass (*Lates calcarifer*), milkfish (*Chanos chanos*), mullet (*Mugil cephalus*), and pearl spot (*Etroplus suratensis*) have been practiced in a traditional way. However, the scientific culture of marine or brackishwater finfish is still in its infancy with limited species, since the seed production remains a bottleneck in expanding mariculture activities to an industrial level. Further, the limited culture of marine fish has taken up after the successful breeding and hatchery technology for seed production of Asian seabass (*Lates calcarifer*) for the first time in the country in 1997. This was followed by the improved hatchery technology by Central Institute of Brackishwater Aquaculture (Chennai, India) for seabass and Rajiv Gandhi Centre for Aquaculture, Sirkazhi (India) for multiple species like seabass, grouper, etc. which enabled large-scale fry production under controlled conditions.

The Recent impetus on coastal aquaculture of marine/brackishwater fish species by National Fisheries Development Board (Hyderabad, India) for augmenting production by demonstration and promotion of open water sea cage culture by Central Marine Fisheries Research Institute (Kochi, India) along the coastline of India started yielding results with total production of 18,000 MT. Thus, Asian seabass has been proven as ideal species for cage and pond culture due to the availability of hatchery-produced seed, market demand, and fast growth. Other species with great potential is cobia (*Rachycentron canadum*); an excellent species for open sea cage culture and silver pompano (*Trachinotus blochii*) reported to be highly suitable for pond farming in vast low saline waters beside sea cage farming. For both fish species hatchery technology has been initiated and on-farm trials are demonstrated in land and cage based culture system at different locations in the country. Many other commercially important brackishwater species like grey mullet, milkfish, and pearlspot are also finding a place as a candidate species for cage and pond culture in brackishwater resources among farmers (**Figure 1 & 2**). In addition to this, capture-based aquaculture uses wild seed or juveniles to stock in aquaculture facilities for grow-out purposes and generate alternative livelihood, income for local coastal communities. Capture-based aquaculture accounts for about 20% of the total quantity of food fish production through aquaculture and includes mainly high valued finfish (e.g., *Lates calcarifer, Etroplus suratensis, Lutjanus argentimaculatus*) with regional preferences.

Like any other productions systems, diseases due to various pathogens of viral, bacterial, fungal and parasitic origin cause considerable economic loss to the sector. Of these viral nervous necrosis (VNN) or viral encephalopathy and retinopa-

thy (VER) is considered to be one of the most serious and emerging viral diseases in hatchery-reared larvae and juveniles, with significant economic impact worldwide. We examine the disease in the context of the massive expansion of marine and brackishwater finfish culture by area, species and types of culture in the country.

VNN –historical perspective

Viral nervous necrosis (VNN) officially denominated as viral encephalopathy and retinopathy (VER) by the Office Internationale des Epizootics (OIE) is one of the main disease constraints in the aquaculture of many fish species across the world. The disease is presently categorized as "significant disease," but not included in the OIE listed diseases of finfish. The etiological agent is a piscine nodavirus, member of the family Nodaviridae causes highly destructive disease with severe mortality reaching up to 100% in hatchery-reared larvae and juveniles. The family Nodaviridae comprises two genera, alphanodavirus (type species-Nodamura virus, NOV), primarily infecting insects, and betanodavirus (type species-striped jack nervous necrosis virus, SJNNV), members of which infect fish. Initial studies described the causative agent of VNN as a "picorna-like virus" [3,4] followed by the first description in Japanese parrotfish, *Oplegnathus fasciatus* in Japan, [5] characterized virus particles isolated from European seabass (*Dicentrarchus labrax*) larvae and juveniles as having a typical icosahedral shape and a diameter of 23 nm as determined by electron microscopy. Since then, the disease has been reported from over 50 fish species (with the greatest impact on seabass, groupers, cods and flat fishes) under 16 families in the Indo-Pacific region, Mediterranean, Scandinavia and North America. In recent times, betanodavirus infection has been reported across habitat barriers from freshwater to the seawater environment.

