

Nodavirus Infection in Freshwater Ornamental Fishes in India: Diagnostic Histopathology and Nested RT-PCR[#]

K. P. JITHENDRAN¹, M. S. SHEKHAR, S. KANNAPPAN and I. S. AZAD²

¹Aquatic Animal Health and Environment Division,
Central Institute of Brackishwater Aquaculture
75, Santhome High Road, Chennai - 600 028, India

²Fish Pathologist, Mariculture and Fisheries Department,
Kuwait Institute of Scientific Research (KISR), Salmiya
Kuwait

[#]Paper presented in 'First International Symposium on Viral Nervous Necrosis of Fish' held at Hiroshima (Japan) during 28 Nov. to 1 Dec., 2006.

Abstract

Betanodaviruses are the causative agents of the disease known as viral nervous necrosis (VNN), or viral encephalopathy and retinopathy (VER) in a variety of cultured marine fishes. Approximately 40 species of marine finfish species are known to be affected with this disease with recent introduction in freshwater and aquarium fish as well. In the present study, we examined two species of freshwater aquarium fishes, viz. gold fish (*Carassius auratus auratus*), rainbow shark (*Epalzeorhynchos frenatum*) and its colour varieties (albino rainbow shark) reported to be affected with nervous system associated symptoms in a fresh water aquarium fish rearing centre near Chennai (India) by histopathology and nested RT-PCR. The only visible clinical symptom was the appearance of a congested brain which appeared as a translucent patch clearer to the naked eye through the transparent skin and cartilage of the young fry of albino forms and the low-grade morbidity among adult fish. Amplification of 430 and 280 bp by 2-step RT-PCR was achieved using primer sets which are known to detect all the genotypic variants of fish nodavirus in the Asian region. Histopathology of these fishes also revealed typical lesions of VNN in eye and brain tissues. No gill lesions or other abnormalities in the internal organs were detected at necropsy examination. To our knowledge, this is the first description of a natural infection of VNN in freshwater aquarium fishes in India causing morbidity and mortality.

Introduction

Fish nodaviruses, members of the family *Nodaviridae*, are the causal agents of a highly destructive disease in approximately 40 species of marine finfish species worldwide (Munday et al. 2002; Maltese and Bovo, 2007). Nodaviruses are small (25-34 nm) icosahedral viruses that infect the central nervous system causing disease known as viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER). VNN outbreaks result in high mortalities up to 100% mainly in hatchery-reared larvae and juveniles, although adults can also be affected (Munday et al. 2002, Pirarat et al. 2009). VNN is generally diagnosed by the demonstration of characteristic histopathological findings such as vacuolation and necrosis of cells of brain and retina combined

*Corresponding author. E-mail address: kpjithendran@yahoo.com

with positive immunohistochemistry for nodaviruses and RT-PCR (OIE, 2006). In the present study, we examined two species of the freshwater aquarium fishes, viz. gold fish (*Carassius auratus auratus*), rainbow shark (*Epalzeorhynchus frenatum*) and its colour varieties (albino rainbow shark) reported to be affected with nervous system associated symptoms by histopathology and nested RT-PCR.

Materials and Methods

Fish samples

Case I : In the present case, two species of the freshwater aquarium fish, viz. gold fish (*Carassius auratus auratus*), rainbow shark (*Epalzeorhynchus frenatum*) and its colour varieties (albino rainbow shark) were reported to be affected with nervous system associated symptoms in a freshwater aquarium fish rearing centre near Chennai (India). Broodstock fish of both species (n= 6) with an average length of about 12 cm and weight 8.5 gm reared at 26°C were brought alive to the laboratory under aeration. Fishes were acclimatized and maintained in rectangular glass tanks of 50 L capacity filled with filtered and aerated freshwater at 28-30°C.

