

FIRST REPORT OF VIRAL NERVOUS NECROSIS IN ASIAN SEA BASS, *LATES CALCARIFER* CULTURED IN SRI LANKA

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ABSTRACT

Viral Nervous Necrosis (VNN) caused by Betanodavirus is a devastating disease in aquatic animals. The virus infects both marine and freshwater fish worldwide. Vacuolating necrosis of neural cells of the brain, the retina of the eye, and the spinal cord of the infected fish are the primary histological lesions of the condition. It causes up to 100% mortality in larvae and juvenile fish and can cause significant death in adult fish. The present study detected viral nervous necrosis in larvae and fry of Asian sea bass (*Lates calcarifer*) with progressive mortality of up to 95% in one week during the Northeast monsoon when the mean water temperature was 27 to 29°C. Histopathological examination of the moribund fish revealed extensive vacuolation and gliosis in the olfactory bulb, the optic lobe of the forebrain, and the inner and outer layer of the retina. Furthermore, tissues of the brain and the retina had intracellular inclusion bodies suggesting viral etiology, further justified by the negative results of the bacterial and parasitic examinations. The Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) test additionally confirms the etiology diagnosis using specific primers designed previously. The histopathology and RT-PCR results suggest that the mortalities of Asian sea bass were due to the VNN. The present finding is the first report of the VNN associated with mass mortalities in Asian seabass cultured in Sri Lanka. These crucial findings emphasize the need for quarantine and control strategies to prevent the spread of the virus and outbreak of the disease.

Keywords: Asian sea bass, RT - PCR, Sri Lanka, Vacuolating necrosis of neural cells, Viral Nervous Necrosis,

INTRODUCTION

Viral Nervous Necrosis (VNN), caused by Betanodavirus, is known as viral encephalopathy, and retinopathy (VER) is a devastating disease in Asian sea bass, especially in larvae and juveniles (Munday *et al.*, 1997; Yothikoshi and Inoue, 1990). The virus damages and destroys the fish's central nervous system, causing up to 100% mortality in marine (Bigarre *et al.*, 2009; Zorriehzahra *et al.*, 2019a), freshwater (Hedge *et al.*, 2003; Binesh *et al.*, 2013; Praveenraj *et al.*, 2018) aquaculture. Betanodavirus is non-enveloped and icosahedral with a diameter of 20–30 nm, with two positive-sense RNA strands known as RNA1 and RNA2. RNA1 encodes RNA-dependent RNA polymerase (RdRp), a mitochondrial enzyme responsible for viral replication (Nopadon *et al.*, 2009), and RNA2 encodes the capsid protein. The first nodavirus infection was detected in Japanese parrotfish (*Oplegnathus fasciatus*) (Yoshikoshi and Inoue, 1990). Then the disease was reported in barramundi (*Lates calcarifer*) farmed in Australia (Glazebrook *et al.*, 1990). The VNN has been found in up to 120 marine and freshwater fish species worldwide, including Asian seabass (*Lates calcarifer*) (Vela-Avitúa *et al.*, 2022).

The transmission of the VNN can be horizontal and vertical. Horizontal transmission happens from diseased fish, virus-carrier animals, contaminated water, trash fish, and cannibalistic fish (Cherif *et al.*, 2009; Mannin and Ransangan, 2011). On the other hand, vertical transmission from broodstock gonads and sperm where the betanodavirus can be detected. Similarly, the virus also presents in fertilized eggs and passes to the next generation (Kuo *et al.*, 2012). VNN can survive without a host for over a month and transmit to fish from water or other animals (Gomez *et al.*, 2011). The virus can infect fish between the temperature ranges of 16 to 30°C (Zorriehzahra *et al.*, 2019a). A temperature closer to 16 °C will result in the sub-acute form of sickness characterized by necrosis on the upper jaw and head areas. The acute phase of the disease has been linked to the elevated sea temperature up to 30°C when nervous indications arise (Le Breton *et al.*, 1997). The high-water temperature of more than 30°C inhibits viral proliferation in fish (Yuasa *et al.*, 2007). The water temperature and viral strains strongly influence the brain's viral load (Toffan *et al.*, 2016).