Disease Status in India

The first observation of VNN in India was made in a batch of hatchery produced seabass larvae in 2003 accounting for 80-90% mortality. Detailed investigation followed, later on, to confirm by histopathology, immunohistochemistry (IHC), immunofluorescent antibody test (IFAT), electron microscopy and nested RT-PCR. In India, the disease is increasingly becoming important in wild and culture conditions across the wide geographical range in almost all coastal states. A wide range of fish species might be susceptible to nodavirus infection, and sub-clinically infected wild fish population could be a primary source of infection for the farmed fish. Betanodavirus infection has been observed in cultured and wild population of brackishwater / marine fish species such as *Lates calcarifer, Rachycentron canadum,* Trachinotus blochii, Mugil cephalus, Liza parsia, Chanos chanos, Epinephelus tauvina, Sardinella longiceps, Amblygaster clupeoides, *Thrissocles dussumieri, Leiognathus splendens, Upeneus sulphureus, Mystus gulio*, etc (**Table 1**). Among aquarium fishes, *Carassius auratus* (Gold fish), *Epalzeorhynchos frenatum* (Rainbow shark), *Danio rerio* (Zebra fish) and *Amphiprion sebae* (Clown fish) were found to be susceptible to betanodavirus. So far, only latent infection has been reported in the wild as sub-clinical form. However, severe disease outbreaks have been reported in hatchery/farms in larval and juvenile stages or nursery and grow-out phase in aquaculture facilities due to the higher density of rearing or environment-related stress factors as often observed in the case with Asian seabass (*Lates calcarifer*). However, economic loss due to this disease is not known.

Different strains

Betanodaviruses are small (25-34 nm), icosahedral, non-enveloped viruses with bipartite positive sense RNA genome. The larger genomic segment RNA1 (3.1 kb) encodes RNA dependent RNA polymerase (protein A) and the smaller genomic segment RNA2 (1.4 kb) encodes coat protein. A small subgenomic component, RNA3 synthesized from RNA1 during viral propagation encode for one or two non-structural proteins called B1 and B2. Currently, there are four genotypes within the genus betanodavirus based on partial nucleotide sequences of the coat protein gene (RNA2). These are striped jack nervous necrosis virus (SJNNV), red-spotted grouper nervous necrosis virus (RGNNV), tiger puffer nervous necrosis virus (TPNNV) and barfin flounder nervous necrosis virus (BFNNV). Furthermore, an additional genotype including a turbot betanodavirus strain (TNV) has been proposed. Viral titration studies using the E-11 cell line revealed differences in the optimal growth temperature among the four genotypes: 25 to 30°C for RGNNV, 20 to 25°C for SJNNV, 20°C for TPNNV and 15-20°C for BFNNV. Correspondingly, RGNNV infects the more number of warm-water fish species. BFNNV and TPNNV are associated with cold-water fish species, whereas SJNNV infects warm as well as and cold water fish species. In India, all betanodavirus related disease outbreaks so far, have been reported to be due to RGNNV genotype.

Disease signs and pathology

The main clinical signs seen during VNN infection are behavioral changes such as anorexia, erratic, spiral or belly-up swimming and dark coloration of the body. Affected fishes remain secluded and moving away from schools. In general,

earlier the disease signs occur; greater is the rate of mortality. In the case of seabass, the earliest onset of clinical signs of the disease begins ninth day post hatch. Recently, the disease has been observed in fishes irrespective of their age. Instances of asymptomatic/sub-clinical infection in which the fishes do not show any clinical signs of disease in the wild may act as potential carriers. The important histopathological changes noticed during this disease are cellular necrosis and vacuolation in the central nervous system (brain and spinal cord) and retina.

Transmission

There is a lack of information on natural routes of transmission of betanodaviruses. While nodaviruses have been regarded as pathogens of marine fish, the natural development of disease has been reported from fishes of low saline and freshwater environments including freshwater aquarium fishes. Salinity tolerance of betanodaviruses is also important in the context of culture of many marine/brackishwater fishes in low saline environments since many nodavirus isolates from marine fishes can infect their freshwater counterparts or other fishes. Although horizontal transmission represents the most common route, the vertical transmission has also been highly suspected. The disease had been reported to transmit from one species to another by cohabitation and waterborne challenges. The exact mode of horizontal transmission and the possibility of inapparent carriers shedding virus in natural conditions are yet to be studied. As betanodaviruses are quite resistant to environmental conditions, it is possible that commercial activities readily translocate them via inlet water, juvenile fish held on the same site and carriage on utensils, vehicles, etc. Possible risk factors associated with translocation of species for aquaculture or stocking purpose from one location to other is not known. The inherent potential for transmission of pathogens in situ via fish seed facilitates the spread to a relatively naive host or environment. The VNN infection in low-value trash fishes raise concerns over the probable horizontal transmission of the virus into the cultured fishes since most of these fish species are used as feed in finfish aquaculture.

