

NOTE

# Mortality due to viral nervous necrosis in zebrafish *Danio rerio* and goldfish *Carassius auratus*

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**ABSTRACT:** Heavy mortality was observed in an experimental lot of 2 ornamental fish species, zebrafish *Danio rerio* (F. Hamilton, 1822) and goldfish *Carassius auratus* (Linnaeus, 1758). The fishes showed typical symptoms of viral nervous necrosis before death. Gross morphological examination revealed no visible lesions except in the brain, visible as a creamy opaque patch through the dorsal side of the head. Parasitic and bacteriological analysis revealed no pathogenic agents. Histopathological analysis revealed severe vacuolation in the brain and spinal cord of the samples. A fragment within the variable region of genomic RNA2 of betanodavirus was amplified from the samples by reverse transcription polymerase chain reaction using specific primers designed previously. The analysis suggests that the observed mortality in the fishes was due to betanodavirus infection. This is the first report of natural infection of betanodavirus in laboratory fishes causing viral nervous necrosis leading to mortality. The observation is alarming, as the ornamental fish culture and trade is being popularized in India where the fatal disease may cause severe setbacks in the industry. It emphasizes the need for quarantine and control strategies to prevent the spread of the virus and outbreak of the disease.

**KEY WORDS:** VNN · VER · Betanodavirus · Zebrafish · Goldfish · RGNNV

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## INTRODUCTION

Viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER) is a threat to commercial finfish aquaculture, causing high mortality in larval and juvenile fish and resulting in important economic losses (Yoshikoshi & Inoue 1990, Munday et al. 2002). The disease is caused by betanodavirus of the family *Nodaviridae* with 4 distinct species recognized thus far (Thiéry et al. 2012) based on the similarities of the variable region in the viral coat protein gene (nt 604–1030) observed previously (Nishizawa et al. 1997). The 4 betanodavirus species show some degree of temperature preference: Red spotted Grouper Nervous Necrosis Virus (RGNNV), Striped Jack Nervous Necrosis Virus (SJNNV), Tiger Puffer Ner-

vous Necrosis Virus (TPNNV) and Barfin Flounder Nervous Necrosis Virus (BFNNV) favour temperatures of 25–30, 20–25, 20 and 15–20°C, respectively, in cell culture conditions. The disease was first reported in hatchery facilities of Japanese parrot fish *Oplegnathus fasciatus* from Japan (Mori et al. 1992). Later it was reported in over 40 fish species from marine, brackish and freshwater environments, and the number of susceptible fish hosts is steadily increasing (Munday et al. 2002, Maltese & Bovo 2007). Goldfish *Carassius auratus* (Linnaeus, 1758) and zebrafish *Danio rerio* (F. Hamilton, 1822) were previously reported to be non-susceptible to betanodavirus (Furusawa et al. 2007), but experimental infection trials for zebrafish were standardized later (Lu et al. 2008). In this paper, I describe the natural susceptibility of

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zebrafish and goldfish to betanodavirus leading to mortality. A standard diagnostic approach was adopted for this study, and the aetiology was confirmed by gross observations, histopathology and molecular diagnosis. An experimental challenge protocol for zebrafish was also standardized.

## MATERIALS AND METHODS

Samples of zebrafish and goldfish were procured from local ornamental fish stores in the Cochin region of India. They were maintained in an aquarium for 2 wk; morbidity and mortality up to 32% began thereafter. Samples were collected for gross observation, histopathology and molecular diagnosis. Wet mounts of skin and whole mounts were prepared and examined for parasites. Aseptically collected samples were analysed for common opportunistic pathogenic bacteria. For histopathology, freshly dead samples were fixed in neutral buffered formalin (NBF) for 24 h, washed with sterile PBS and re-fixed in NBF for another 24 h. Slides for light microscopy were prepared following standard histopathology procedures (Bullock 2000). Total RNA was extracted from the samples using TRIZOL™ Reagent (Invitrogen) as per the manufacturer's instructions. Molecular analyses were performed following the specific nested reverse transcription PCR (rt-PCR) assay described by Gomez et al. (2004). The amplified PCR products were resolved in 1.5% TBE agarose gel stained with ethidium bromide. Specific DNA bands were excised, extracted using PCR/gel extraction columns (Qiagen) and sequenced. The nucleotide sequences were analysed by nucleotide BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), aligned with representative nucleotide sequences from the 4 species of betanodavirus: SJNNV (NC\_003449), TPNNV (NC\_013461), BFNNV (NC\_013459) and RGNNV (AY324870, AB373029) retrieved from the NCBI GenBank database. An unrooted tree (unweighted pair group method with arithmetic mean, UPGMA) was created in MEGA version 5 (Tamura et al. 2011) to assess the phylogenetic relationship of the isolates. The full length sequences of viral RNA2 of the present isolates were deposited in GenBank (GenBank IDs: BVNZ1, KC136294; BVNZ2, KC136295).