Clinical history

The main clinical signs include less feed intake, descaling and the infected fish settled at the bottom with dropsy in the abdominal region. A couple of fish showing clinical symptoms were examined thoroughly for any bacterial and parasitic infections. On necropsy examination, the only visible clinical symptom was the appearance of congested brain which appeared as a translucent patch clearer to the naked eye through the transparent skin (Fig. 1) and cartilage of the young fry of albino forms and the low-grade morbidity among adult fish. The fish in different tanks with various age groups exhibited low-grade morbidity and mortality of 2-3 fish per day over a period of 14 days. About 14 fishes died from a stock of 280 fishes. The clinical course before mortality includes erratic swimming behavior, isolation, change of body coloration, loss of appetite and emaciation before succumbing to death.

Case II : Apparently healthy specimens of gold fish larvae bred naturally in an aquarium tank were collected. One of the adult fish found lethargic in the same tank among eight adult fish with symptoms such as erratic swimming, whirling with belly upside were also collected for laboratory investigation.

**A** Gold fish**B** Rainbow shark**C** Albino form of rainbow shark

Fig. 1. Different species of aquarium fishes with nervous system associated disease (A, Gold fish (*Carassius auratus auratus*); B, Rainbow shark (*Epalzeorhynchus frenatum*) and C, albino form of the Rainbow shark (*Epalzeorhynchus frenatum*). [Note clearly visible whitish patches above the area of brain]

Laboratory Investigation

Wet mounts of scrapings from the skin, fins and gills of different species and age group of fish were prepared and examined under stereo zoom microscope. Similarly, all the visceral organs were also dissected out and examined for any internal parasites and subjected to bacteriological studies. For histological studies, the brain, eye, kidney, spleen, gut, heart, liver and gills were fixed in 10 % neutral buffered formalin and processed for paraffin wax sections. Sections (4-5 μm) were stained using haematoxylin and eosin and examined under light microscope. Photomicrographs were taken using an Olympus digital camera C7070 fitted to Olympus CX41 microscope. Electron microscopy of brain sample was done as per the protocol described by Azad et al. (2005) at Kuwait Institute of Scientific Research (KISR), Kuwait using Joel microscope JEM-1200. The brain or other pooled organs were also collected aseptically from the remaining fish samples, and stored at -80°C until use. *RT-PCR*.

Total RNA was extracted as per manufacturer's instruction from the brain, spleen and gills of adult fish and whole larvae using Nucleospin RNA II kit (Macherey-Nagel). cDNA synthesis was carried out using Robust II RT-PCR kit (Finnzymes) with M-MuLV reverse transcriptase at 42°C for 1 hr followed by PCR amplification. PCR amplification was done using primers (Table 1) designed by Nishizawa et al. (1994) and Thiery et al. (1999). Amplification cycle consisted of initial denaturation for 94°C for 2 min, followed by 30 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and final extension at 72°C for 5 min. Nested PCR was conducted using the protocol described above using primer set NFRG and NRFG with the target product of 280 bp. The PCR products were then analysed by 2% agarose gel electrophoresis.

Table 1. Primers used for RT-PCR

Primer	Sequence	Amplicon size (bp)	Reference
F2	5' CGT GTC AGT CAT GTG TCG CT 3'	430 (RT-PCR)	Nishizawa et al. (1994)
R3	5' CGA GTC AAC ACG GGT GAA GA 3'		
NFRG	5' ACC TGA GGA GAC TAC CGC TC 3'	280 (Nested)	Nishioka et al. (2010)
NRFG	5' CAG CGA AAC CAG CCT GCA GG 3'		
FP	5' GTT CCC TGT ACA ACG ATT CC 3'	294 (RT-PCR)	Thiery et al. (1999)
RP	5' GGA TTT GAC GGG ACT GCT CA 3'		

Results and Discussion

In the present study, the occurrence of a nervous system associated disease was investigated in a stock of freshwater aquarium fish species (*viz.* gold fish, *Carassius auratus auratus*, rainbow shark (*Epalzeorhynchus frenatum*) and its colour varieties (albino rainbow shark). On microscopic examination, no parasitic infections were detected. No gill lesions or other abnormalities in the internal organs were detected on necropsy examination. Similarly no dominant bacteria were isolated. Under light microscope, mild to moderate vacuolation was observed in the brain and retina of both affected fishes gold fish and rainbow shark (Figs. 2 and 3), though the extent of lesions varied considerably from fish to fish. Histologically, all other organs were apparently normal.

