

Detection of betanodavirus in wild caught fry milk fish, *Chanos chanos*, (Lacepeds 1803)

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Betanoda virus was detected in wild caught milk fish fry (*Chanos chanos*) exhibiting Beta noda virus was detected in wild caught milk fish fry (*Chanos chanos*) showing typical clinical symptoms and signs of viral nervous necrosis (VNN)/ Viral encephalopathy and retinopathy, (VER) from the bank of Matchlipattinam, Andhra Pradesh, India during March 2014. Mortality of these fry was observed within a few days after stocking and attained 100 % in next few days. The larvae infected by the virus showed typical swimming behavior which included positioning in a vertical manner with a whirling type movement; sinking to the bottom, darting or swimming in a corkscrew fashion; belly-up at rest, abnormal body coloration (pale or dark) and over inflation of swim bladder. Severe pathological changes in the form of vacuolation and necrosis in brain and other organs such as spinal cord, and retina of the eyes further confirmed the infection by this virus. The earliest occurrence of diseases was less than 30 days of post-hatch, less than 35 mm total length. Usual mortality rate varied from 50-80 % with highest mortality rate up to 100 % in wild caught milk fishes. Amplification of the virus RNA2 region by RT-PCR of Beta noda virus yielded a product of 430 bp.

[**Keywords:** White spot syndrome virus, *Penaeus monodon*. Brood stock, Andaman.]

Introduction

The commonly known as milkfish, *Chanos chanos* is belongs to family Chanidae, and seven extinct milk fish species in five additional genera have been reported globally. This fish is the national fish of Philippines and locally known as *bangus*. It is one of the important food fishes in brackishwater aquaculture in India, little is known about its parasites and their potential to cause disease.

Spiral swimming pattern and vacuolation of nerve and retina cells are the major character of viral nervous necrosis (VNN) virus infection, and has been reported as a major pathogen from a number of commercially important finfishes species throughout the world. Based on several properties such as the type of nucleic acid it contain, typical genomic structure, properties of the proteins and serological reactions, the virus has been placed in the family Nodaviridae^{1,2}. Except some differences in the nucleotide sequences, majority of VNNs share a similar genomic structure^{3,4}. This viruses have been classified into four genotypes based on the RNA sequence of the T4 variable region of their capsid protein, and were

named after the fish species from which they were first isolated i.e. Tiger puffer nervous necrosis virus (TPNNV), Barfin flounder nervous necrosis virus (BFNNV), Striped Jack nervous necrosis virus (SJNNV) and Red-spotted grouper nervous necrosis virus (RGNNV). During 2000, Nodaviridae family were classified into two genera, the Betanodavirus group that genus nodaviruses affecting fish as host, and the second genus Alphadodavirus that includes all the insect host nodaviruses⁵. It has been reported in larvae of different fishes including Japanese parrotfish, *Oplegnathus fasciatus*⁶, Barramundi, *Lates calcarifer* Bloch⁷, from hatchery produced sea bass larvae as a first report from India⁸, Redspotted grouper, *Epinephelus akaara*⁹, Turbot, *Scophthalmus maximus* (L.)¹⁰, and European sea bass, *Dicentrarchus labrax* (L.)¹¹. Mass mortality of fish larvae due to VNN infection in the brackishwater hatchery system has been reported which ultimately leads serious economic losses. In this present study a betanoda virus infection from wild milk fish, *Chanos chanos* were identified using RT-PCR technique and histopathological techniques.

Materials and Methods

About two hundred fifty numbers of fries of *Chanos chanos* were collected from the bank of Matchlipattinam of the Bay of Bengal, Andhra Pradesh, India during March 2014. Live fish were brought to the laboratory and immediately examined for parasites. The infected fries with spiral movement behavior and skeletal deformities were stored in 10 % Neutral Buffered Formalin solution for regular histology. Eyes & brain of fish specimens were used for viral detection through PCR technique.

Fish nodavirus contains two single-stranded, positive-senses, nonpolyadenylated RNAs (RNA1 and RNA2). One of the structural proteins of the virus is encoded by RNA2. Two of the already published primers; consisting of a reverse primer (5'-CGA-GTC-AAC-ACG-GGT-GAA-GA-3'), and a forward primer (5'-CGT-GTC-AGT-CATGTG-TCG-CT-3'), were used for the amplification of a sequence (about 430 bases) targeting the RNA2 by RT-PCR.