Several steps are involved in diagnosing the VNN in fish, including clinical signs, necropsy findings, molecular methods, and histopathology (Munday *et al.*, 2002; Hegde *et al.*, 2003; Yuasa *et al.*, 2007). The affected fish swim rapidly, spiralling, whirling, lying down at the bottom, and becoming dark-skinned (Yoshikoshi and Inoue, 1990). Further, the swim bladder contains severe hyperinflation in diseased juveniles (Zorriehzahra *et al.*, 2019b). Hemorrhages are found in the brain, liver, and spleen tissues (Yang *et al.*, 2022). Many reports showed the numerous molecular tools for detecting VNN in fish, which have become an effective and precise method for virus detection. The primary molecular method for diagnosing VNN is the laboratory's reverse transcription polymerase chain reaction (RT -PCR) (Grotmol *et al.*, 2000). The World Organization for Animal Health (OIE) consents to amplify RNA2 fragments as a routine diagnostic of VNN disease. However, due to low sensitivity, the nested RT - PCR could only detect the virus if the sample has a high viral load. Real-time quantitative RT-PCR assay (qRT-PCR) is a precise and powerful tool for the detection and quantification of betanodavirus (Dalla Valle *et al.*, 2005; Kuo *et al.*, 2012; Liu *et al.*, 2012). The characteristic histopathological lesions of VNN are severe degeneration, pyknosis, shrinkage, and basophilic cells in affected tissues and vacuolation of the central nervous system and retina of the affected larvae and juvenile fishes showing abnormal swimming behavior (Fukuda *et al.*, 1996; Bigarré *et al.*, 2009; Liu *et al.*, 2012). Zorriehzahra *et al.* (2019a) reviewed that the vacuolated cells and vacuoles are mainly present in the bipolar and ganglionic nuclear layer of the retina in the eyes. Gliosis in the central nervous system is another typical histological lesion associated with VNN in fish (Costa and Thompson, 2016). Histopathological examination of the tissues is a vital diagnostic tool for identifying infectious diseases, as conventional methods often fail to identify the organism for various reasons (Gupta *et al.*, 2009).

Mass mortality of Asian sea bass fries in hatcheries in Sri Lanka is suspected to be caused by Viral Nervous Necrosis (VNN), which can have significant ecological, economic, and scientific implications. This can result in substantial financial losses for hatcheries and aquaculture operations, impacting hatchery owners and the broader aquaculture industry. Identifying and mitigating VNN's causes can help safeguard the economic sustainability of the aquaculture sector and contribute to our understanding of aquatic diseases and their transmission. Controlling VNN in Asian sea bass fries can prevent the virus from spreading to other aquatic species, reducing the risk of disease outbreaks in the broader aquatic ecosystem. Addressing this complex issue is essential for the sustainability of aquaculture, fish well-being, and the availability of Asian sea bass as a food source.

Therefore, the investigation was carried out to identify the causative agents for mass mortality of Asian sea bass fry in the Sri Lankan hatchery and underlying factors influencing mortality.

METHODOLOGY

Mortalities of similar clinical signs were observed in the Asian sea bass hatchery located on the East coast of Sri Lanka from the first week of November 2018 to February 2019. There were two incidences of the outbreak during the sampling period. The first outbreak was observed in Mid November 2018, when the larvae on 14 days old. Twenty larvae with an average body size of (3-5 mm in length) were collected during the first outbreak, with significant progressive mortalities starting from 20 to 95% in one week. During the second outbreak in early January 2019, we collected another 20 Asian sea bass fries (28 days old) with an average body weight of 1.8 ± 1.6 g. Wet mount of the whole body of larvae and fries from both outbreaks were examined by light microscopy for the presence of parasites. The bacterial examination was also performed in larvae and fries by plating tissue samples on TSA (Oxoid, England) supplemented with 1.5% (w/v) NaCl and TCBS (Himedia, India) medium and incubated at room temperature and read every 24 hours for five days as described by Frerichs (1989). The larvae and fries suspected of VNN were brought to the laboratory in Liquid nitrogen. The Committee of Ethical Clearance of the Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Sri Lanka, granted approval for the study. The ethical clearance's approval number is VER 15-008.

