JARQ 38 (1), 69 – 73 (2004) http://www.jircas.affrc.go.jp

Mass Mortalities Associated with Viral Nervous Necrosis in Hatchery-Reared Sea Bass *Lates calcarifer* in the Philippines

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Abstract

Viral nervous necrosis (VNN), also known as viral encephalopathy and retinopathy (VER), is an emerging disease affecting larvae and juveniles of many farmed marine fish species in Asia, Australia, Europe and North America. Mass mortality occurred in 14-day old larval sea bass *Lates calcarifer* at a hatchery in the Philippines associated with clinical signs such as abnormal swimming behavior and pale-gray discoloration of the body. Histological investigations in moribund fish revealed marked vacuolation in the retina and brain. Cytopathic effects (CPE) were observed in SSN-1 cells inoculated with the tissue filtrate of affected sea bass. A piscine nodavirus, the causative agent of VNN, was detected in the affected tissues and SSN-1 cells inoculated with the tissue filtrate of affected fish by RT-PCR. Electron microscopy revealed non-enveloped viral particles, 22–28 nm in diameter, in the cytoplasm of the brain and retina of affected fish and in the cytoplasm of VNN-infected SSN-1 cells after CPE appeared. These results indicate that mass mortality of sea bass larvae in the Philippines was caused by a piscine nodavirus.

Discipline: Aquaculture
Additional key words: VNN, VER, piscine nodavirus

Introduction

In Southeast Asia, sea bass *Lates calcarifer*, orange-spotted grouper *Epinephelus coioides*, and mangrove red snapper *Lutjanus argentimaculatus* are important species as they command a high market price[8]. In cultured larvae and juveniles of sea bass, a disease characterized by abnormal swimming behavior and heavy mortalities has been reported in Australia, Indonesia and Singapore[2,6,10,15,17]. The disease marked by vacuolations in the brain and retina is associated with viral infection, and has been described as viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER)[14,16].

In the Philippines, hatchery-reared sea bass larvae had been suffering from a disease characterized by similar clinical signs and heavy mortalities since 1991 (Lavilla-Pitogo, personal communication). However, because diagnostic techniques for VNN were not yet established, the causative agent could not be identified at that time. In June 2001, at the hatchery of the Aquaculture Department of the Southeast Asian Fisheries Development Center (SEAFDEC AQD), acute mortality characterized by anorexia, spiral swimming behavior and pale-gray discoloration of the body were observed in sea bass larvae. Viral etiological studies using histopathology, cell culture utilizing SSN-1 cell line, reverse transcriptase polymerase chain reaction (RT-PCR) and

This study was supported by funds of the Japan International Research Center for Agricultural Sciences (JIRCAS) Research Project "Studies on Sustainable Production Systems of Aquatic Animals in Brackish Mangrove Areas" and the Japanese Trust Fund (GOJ-TF) to SEAFDEC AQD.

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Received 27 August 2003; accepted 4 November 2003.

electron microscopy demonstrated that the mortality was caused by VNN. This is the first documented report on VNN in sea bass in the Philippines.

Materials and Methods

1. Fish samples

Sea bass larvae were reared in 250 L fiberglass tanks at a stocking density of 20 fish/L. The larvae were fed on rotifer *Brachionus* sp. enriched with *Chlorella* starting at day 2, enriched *Artemia* at day 12 and minced fish according to their developmental stage. The rearing water was not changed for the first 6 days and from the 7th day onward, 50% was changed by bottom siphoning.

2. Parasitological and microbiological examination

The skin, fins and gills of moribund fish were subjected to parasitological examination by light microscopy. Bacteriological examination was carried out by plating kidney material on Nutrient Agar (NA: Merck and BBL) supplemented with 1.5% NaCl and thiosulphate citrate bile salt sucrose agar (TCBS: Merck). Plates were incubated at 28ºC for 24 h.

3. Histological examination

Moribund fish were fixed in 10% buffered-formalin and tissues were processed by routine methods for paraffin-embedding and sectioned at 5 µm prior to staining with haematoxylin and eosin (H & E).