## RESULTS AND DISCUSSION

The fishes exhibited typical VNN symptoms like erratic swimming behaviours; however, no lesions

were noticed in gross examination. Bacterial studies ruled out the presence of opportunistic bacterial pathogens. Wet mounts revealed that both ecto- and endoparasites were absent from the specimens. 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 (Yoshikoshi & Inoue 1990, Jung et al. 1996, Grotmol et al. 1997, Johansen et al. 2004, Tanaka et al. 2004, Azad et al. 2006). In the present study, prominent vacuolations were seen in the brain and bipolar and ganglion layer of the eye (Fig. 1a,b), and a specific rt-PCR confirmed the aetiological agent as betanoda-

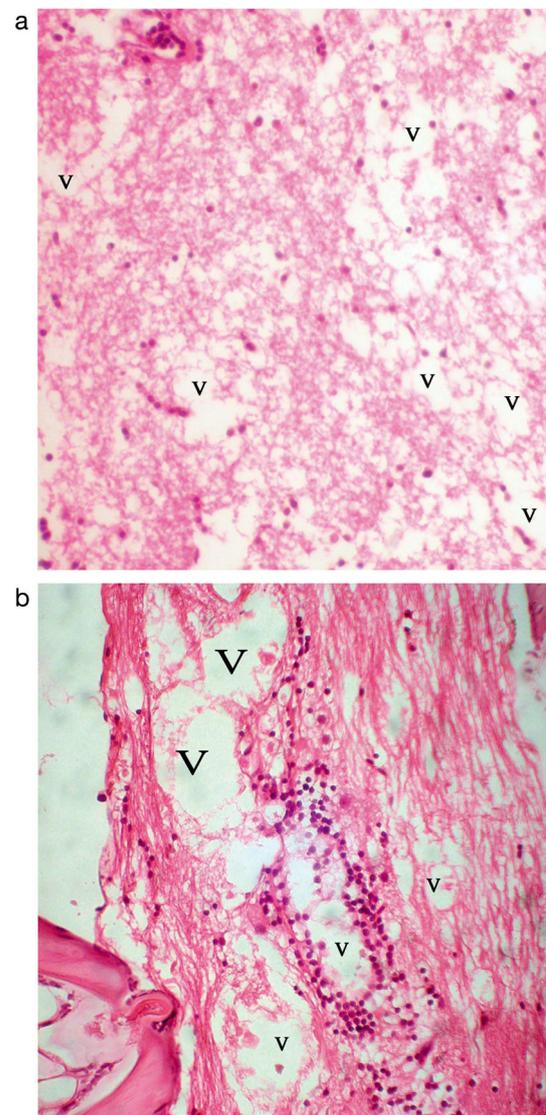


Fig. 1. *Danio rerio* and *Carassius auratus*. Severe vacuolation (V) in (a) the brain of zebrafish (200×) and (b) the spinal cord of goldfish (400×)

virus (Fig. 2). The specific rt-PCR amplified the variable region of viral RNA2, which has extensively been used to infer the relatedness and phylogeny of betanodavirus (Nishizawa et al. 1997, Sakamoto et al. 2008). Nucleotide sequencing of PCR products followed by BLAST analysis confirmed the origin of the sequence to betanodavirus with high similarity hits (97–98%). In the phylogenetic tree constructed based on this segment with similar segments from the members of the 4 known species of betanodavirus, the present isolates were placed with the RGNNV cluster (Fig. 3). The affinity of the present isolates to RGNNV was again supported, as they were isolated from an environment with a temperature of  $30 \pm 2^\circ\text{C}$ . Among the 4 species of betanodavirus, only RGNNV prefers this temperature range (OIE 2012).

The present observation of viral nervous necrosis in 2 freshwater ornamental fish species raises concerns regarding the host range of the virus in India where VNN has rarely been reported. This is the first report of natural susceptibility of these fishes to betanodavirus causing acute VNN leading to mortality. The study suggests the need for a proper surveillance protocol for ornamental fish breeding and trade considering the high risk of introduction of the virus to otherwise naïve areas by translocation.

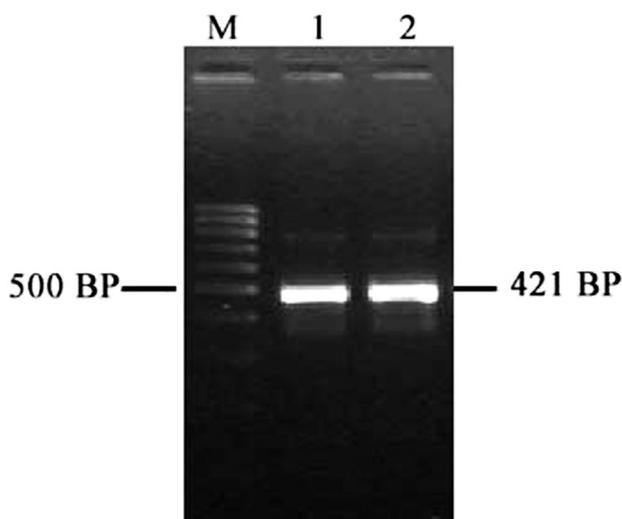


Fig. 2. Agarose gel electrophoresis showing betanodavirus specific amplicons. Lane M: 100 bp marker, Lane 1: zebrafish, Lane 2: goldfish

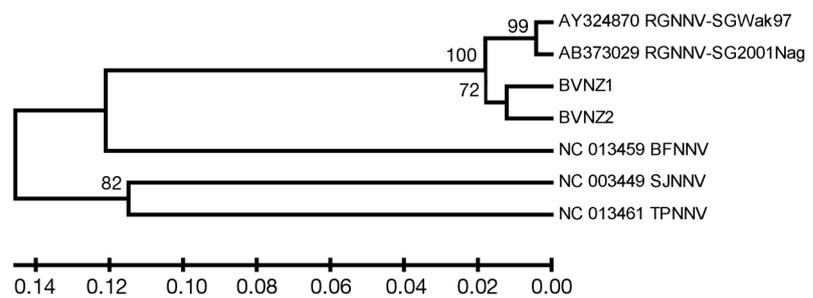


Fig. 3. Unrooted UPGMA tree showing the comparative phylogenetic position of betanodavirus isolates in this study (BVNZ1 and BVNZ2). The isolates were clustered together with the Red spotted Grouper Nervous Necrosis Virus (RGNNV) species with a high bootstrap value

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