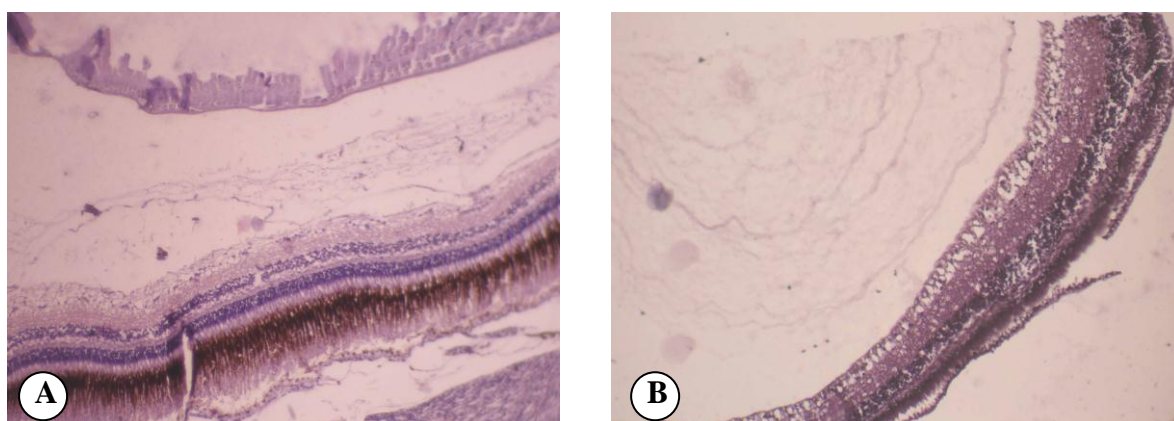


Fig. 2. Histological section of the eye showing mild vacuolation in the retina of the infected rainbow shark (B) along with control (A) - 100x