4. Virus isolation

Virus isolation trials were performed by inoculation of the filtered homogenate of the head portion on a 24 h fish cell line monolayer (SSN-1, BF-2, EPC) according to standard procedures, and incubation at 25ºC for 10 days.

5. Virus detection

The RNAs from eye and brain samples of sea bass larvae and the supernatant of SSN-1 cells inoculated with the tissue filtrate of affected sea bass were extracted using TRIzol (Gibco) following the manufacturer's protocol.

Detection of piscine nodavirus was performed by RT-PCR amplification as previously described[12]. The T4 (430 bp) region of SJNNV (striped jack nervous necrosis virus) coat protein gene was selected as the target sequence for PCR amplification. The PCR products were analyzed by gel electrophoresis on 2% agarose (Agarose 1000, Gibco) and stained with ethidium bromide.

6. Electron microscopy

For transmission electron microscopy (TEM), the brain and eyes of affected fish and the VNN-infected SSN-1 cells showing cytopathic effects (CPE) were fixed in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde, post-fixed with 1% osmium tetroxide and embedded in Quetol 812. Sections were stained with 1% uranyl acetate and 1% lead citrate and examined with an electron microscope (JEOL-100S).

Results

1. Fish samples

During the rearing period, the larvae started to show disease signs at day 14 after hatching (body length: 4–7 mm). Mortality reached 100% within 3 days. The larvae displayed anorexia, pale-gray pigmentation of body, loss of equilibrium and corkscrew-like swimming behavior prior to death. At necropsy, no marked gross pathological lesions were observed. No pathogenic bacteria and parasites were observed in affected fish.

2. Histological examination

Histopathological changes were observed in the retina and brain (Fig. 1). The brain showed severe vacuolation, necrosis and degeneration of nerve cells. Vacuolation characterized the pathological change in the retina. No significant histological lesions were found in the gills, fins, skin and other internal organs of affected fish.

3. Isolation of virus

No CPE were detected in both BF-2 and EPC cell cultures inoculated with the filtrate of tissue samples from moribund fish. However, CPE were observed in SSN-1 cells 4 days after inoculation with the tissue fil-

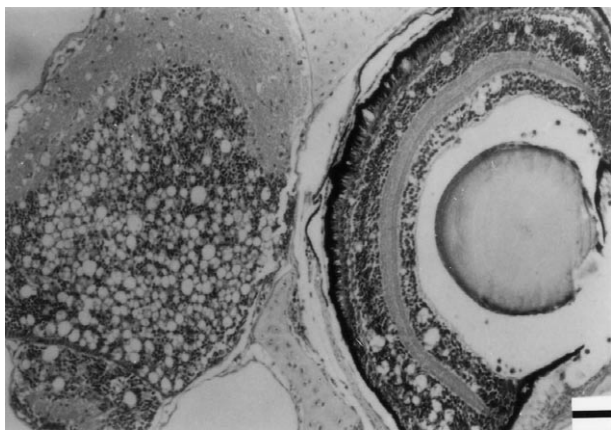

Fig. 1. Extensive vacuolation in the retina and brain of naturally infected sea bass larvae
H & E stain. Scale bar = 100 µm.

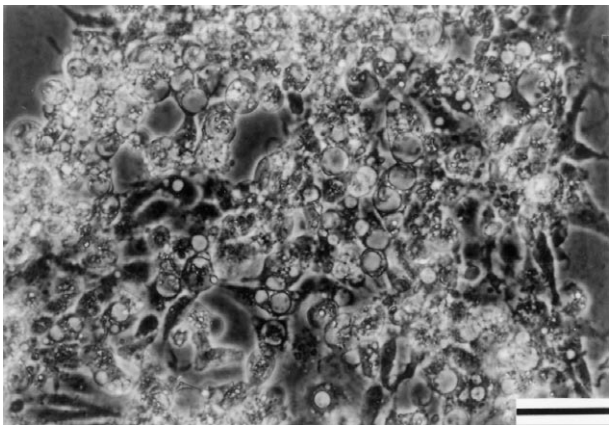

Fig. 2. Cytopathic effects in SSN-1 cells caused by a virus isolated from naturally infected sea bass larvae
SSN-1 cells were inoculated with the filtrate of diseased fish tissue samples. Scale bar = 50 μm.

