ABSTRACT—Mass mortality occurred in 34-day old larval orange-spotted grouper *Epinephelus coioides* reared at a hatchery in the Philippines with clinical signs such as anorexia and abnormal swimming behavior. Histopathology of moribund fish demonstrated marked vacuolation of the brain, spinal cord and retina. Cytopathic effects were observed in SSN-1 cells inoculated with the tissue filtrate of affected grouper. Electron microscopy revealed non-enveloped virus particles measuring 20 to 25 nm in diameter in the cytoplasm of degenerated SSN-1 cells. Piscine nodavirus (betanodavirus), the causative agent of viral nervous necrosis (VNN), was detected in the affected tissues and SSN-1 cells inoculated with the tissue filtrate of affected fish by RT-PCR. This is the first record of VNN in the Philippines.

**Key word:** betanodavirus, *Epinephelus coioides*, VNN, VER, Philippines

Viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER) caused by piscine nodavirus was first described in Japanese parrotfish *Oplegnathus fasciatus* in Japan and barramundi *Lates calcarifer* in Australia. Since then, the disease has been reported in a variety of cultured marine fish species in Europe, Asia, Australia and North America. The disease usually occurs in larvae and juveniles resulting in high mortalities. Affected fish exhibits erratic swimming behavior with associated necrosis and vacuolation of the brain, spinal cord and retina. At present, the causative agents of VNN or VER are classified to the Betanodavirus of Nodaviridae.

The Aquaculture Department of the Southeast Asian Fisheries Development Center (SEAFDEC AQD) in Iloilo, Philippines is culturing different marine fish species including orange-spotted grouper *Epinephelus coioides*. In April 2001, high mortality has been observed among hatchery-reared larvae of orange-spotted grouper. Mortality was 5–10% daily and reached 100% within 10 days. Viral etiological study using histopathology, cell culture with SSN-1 cell line, reverse transcription polymerase chain reaction (RT-PCR) and electron microscopy demonstrated that the mortality was caused by VNN.

Grouper larvae were reared in 5 metric ton concrete tanks at a stocking density of 50,000/tank. The temperature and salinity of the rearing water were 24.5–28.0°C and 22–27‰, respectively. At day 34 after hatching, the larvae began to show disease signs which included reduced feeding activity, darkened pigmentation, lethargy and abnormal swimming behavior such as rotating, spinning, and horizontal looping. However, at necropsy, no marked gross pathological lesions were observed.

The fish was fixed in 10% buffered formalin. The samples were processed using standard histological technique, embedded in paraffin wax, sectioned at 5 μm and stained with haematoxylin and eosin (H & E). Histopathological examination by light microscopy revealed heavy vacuolation in the brain, spinal cord and retina (Fig. 1). Vacuolation in the retina was more prominent than that of the brain. There were no apparent histopathological changes in the gills and other internal organs of affected fish.

The head portion of affected fish were dissected and homogenized at a dilution of 1:10 w/v in Hanks’ balanced salt solution. Homogenates were centrifuged (1500 g, 10 min at 4°C) and the resulting supernatants passed through 0.45 μm membrane filter before inoculation on cell cultures. The EPC and BF-2 cell lines were cultured in a 24-well tissue culture plate at 20°C using...
Eagle’s Minimum Essential Medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin). The SSN-1 cell line was cultured in a 24 well tissue culture plates at 25°C using Leibovitz L-15 medium containing 10% FBS and antibiotics. The monolayer covering 70–80% of the plate was inoculated with the tissue filtrate. The cultures were thereafter incubated at 25°C and observed daily for cytopathic effects (CPE). No CPE were detected in both BF-2 and EPC cell cultures inoculated with the filtrate of tissue samples from moribund fish. CPE were observed in SSN-1 cells two days after inoculation with the filtrate of affected grouper. This was characterized by cytoplasmic vacuole formation and eventual lysis of the monolayer beginning from 4 to 5 days post-inoculation. Following 2 to 4 times inoculation of supernatants to fresh cultures, the same pattern of CPE was observed.

Virus titration of fish homogenate was carried out using SSN-1 cells by limited dilution assay. Based on the appearance of CPE after a 10-day incubation at 25°C, the 50% tissue culture infectious dose (TCID$_{50}$) was calculated. Virus infectivity of the filtrated tissue homogenates of moribund fish was 10$^{10.0}$ TCID$_{50}$/g.

The RNAs from eye and brain samples of grouper larvae and the supernatant of SSN-1 cells inoculated with the tissue filtrate of affected grouper were extracted using TRIZol (Gibco) following manufacture’s protocol. Detection of the virus by RT-PCR amplification was carried out according to the procedure described. The T4 (430 bases) region of the coat protein gene of betanodavirus was used as the target sequence for PCR amplification. The PCR products were analyzed by gel electrophoresis using 2% agarose (Agarose 1000, Gibco) and stained with ethidium bromide. An expected PCR product of about 430 base pairs was observed in samples from naturally affected fish and the culture supernatant of SSN-1 cells inoculated with the tissue filtrate of affected fish (Fig. 2).

The VNN-infected SSN-1 cells showing CPE were fixed for electron microscopy, and processed using standard technique. Sections were stained and examined with an electron microscope (JEOL-100S). Small, spherical, non-enveloped virus particles, 20–25 nm in diameter, arranged in paracrystalline arrays or in membrane-bounded vesicles were abundantly observed in the cytoplasm of SSN-1 cells on 5 days after inoculation (Fig. 3). The size and intracellular location of viral particles detected in cell cultures agree with those described for piscine nodaviruses in SSN-1 cells.

The clinical signs and histopathological lesions observed in the present orange-spotted grouper are very similar to those described in other VNN-affected fish species. 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. Furthermore, challenge tests using the isolated virus reproduced the same clinical signs and the virus was reisolated from experimentally infected fish (Maeno et al., unpublished data). Taken together, these results clearly indicate that the mass mortality of the larval grouper was caused by VNN. This paper is the first documented outbreak of VNN among hatchery-reared larvae of $E$. coioides in the Philippines.

Piscine nodavirus infection has been associated with high mortalities in cultured grouper species in Southeast Asia, such as brown-spotted grouper $E$. malabaricus, greasy grouper $E$. tauvina, red spotted grouper $E$. akaara and carpet cod $E$. fuscogutatus, and humpback grouper $Cromileptes altivelis$, and consequently, has the potential to cause severe economic loss in aquaculture. In this connection, examination of the susceptibility of other important aquaculture fishes to the present virus is urgently needed. The study is also

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**Fig. 2.** Agarose gel electrophoresis of PCR amplification products (T4 region). Lanes: (1) DNA marker; (2) tissue homogenate 1 (naturally affected orange-spotted grouper); (3) tissue homogenate 2 (naturally affected orange-spotted grouper); (4) the supernatant of culture of SSN-1 cells inoculated with the filtrate of tissue samples; (5) positive control (SJNNV-infected striped jack larvae); (6) negative control

**Fig. 3.** Electron micrograph of SSN-1 cells on 5 days after inoculation with the filtrate of diseased fish tissue samples. bar = 1 μm, [bar = 50 nm in insetted figure]
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...ongoing in our research group. The results of the present study also highlight the need for continued epidemiological surveillance of piscine nodaviruses and the development of effective control measures. Thus, screening of broodstock and their corresponding larvae for VNN is imperative to prevent the further spread of this disease.

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