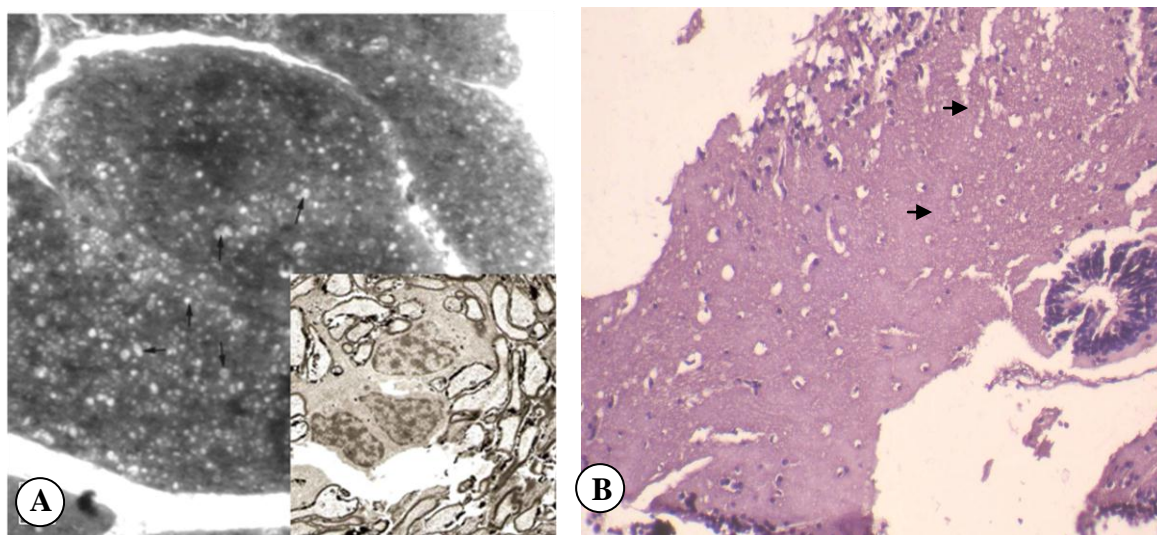


Fig. 3. (A). Extensive vacuolation of the brain tissue (arrows) in semi-thin sections (uranyl acetate stained) 10x; Inset, SEM of nodavirus infected fish brain; (B). Vacuolation of the brain tissue (arrows) in histological section (H&E) 200x

RT-PCR

The results of the PCR using different primer sets are shown in Fig. 4. Majority of the fish samples drawn from Case I, were negative on RT-PCR, but showed single DNA band of the expected amplicon at 280 bp in two-step PCR (Table 2). However, the 430 bp PCR products failed to re-amplify on the same primer sets though the amplification of 280 bp products in nested PCR was consistent using the first step product as template. These results suggest that the fishes were latently infected with betanodavirus. Both the larvae as well as adult gold fish from Case II also showed positive result in RT-PCR showing VNN specific amplicon of 294 bp using the primer developed by Thierry et al. (1999).

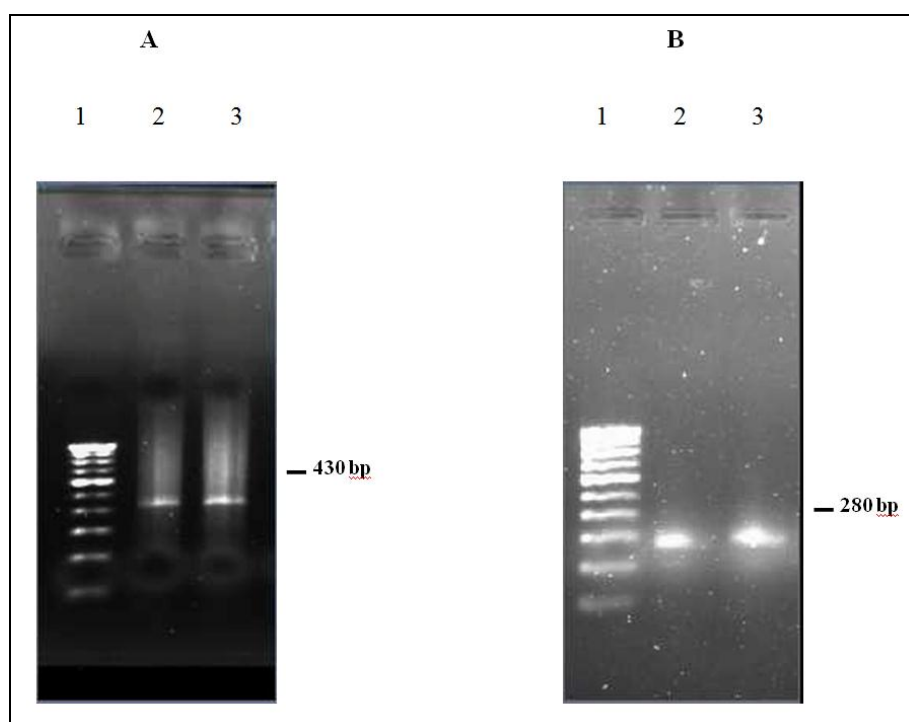


Fig. 4. Detection of VNN by RT-PCR (A) and nested PCR (B) amplifying products of 430 and 280 bp (Lane 1, 100 bp ladder, 2 and 3 RNA samples from brain and spleen, respectively).