Interaction between wild and farmed fish

Wide range of susceptible hosts, the existence of vertical and horizontal transmission, distribution of virus in both farmed or wild population and commercial transportation of fish for aquaculture activities should be considered to minimize the effect of risk factors involved in the farming of marine fish species. Latent infection among wild fishes also serves as a source of infection to farmed fish. The possible mechanisms that may facilitate or enhance potential virus transmission between wild and cultured fish include the following: (i) the use of larvae, juveniles, or broodstocks from wild sources for rearing; (ii) the use of water supplies harboring infected wild fish; (iii) rearing fish in close proximity to wild virus reservoirs; (iv) the use of contaminated fish or fish products as feed for cultured animals; (v) stress or immunosuppressive conditions in cultured fish populations; (vi) continuous high-density culture; (vii) the culture of non-native fish in areas where this virus is endemic; (viii) the culture of multiple fish species in close proximity.

Implications of transporting live fish

The high resistance of betanodavirus to varying environmental situations and husbandry related stress assume more significance towards the spread among the wide variety of cultured organism. Transportation of susceptible fish seeds for aquaculture from one location to other may be contributing to the spread of this disease, which needs study and validation in the field. There are pieces of evidence to support both vertical and horizontal transmission of VNN. Viral nervous necrosis is a complex disease in which the outcome of betanodavirus infection can be influenced by host factors such as age and environmental factors such as water temperature. An association between VNN outbreaks and husbandry-related stress is commonly reported. Stress factors observed include suboptimal feed, suboptimal water quality, crowding, transport and repeated spawning of broodstock.

Disease Diagnosis

The first step in combating an infectious disease is detection and identification of the causative agent using a reliable diagnostic method followed by better surveillance techniques. Presumptive diagnosis is made based on the clinical signs and by the light microscopic changes like the appearance of vacuoles in brain, spinal cord, and retina. In electron microscopy, the icosahedral virus is seen as associated with vacuolated cells and arranged intra-cytoplasmically in paracrystalline arrays or as inclusion bodies. Confirmatory diagnosis is made by fluorescent antibody test (FAT) and enzyme-linked immunosorbent assay (ELISA) using polyclonal or monoclonal antibodies.

Reverse-transcriptase polymerase chain reaction (RT-PCR) is the most rapid and convenient method of diagnosing clinically affected fish (**Figure 3**). The first described PCR protocol for betanodavirus targeting the capsid protein sequences

(Nishizawa et al. 1994) is now accepted as the gold standard for its confirmatory diagnosis [6]. Recently, it was reported that nested PCR was 10-100 folds more sensitive than the RT-PCR. It is particularly useful to diagnose asymptomatic broodstock fish. However, for confirmative diagnosis, PCR must be followed by other techniques like histopathology with immuno-staining or virus isolation in cell culture (**Figure 4 & 5**). Virus identification can also be done in cell culture exhibiting characteristic cytopathic effects (CPE) or by performing serological test like virus neutralization tests. Many permissive cell lines are available for virus isolation and successful propagation in the laboratory. A combination of cell culture replication followed PCR has been practiced by many workers to detect subclinical infections. Betanodavirus detection by PCR in sub-clinically infected spawners is considered difficult as the viruses are not always detectable in the gonad, but can be detectable after stressful repeated spawning. Several techniques of non-destructive screening have been developed and an exclusive method involves the antibody-based detection of VNN in the available stocks but does not prove infection in fish. The Immunoassays such as ELISA and immunodot have been used for screening the broodstock.

Strategies for controlling VNN in hatcheries

To control nodavirus infections in aquaculture, it is important to understand the epidemiology of the virus. In theory, the virus can be transmitted through the inlet water or the feed, or from the broodstock via the eggs/sperm. Many studies demonstrated a high concentration of virus in the environment around infected larvae. However, the likelihood of acquiring infection through water is low due to the enormous dilution of the virus in seawater. As there are no effective treatment methods available for controlling the viral disease, several management measures have to be applied for a sustained production and containment of the disease-related fish losses. This may be achieved through the production and procurement of quality seed or stocking material and establishment of a healthy broodstock. Vertical transmission of NNV allows virus spread from generation to generation, which is the most challenging issue in fish farming. VNN-free larvae can be produced by the establishment of highly biosecured VNN-free broodstock facilities in the first place, by careful screening and monitoring of health status. Antibody based assays or PCR based tests to identify infected carrier fish using biopsy or reproductive fluids have been successfully practiced. Any re-stocking of new broodstock should be made after adequate quarantine and screening to reduce the potential introduction of the virus. However, this technique has the serious limitation as the brooder fish need not necessarily show a positive reaction in latent stage of infection. However, screening of the offspring could increase the likelihood of identifying possible infected animals. Hence a screening strategy should consequently consist of both broodstock screening by non-lethal means and multiple screening of offspring to reveal any vertical transmission to egg and larvae. The mass collection of field samples, transport, and screening using modern tools can facilitate the quick diagnosis of broodstock and batch screening of larvae in hatcheries. In the hatchery, larvae and juveniles would be vulnerable to horizontal transmission of VNN. The source of the virus could be from within the aquaculture facility itself or by using untreated or improperly disinfected water.