Histopathological examination

The samples of moribund larvae and fry were fixed in 10% phosphate-buffered saline (PBS), and tissues were prepared according to standard procedures described by Gupta *et al.* (2009) for paraffin embedding and sectioned at 5 μ m before being stained with Haematoxylin and Eosin (H&E), particularly on the retina of the eye and the brain.

Reverse Transcriptase Polymerase Chain Reaction (RT -PCR)

Total RNA was extracted from whole Asian seabass larvae and fries using the Trizol reagent (Invitrogen, Carlsbad, USA). First-strand cDNA was synthesized using a SuperScript™ VILO™ cDNA Synthesis Kit (Invitrogen, USA). We used the primers (VNN -F1 : 5'GGATTTGGACGTGCGACCAA 3' and VNN -R1 : 5'CTGAATTTCGAACTCCAGTG 3') published by Intamaso *et al.* (2018). One microliter of reverse-transcribed cDNA was used as a template

in the polymerase chain reaction amplification. An aliquot of 25 µl of the overall reaction mixture was made up of 0.1 pg - 1 mg of RNA template, 0.4 M forward and reverse primer, 0.4 mM MgSO₄, 12.5 µl of reaction mix, and 1µL of Super Script® III One-Step RT-PCR System with Platinum® Taq DNA polymerase (Invitrogen, USA) as per the manufacturer's instructions. The PCR amplification reaction started with a denaturation stage at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min respectively, with the final extension step took place at 72 °C for 5 min. The PCR product was visualized by using 2% agarose gel electrophoresis stained with ethidium bromide. Materials from already confirmed VNN isolates, and healthy Asian sea bass fish served as the positive and negative controls.

RESULTS AND DISCUSSION

The catastrophic deaths of Asian sea bass larvae and fry at a hatchery in Sri Lanka were attributed to viral nervous necrosis because of the disease's clinical symptoms, histological findings, and PCR results. The clinical signs of the first outbreak on the 14 days old larvae were characterized by becoming transparent with the chromatophores retracted and floating on their side at the surface with a hyperinflated swim bladder (Figure 1 A), followed by progressive mortality from 20 to 95% in one week. In addition to these findings, the affected fish showed abnormal swimming backward, an enlarged swim bladder, uneven skin colour (Figure 1 B), and abdominal distension in another outbreak. These findings are similar to reports in the literature (Fukuda *et al.*, 1996; Bigarré *et al.*, 2009; Yang *et al.*, 2022). Also, mortalities were recorded during the Northeast monsoon in Sri Lanka, where the seawater temperature ranged from 27 to 29°C. The VNN virus can thrive in this temperature range and produce clinical illness (Zorrihahra *et al.*, 2019). However, the water temperature became more than 30°C during the dry season, and the virus could have been inactive to cause disease. Therefore, we did not observe any outbreaks when temperature exceed 29°C. However, Toffan *et al.* (2016) showed that nodavirus replication in vivo is a composite process regulated by both the genetic features of the viral strain and water temperatures. Based on similarities in the partial RNA2 sequences, betanodaviruses can be classified into four genotypes: 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), and the optimal temperatures for the growth of these viruses are 20–25°C (SJNNV), 25–30°C (RGNNV), 20°C (TPNNV), and 15–20°C (BFNNV) (Nakai *et al.*, 2009; Hata *et al.*, 2010). Generally, Asian sea bass, European sea bass, red spotted groupers, and other groupers are more

susceptible to RGNNV (Nakai *et al.*, 2009). Although we could not do the sequencing of the PCR isolates of our samples due to various limitations, anyone can come to the idea that the strain of betanodaviruses associated with mass mortalities in Sri Lanka could be an RGNNV as we found the outbreak when the temperature is 27 to 29 °C and the PCR product of 198 bp (Intamaso *et al.*, 2018) based on the recommendations by the OIE's (2019) for the confirmatory diagnosis of VNN. We used RT-PCR to extract total RNA from afflicted larval tissues and fries with clinical symptoms attributed to VNN. The amplification of tissues from 10 fries and ten larvae produced a target cDNA band on agarose gel electrophoresis of about 198 bp. The previously positive samples that produced 198 bp were used as a positive control for the subsequent PCR reaction (Figure 2). We used the previously published primers to identify RGNNV yielding 198 bp (Intamaso *et al.*, 2018).