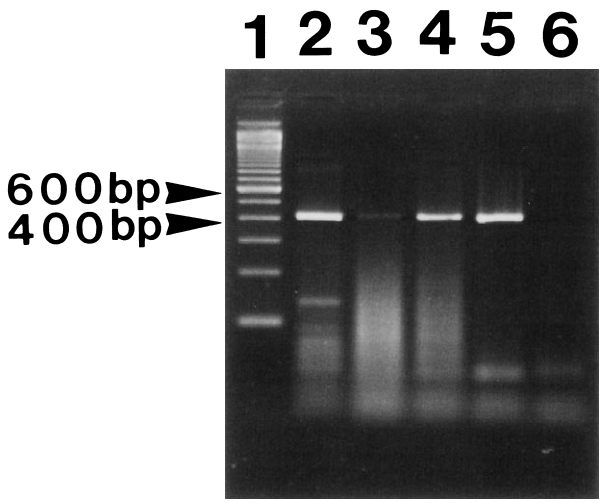

Fig. 3. Agarose gel electrophoresis of PCR amplification products (T4 region)
Lanes: 1, DNA marker; 2, positive control; 3, tissue homogenate of naturally infected sea bass larvae; 4 & 5, supernatants of SSN-1 cell culture inoculated with the tissue filtrate; 6, negative control.

trate of affected fish, characterized by cytoplasmic vacuole formation (Fig. 2) and eventual lysis of the monolayer beginning from 5 to 6 days post-inoculation. Following 2 to 4 passages of supernatants to fresh cultures, the same pattern of CPE was observed. Virus titer of the filtered tissue homogenates of moribund fish was $10^{8.3}$ $TCID_{50}$ /g.

4. Detection of virus

By RT-PCR amplification with primers for SJNNV RNA 2 gene, a 430 bp amplicon (Fig. 3) was detected in naturally infected fish and the culture supernatant of SSN-1 cells inoculated with the tissue filtrate of affected fish.

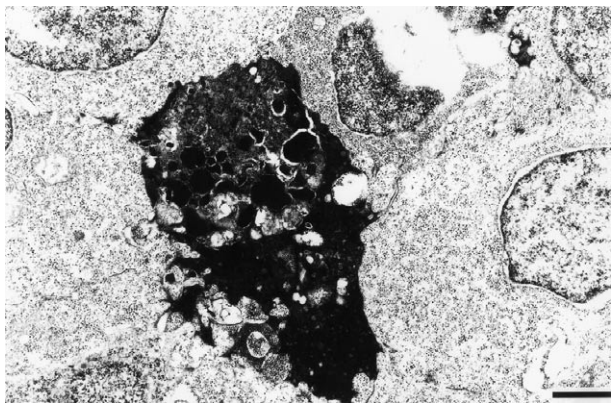

Fig. 4. Electron micrograph of the brain of naturally infected sea bass larvae showing free virus particles in the cytoplasm of nerve cell
Scale bar = 1 μm.

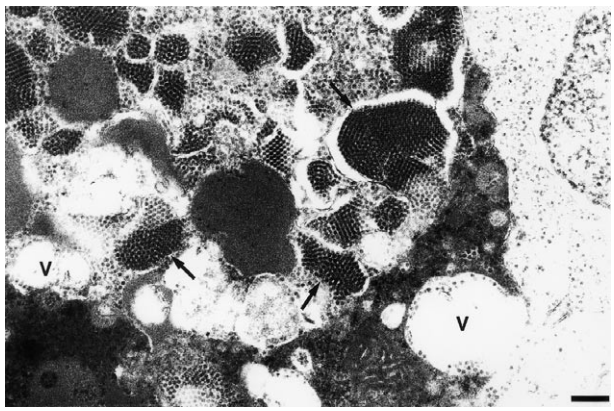

Fig. 5. Electron micrograph of the brain of naturally infected sea bass larvae showing viral aggregates (arrows) with vacuolation (V)
Scale bar = 200 nm.