The whole fish sample (0.1 g) was homogenised with 0.5 ml double distilled water previously treated with diethyl pyrocarbonate (0.1%) and was subjected to centrifugation for 10 mins at 10,000 g. The supernatant was mixed with 0.04 ml proteinase K (1mg/ml) and 0.04 ml 1 % sodium dodecyl sulfate (SDS), and again was incubated at 37°C for 30 minutes. After centrifugation, total nucleic acids were extracted using phenol-chloroform method. The total nucleic acids were preheated at 90°C for 5 minutes and further incubated at 42°C for 30 minutes in 20 µl of PCR buffer (10 mM Tris/HCl, pH 8.3, 50 mM KCl) containing 2.5 U murine leukaemia virus (M-MLV) reverse transcriptase (USB), 1.0 U ribonuclease inhibitor, 0.5 µM reverse primer, 1 mM each of four deoxynucleotide triphosphates (dNTP) primers and 5 mM MgCl₂. The mixture was incubated at 99°C for 10 minutes to inactivate the reverse transcriptase, and was then diluted five-fold with PCR buffer containing 0.1 µM forward primer, 2.5 U Taq polymerase and 2 mM MgCl₂. The mixture was incubated in thermal cycler programmed for one cycle at 72°C for 10 minutes and 95°C for 2 minutes, then 25 cycles at 95°C for 40 seconds, 55°C for 40 seconds, and 72°C for 40 seconds, and finally held at 72°C for 5 minutes. Amplified DNA (430 bp) products were analyzed by agarose gel electrophoresis.

Results

Milk fish fries infected by the virus showed typical swimming behavior which included positioning in a

vertical manner with a whirling type movement; sinking to the bottom, darting or swimming in a corkscrew fashion; belly-up at rest, abnormal body coloration (pale or dark) and over inflation of swim bladder¹². The earliest occurrence of diseases was less than 30 days of post-hatch, less than 35 mm total length. Usual mortality rate varied from 50-80 % with highest mortality rate up to 100 % in wild caught milk fishes. (Fig.1, A-D) & Table. 1). Clinical symptoms showed the targeted system is nervous system. Infected wild milk fish fries showed typical abnormal swimming patterns as clinical signs (Fig.1, A-D). Due to hyperinflation of the swim bladder, the infected animals were noticed at the water surface. (Fig.1.C). Uncontrolled swimming/ spinning symptomatology



(A)



(B)

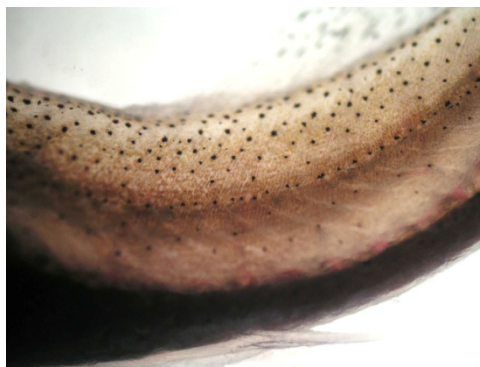


(C)

were clearly exhibited as traumatic lesions in many of the fishes. Abnormal body pigmentation with either lightening or darkening was also observed in the fishes of present investigation (Fig.1, D).



(D)



(E)

Fig.1 — (A&B) Deformity of vertebra column of milk fish infected with betanoda virus. (C) Surfacing, Darkening, traumatic lesions caused by swimming into structures, hyperinflation of the swim bladder in younger stages, and moderate to high mortalities in tanks (D) Changes in Eye, skin pigmentation, either darkening or lightening were seen in present milk fishes.

Histopathology by Light Microscopy:

As has been reported earlier, vacuolation associated with necrosis of the central nervous systems located in eye and brain were clearly observed through histological investigation (Fig. 2A). In Atlantic halibut, vacuolation of neurons both in spinal ganglia and cephalic ganglia of the sympathetic nervous system were reported¹³. However, in another observation the optic tectum was found to be very rarely affected in adult European seabass¹⁴ whereas the Japanese parrot fish exhibited lesions in the spinal ganglia also⁶. Milk fish larvae/fries were more severely affected than juveniles. The most important characteristic of VNN infection at cell level is the presence of vacuoles, lesions in the grey matter of the brain and other lesions includes pyknosis, karyorrhexis and karyolysis of neural cells^{6,15}. Cerebral blood vessel lesions were reported in barramundi, *L. calcarifer*^{16, 7, 14} reported swelling of the endothelium. Basophilic, intracytoplasmic inclusions, in Japanese parrot fish⁶ and, Brown spotted grouper, *Epinephelus malabaricus* (Bloch & Schneider)¹⁷ and European sea bass¹⁸ have been reported in brain cells.

In present studies the most distinctive finding is the presence of vacuolation and necrosis in the brain, and retina of the eyes of milk fishes (Fig. 2A & 2B).

Thirty infected milk fish fries were tested betanodavirus positive by PCR test. All samples showed a product at the expected size of about 430 bp as shown as betanodavirus specific RT-PCR confirmed VNN samples in Fig.3. In this present studies, Betanodavirus (VNN) was first reported in wild caught milk fish fry through RT-PCR test (Fig.3).