Table 2. Details of the freshwater aquarium fishes examined by PCR

Fish species	Group	Source	No. examined	No (%) of positive sample
Gold fish	Adult	Hatchery	6	2
Rainbow shark	Adult	Hatchery	6	4
Gold fish	Adult	Aquarium	1	1
Gold fish	Larval sample	Aquarium	1	1
Total			14	8 (57%)

Recently, latent infection of the nodavirus has been reported in various parts of the world and is considered as a major source of viral infections under culture conditions. The virus has started showing its presence in various low value fishes (trash fish), fresh water fish species and even marine invertebrates like mussels, lobsters etc. (Hegde et al. 2003, Chi et al. 2003; Gomez et al. 2006) without any apparent clinical symptoms. Furusawa et al. (2006) and Lu et al. (2008) also reported betanodavirus infection in the freshwater model fish medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*), respectively. Recently, Furusawa et al. (2007) screened 21 freshwater ornamental fish species, for their susceptibility to red spot grouper necrosis virus (RGNNV) and demonstrated that four were susceptible, ten less susceptible and seven resistant to the viral infection. The virus was specifically located in the brain, spinal code, and retina of the infected fish, similar to the pattern of infection in naturally infected marine fish. It was also reported that most of the freshwater species studied to date revealed no obvious lesions in the central nervous system or retina except in one, threadfin rainbow fish sample, though clinical symptoms of erratic swimming and considerable morbidity and viral titers were found in susceptible fish. Collectively, rainbow fish and medaka appear suitable for good experimental hosts for betanodavirus studies.

In India, nodavirus infection has been reported in hatchery reared seabass larvae (Azad et al. 2005; Parameswaran et al. 2008). Recently, many unexplained mortality among larval and juvenile stages has been reported in hatchery facilities in India. Such unexplained mortality associated with increase in water temperature was suspected to be due to viral infections with characteristic swimming behaviour has been noticed in captive broodstock of seabass. However, in India, the nodavirus infections and its epizootiology in wild population of fish or in captivity have not been well studied and documented. A cell culture system using sea bass kidney cells has been reported to be permissive to the nodavirus strain isolated from adult sea bass (Sahul Hameed et al. 2006).

The present clinical symptoms resembling that of nodavirus infection in aquarium fish is of significance, which is likely to play a crucial role in the spread of VNN due to the commercial movement of live animals from one region to the other. Further, many broodstock fishes were found to be with sub-clinical symptoms and bred on commercial basis. It is often a practice to culture seabass (*Lates calcarifer*) and pearl spot (*Etroplus suratensis*) in freshwater and low-saline conditions. Moreover, juvenile *Etroplus suratensis* is being used as freshwater aquarium fish as well. Under these conditions, the possible cross infectivity of virus between freshwater

and brackish water fish species needs to be ascertained under natural conditions. In the present study, we have no evidence on the source of the virus.

Viral nervous necrosis is considered as one of the emerging diseases in marine fishes in Asiatic region. Their invasion in freshwater and brackish water aquaculture has become evident in many areas. Rainbow shark and gold fish are ornamental fish species of the family Cyprinidae that are economically important as aquarium fish in the region. They are spawned locally and sold marketable sizes (~3-5 cm of total length) mainly for ornamental purpose. Sub-clinically infected fish may pose a threat to the ornamental fish industry due to the potential source of infection to the young ones and other susceptible species. To our knowledge, this is the first description of natural infection of VNN in freshwater aquarium fishes in India causing low grade morbidity and mortality in the brood stock. Further studies are required to investigate the potential role of the sub-clinically infected wild fish population and epizootiology in the context of the farmed fish in the region.

Acknowledgements

The authors are thankful to the Director, Central Institute of Brackish water Aquaculture (Chennai) for providing facilities. Thanks are also due to Mr. V. Rajaraman, Raj Aqua Clinic for providing the experimental animals from aquarium fish rearing centre at Kolathur, Chennai (India).