Natural infections of nodaviruses in marine fish occur within a wide range of water temperatures. Issues of concerns is the occurrence of betanodavirus carriers in the form of adult marine, brackishwater and freshwater fishes and many invertebrates as the potential source of infection. Further, the combination of stress like repeated spawning, transportation, elevated temperatures, salinity variations, etc. are known to express the disease and entail economic losses. It is also likely that sub-clinically infected samples may constitute a persistent source of nodavirus from exporting countries for susceptible fish species elsewhere through the import of seeds. Diagnostic assays for VNN may be conveniently used not only to eliminate virus-positive brooder fish but also to check the fish status during the grow-out phase in floating or submersible cages when contacts between wild and cultured fish are most likely to occur.

Broodstock fish act as the most important source of the virus to their larvae by vertical transmission. This finding led to the successful control of VNN of larval fish, by eliminating virus-carrying broodstock by RT-PCR screening and disinfection of fertilized eggs with iodine or ozone, etc. in many Asian countries. Strict hygiene within hatcheries assisted in the control of VNN. Disinfection of hatchery/farm materials with chlorine; rearing of each batch of larvae/juveniles in separate tanks supplied with sterilized (UV or ozone) seawater; and rigorous separation of larval and juvenile fish from brood fish were also found useful.

Disease prevention and control

The first step in combating a disease like VNN is detection and identification of the causative agent using a reliable diagnostic method followed by better control measures. No treatment is available against the VNN. Therefore, preventive measures are the primary strategy to control this disease. Screening of broodstock by RT-PCR to eliminate carriers, wash-

ing fertilized eggs in ozone-treated sea water and disinfection of rearing water by ozone, etc. efficiently control the disease in larval production. To prevent horizontal transmission, disinfectants like sodium hypochlorite, benzalkonium chloride, iodine and ozone are effective in inactivating the virus. Washing fertilized eggs in ozone-treated sea water and disinfection of rearing water by ozone efficiently control the disease in larval production of striped jack, barfin flounder, Atlantic halibut, and seven-band grouper. Separate rearing facility of larvae/juveniles from brooder should be maintained with each batch of larvae and juveniles in the different tanks supplied with sterilized (UV or ozone) seawater. Till date, no vaccine is available against any of the affected species. Efforts are being made to develop a vaccine using recombinant viral coat protein expressed in Escherichia coli, virus-like particles expressed in a baculovirus, inactivated virus or using avirulent aquabirnavirus.

Table 1. Detection of betanodavirus infection in freshwater ornamental, brackishwater and marine fishes in India