Table 1 — Important features of VNN/VER of larval and juvenile fishes.

Fin fishes	Earliest occurrence of disease	Usual onset of disease	Latest occurrence of new outbreaks	Usual mortality rate	Highest mortality rate
<i>Lates calcarifer</i>	9 days post-hatch	15-18 days post-hatch	≥24 days post-hatch	50-100%/ month	100% in <1 month
<i>Dicentrarchus labrax</i>	10 days post-hatch	25-40 days post-hatch	Body weight 400-580 g	10%/month	-
<i>Oplegnathus fasciatus</i>	6-25mm total length	-	<40 mm total length	-	Up to 100%
<i>Epinephelus akaara</i>	14 days posthatch (7-8 mm total length)	9-10 mm total length	<40 mm total length	80%	Up to 100%
<i>Epinephelus malabaricus</i>	-	20-50 mm total length	-	50-80%	-
<i>Pseudocaranx dentex</i>	1 day post-hatch	1-4 day post-hatch	<20 days post-hatch (8 mm total length)	100%	-
<i>Scophthalmus maximus</i>	<21 days post-hatch	-	Body weight 50-100 mg	-	Up to 100%
<i>Chanos chanos</i> (Present study)	<30 days post-hatch	20-30 day Post-hatch	<35 mm total length	50-80%	Up to 100%

#: Source: Manual of Diagnostic Tests for Aquatic Animals Fourth Edition, 2003, Office international des épizooties •12, rue de Prony • 75017 Paris • France

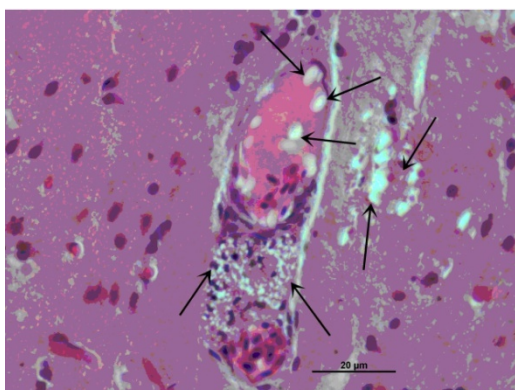
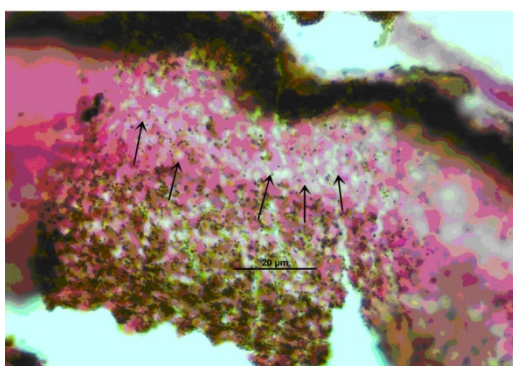


Fig. 2 — A. Light microscopy of brain tissue sections of milk fish, *Chanos chanos* showed severe vacuolation (arrows) in larva (H&E Staining, 100 x).



(B)

Fig. 2 — B Light microscopy of eye tissue sections of milk fish, *Chanos chanos* showed severe vacuolation (arrows) in larva (H&E Staining, 100 x).

Gel picture VNN OIE RT- PCR:

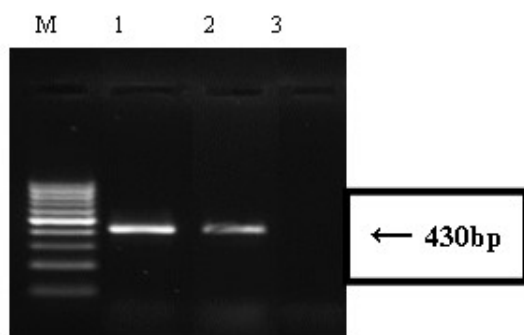


Fig. 3 — Agarose gel electrophoresis visualization of amplified (430 bp) RT-PCR product of the T4 region of the nodavirus: Positive control (Lane 1), Milk fish fries sample (Lane 2), Negative control (Lane 3) and Molecular marker size (100 bp DNA ladder) (M).

Conclusion

In present studies the most distinctive histopathological finding of betanodaviruses is the

presence of vacuolation and necrosis in the brain and retina of the eyes of milk fishes. Molecular detection of Betanodaviruses (VNN) using RT-PCR in wild caught milk fish fries. This is the first report of natural susceptibility of milk fish to betanodavirus, causing acute VNN leading to mass mortality. The study suggests the need for a proper surveillance protocol and biosecurity protocol for milk fish breeding and seed production. Biosecurity measures for milk fish includes use of chlorinated water sources; testing of potential parent fishes through advanced molecular techniques and frozen or live foods; disinfection of fertilized eggs; and testing of diseased fish / moribund fish are to be implemented in milk fish hatchery.

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