References

- Azad, I.S., M.S. Shekhar, A.R. Thirunavukkarasu, M. Poornima, M. Kailasam, J.J.S. Rajan, S.A. Ali, M. Abraham and P. Ravichandran. 2005. Nodavirus infection causes mortalities in hatchery produced larvae of *Lates calcarifer*: first report from India. *Diseases of Aquatic Organisms* 63: 113-118.
- Chi, S.C., J.R. Shieh and S.J. Lin. 2003. Genetic and antigenic analysis of betanodaviruses isolated from aquatic organisms in Taiwan. *Diseases of Aquatic Organisms* 55: 221-228.
- Furusawa, R., Y. Okinaka, and T. Nakai. 2006. Betanodavirus infection in the freshwater model fish Medaka (*Oryzias latipes*). *Journal of General Virology* 87: 2333-2339.
- Furusawa, R., Y. Okinaka, K. Uematsu and T. Nakai. 2007. Screening of freshwater fish species for their susceptibility to a betanodavirus. *Diseases of Aquatic Organisms* 77: 119-125.
- Gomez, D.K., D.J. Lim, G.W. Baek, H.J. Youn, N.S. Shin, H.W. Youn, J.H. Hwang and S.C. Park. 2006. Detection of betanodaviruses in apparently healthy aquarium fishes and invertebrates. *Journal of Veterinary Science* 7: 369-374.
- Hegde, A., H.C. Teh, T.J. Lam and Y.M. Sin. 2003. Nodavirus infection in freshwater ornamental fish, guppy, *Poecilia reticulata*-comparative characterization and pathogenicity studies. *Archives in Virology* 148: 575-586.
- Lu, M.W., Y.M. Chao, T.C. Guo, N. Santi, O. Evensen, S. K. Kasani, J.R. Hong, J.L. Wu. 2008. The interferon response is involved in nervous necrosis virus acute and persistent infection in zebrafish infection model. *Molecular Immunology* 45: 1146-1152.
- Maltese, C. and G. Bovo. 2007. Viral encephalopathy and retinopathy. *Ittiopatologia* 4: 93-146.
- Munday, B.L., J. Kwang, and N. Moody. 2002. Betanodavirus infections of teleost fish. A review. *Journal of Fish Diseases* 25: 27-142.

- Nishioka, T., K. Mori, T. Sugaya, N. Tezuka, T. Takebe, H. Imaizumi, K. Kumon, S. Masuma and T. Nakai. 2010. Involvement of viral nervous necrosis in larval mortality of hatchery-reared Pacific bluefin tuna *Thunnus orientalis*. *Fish Pathology* 45: 69-72.
- Nishizawa, T., K. Mori, T., Nakai, I. Furusawa and K. Muroga. 1994. Polymerase chain reaction (PCR) amplification of RNA of striped jack nervous necrosis virus (SJNNV). *Diseases of Aquatic Organisms* 18: 103-107.
- OIE. 2006. *Manual of Diagnostic Tests for Aquatic Animals* (Fifth Edition) Office International des epizooties, Paris, France. pp. 169-175.
- Parameswaran, V., S. Rajesh Kumar, V.P. Ishaq Ahmed and A.S. Sahul Hameed. 2008. A fish nodavirus associated with mass mortality in hatchery-reared Asian Sea bass, *Lates calcarifer*. *Aquaculture* 275: 366-369.
- Pirarat, N., A. Ponpornpisit, T. Traithong, T. Nakai, T. Katagiri, M. Maita, M. Endo. 2009. Nodavirus associated with pathological changes in adult spotted coral groupers (*Plectropomus maculatus*) in Thailand with viral nervous necrosis. *Research in Veterinary Science* 87: 97-101.
- Sahul Hameed, A.S., V. Parameswaran, R. Shukla, I.S. Bright Singh, A.R. Thirunavukkarasu and R.R. Bhonde. 2006. Establishment and characterization of India's first marine fish cell line (SISK) from the kidney of sea bass (*Lates calcarifer*). *Aquaculture* 257: 92-103.
- Thiery, R., J.C. Raymond, J. Castric. 1999. Natural outbreak of viral encephalopathy and retinopathy in juvenile sea bass, *Dicentrarchus labrax*: study by nested reverse transcriptase-polymerase chain reaction. *Virus Research* 63: 11-17.