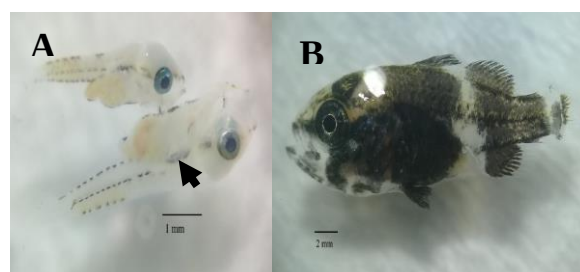


Fig. 1. The 14 days old Asian seabass larvae attributed the clinical signs suspected for VNN with transparent bodies floating on their side at the surface with a hyperinflated swim bladder (A) and 28 days old fry with uneven skin colour (B).

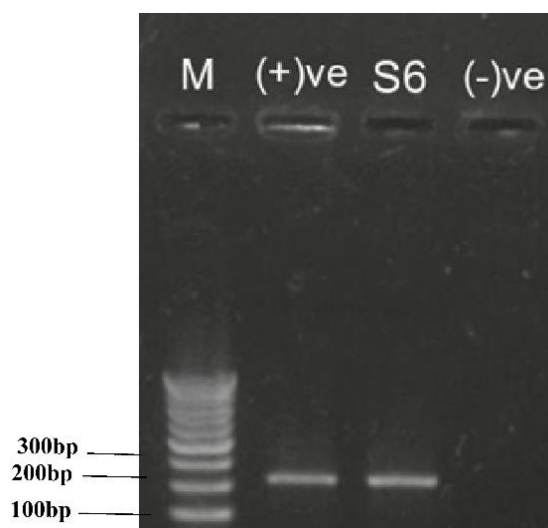


Fig. 2. Gel electrophoresis (2% agarose) for screening of VNN in affected Asian sea bass in hatchery by RT-PCR. Lane M- 100bp marker, Lane (+)ve: cDNA positive control, Lane S6: tissue from VNN infected fish, and Lane (-)ve: Negative control.

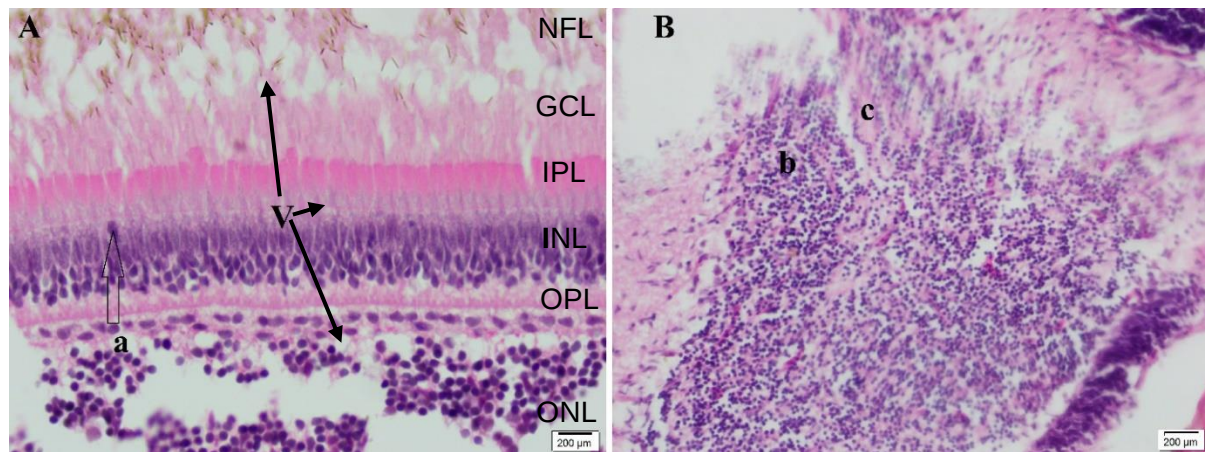


Fig. 3. Histopathological changes induced by viral nervous necrosis (VNN) in Asian sea bass (*Lates calcarifer*). A- shows the vacuoles (v) in the retina's nuclear, plexiform, and ganglion cell layers, and basophilic inclusion bodies in the outer nuclear layer (a). B- shows the presence of gliosis (b) and neuronophagia (c) in the brain. NFL – Nerve fiber layer, GCL- Ganglion cell layer, IPL- Inner plexiform layer, INL- Inner nuclear layer, OPL- Outer plexiform layer, ONL- Outer nuclear layer.