5. Electron microscopy

In the brain and retina of naturally infected sea bass, intracellular viruses occurred as free single particles (Fig. 4), aggregates (Fig. 5) or crystalline arrays (Fig. 6) with cytoplasmic inclusions. The viruses were non-enveloped, spherical to icosahedral and measured 22 to 28 nm in diameter. In the cytoplasm of SSN-1 cells showing CPE, virus particles containing similar morphological characteristics were observed in abundance (Fig. 7).

Discussion

This paper describes the causative agent associated with the mortalities of hatchery-reared sea bass *Lates calcarifer* larvae cultured in the Philippines. The clinical

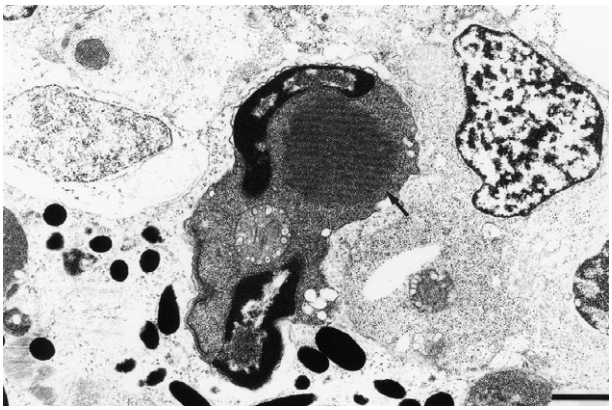

Fig. 6. Electron micrograph of a crystalline array of virus (arrow) with cytoplasmic inclusion in affected retina of naturally infected sea bass larvae
Scale bar = 1 μm.

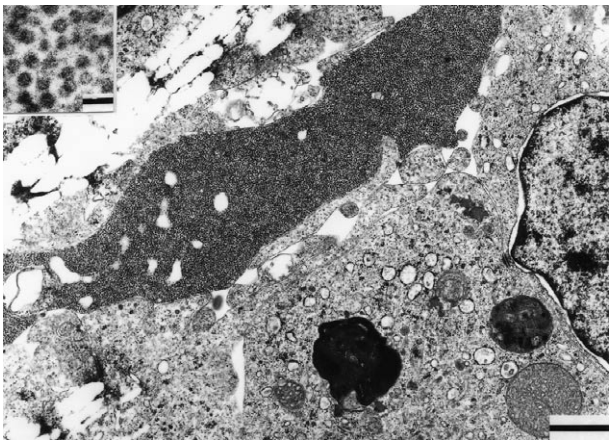

Fig. 7. Electron micrograph of SSN-1 cells on 5 days after inoculation with tissue filtrate from naturally infected sea bass larvae
Scale bar = 1 μm. Inset figure shows virus particles (Scale bar = 50 nm).

signs and pathological changes observed in affected sea bass larvae are consistent with those described in other VNN-affected fish species[9]. Infected SSN-1 cell cultures developed extensive CPE, and the isolated virus was identified as a piscine nodavirus based on the results of RT-PCR and electron microscopy performed on the brain and retina of naturally infected fish and infected cell cultures. The size of the PCR product is consistent with that of other piscine nodaviruses using the same primer set. The size and intracellular location of viral particles detected in the brain and retina of naturally infected sea bass and cell cultures agree with those described for piscine nodaviruses[11]. Taken together, these results clearly indicate that the mass mortality of the sea bass larvae was caused by VNN. Piscine nodaviruses are divided into 4 genotypes based on the sequence of the coat protein gene[13]. Genotyping of the present isolate is now being carried out.

In the Asia-Pacific region, piscine nodavirus infection has been associated with high mortalities in cultured species such as sea bass[2,6,10,15,17] and grouper[1,3–5,18]. In the Philippines, the first documented outbreak of VNN occurred in orange-spotted grouper[7]. Consequently, this disease has the potential to cause severe economic losses in the region. In this regard, examination of the susceptibility of other aquaculture-important fish species to this piscine nodavirus is urgently needed. At present, we have an ongoing study on this aspect.

In order to gain a better understanding of infection mechanisms and to establish suitable control strategies, future investigations must focus on the epidemiology and genotyping of the piscine nodavirus in the Asia-Pacific region.

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