Ecosystem	Sources	Fish species (common name)	No of positive sample	Reference(s)
Freshwater	Aquarium fish breeding centre, Chennai	*Carassius auratus* (Gold fish)	Sub clinical	Jithendran *et al.* (2011)
Freshwater	Aquarium fish breeding centre, Kochi	*Carassius auratus* (Gold fish)	Clinical	Binesh (2013)
Freshwater	Aquarium fish breeding centre, Chennai	*Epalzeorhynchos frenatum*(Rainbow shark)	Sub clinical	Jithendran *et al.* (2011)
Freshwater	Aquarium fish breeding centre, Kochi	*Danio rerio* (Zebra fish)	Clinical	Binesh (2013)
Brackishwater/Marine	Hatchery/farm/wild	*Lates calcarifer*(Asian seabass)	Clinical & sub clinica	Azad *et al.* (2005) Parameswaran *et al.* (2008) Binesh and Jithendran (2013) John *et al.* (2014) Banerjee *et al.* (2014) Rajan *et al.* (2016) Jithendran *et al.*(2016)
Brackishwater	Farm/wild	*Mugil cephalus* (Mullet)	Clinical & sub clinical	Rajan*et al.* (2016)
Brackishwater	Farm/wild	*Chanos chanos*(Milk fish)	Sub clinical	Rajan*et al.* (2016)
Brackishwater	Farm	*Mystus gulio*	Sub clinical	Jithendran and Binesh (2013)
Brackishwater	Farm	*Liza parsia*	Sub clinical	Rajan *et al.* (2016)
Marine	Wild	*Lutjanus argentimaculatus* (Red snapper)	Sub clinical	CIBA (2017)
		Rachycentron canadum (Cobia)	Clinical & sub clinical	Unpublished data (2016)
		Trachinotus blochii (Silver pompano)	Clinical & sub clinical	Unpublished data (2016)
		Epinephelus tauvina (Grouper)	Clinical & sub clinical	Jithendran and Binesh (2013)
		*Sardinella longiceps**	Sub clinical	Jithendran and Binesh (2013)
		*Amblygaster clupeoides**	Sub clinical	Jithendran and Binesh (2013)
		*Thrissocles dussumieri**	Sub clinical	Jithendran and Binesh (2013)
		*Leiognathus splendens**	Sub clinical	Jithendran and Binesh (2013)
		*Upeneus sulphureus**	Sub clinical	Jithendran and Binesh (2013)
Marine	Hatchery	*Amphiprion sebae* (Clownfish)	Clinical	Binesh *et al.* (2013)

Figures

Figure1. Cage facilities of seabass nursery rearing

Figure 2. Pond culture of milkfish

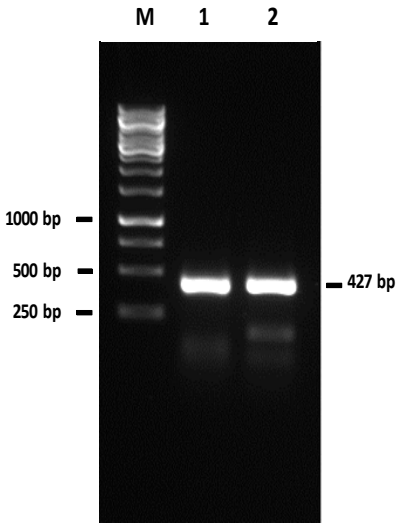

Figure 3. RT-PCR diagnosis is the most popular technique for confirmatory diagnosis of VNN in fish samples.

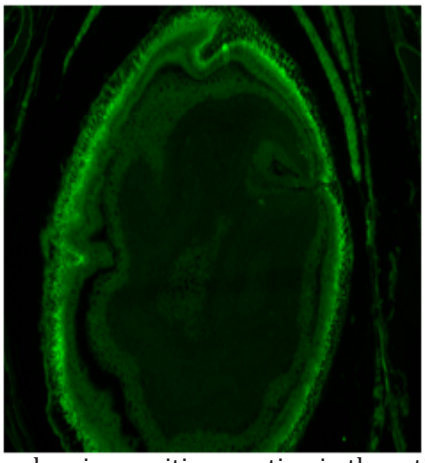

Figure 4. IFAT reaction showing positive reaction in the retina of the eye ball of seabass larva.

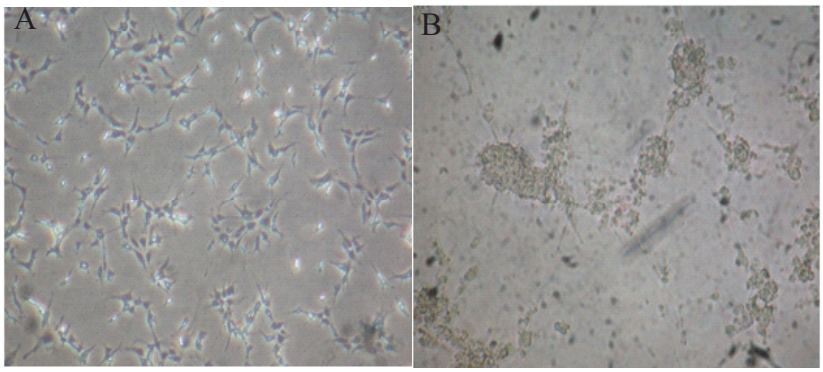

Figure 5. Monolayer cells of E-11 cell line inoculated with betanodavirus. Cells after 24 h (A) and 96 h (B) after inoculation of betanodavirus showing the progression of CPE.

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