Furthermore, we only identified these viruses in the larvae and fries below five weeks of age, with a similar finding to Jaramillo *et al.* (2017). As the virus is age dependent, the mortality occurs up to 100% only in the larvae and fries when their age is up to five weeks of age. Afterward, the diseases remain subclinical, or the viruses in lower doses in juvenile and adult fish (Ariff *et al.*, 2019).

The histopathological examination of the present study revealed extensive vacuolation and neuronal degeneration of the retina of the eye and the olfactory lobe of the forebrain in all affected fish (Figure 3), as indicated by Azad *et al.* (2006). Interestingly, no parasites or bacteria associated with the disease were found in any of our samples. The lesions caused by betanodavirus can be seen in the histopathological section of different cells, including nerve cells, epithelial cells and myocardial cells and blood cells of juvenile and larval fish (Grotmol *et al.*, 1997; Tanaka *et al.*, 2001). However, the present study document the histopathological lesions in the eye and the brain as the primary target tissue of the fish is the nerve cells in the brain and eye (OIE, 2019). The retina of some infected fish showed marked necrosis in the inner nuclear layer, outer and inner plexiform layers, and ganglion cell layer in the retina (Fig 3-A). The outer and inner nuclear layers, outer and inner plexiform layers, and ganglion cell layers of the retina had numerous large vacuoles formed by fragmentation and degeneration of infected cells. The outer nuclear layers of the retina had characteristic homogenous basophilic intracytoplasmic inclusion bodies (Figure 3 A -a). Further to this finding of the inclusion bodies in eye tissues in the present study, Yuwanita *et al.* (2013) found characteristic inclusion bodies in the liver, kidney, and eye tissues. The brain histopathological section showed clear gliosis in the olfactory lobe, a common finding in the CNS of fish affected by VNN (Shetty *et al.*, 2012; Costa and Thomson, 2016). Vacuolar degeneration was severely

detectable in dendritic cells extending into the outer molecular layer of the cerebellum. Gliosis and neuronophagia, characterized by the accumulation of microglia around degenerated or necrotic neurons, were seen in the olfactory lobes of the forebrain, as mentioned by Zorriehzakra *et al.* (2019b). We observed the necrosis of the brain at 14 DPH (Fig 3-B), which is supported by the finding of Azad *et al.* (2006), where the first signs of brain necrosis can be observed from 6dph. In the present study, the retina of affected fish had a large number of vacuoles in the inner and outer layers of nuclear and plexiform, and ganglion cell layers, as identified by Zorriehzakra *et al.* (2019a) and Azad *et al.* (2006).

To our knowledge, this is Sri Lanka's first report of Asian sea bass VNN infection. Control strategies such as vaccination quarantine measures, biosecurity, and getting fry and fingerlings from disease-free stock must be adapted to control and eradicate the disease (Jeney, 2017; Hegde *et al.*, 2003). Therefore, there is a possibility that VNN can be spread to ornamental fish, and it is essential to take necessary control measures to eradicate the disease in other fish species.

CONCLUSIONS

The findings of histopathology, RT-PCR, and history and clinical signs confirm that VNN is associated with mass mortality of the Asian sea bass larvae and the fry on the Eastern coast of Sri Lanka. In the present study, diseased larvae and fries collected during mass mortalities were characterized by degeneration of nervous tissues (necrosis, vacuolation, and gliosis) and the presence of inclusion bodies in the histopathological examination of whole larvae and fries. However, the occurrence is highly related to the water temperature. The findings are emphasized that the Asian sea bass hatcheries should be aware of this disease, especially during the Monsoon in Sri Lanka.

It is necessary to conduct further research, including the other freshwater and marine species, and should be related to detailed molecular characterization.

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