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Nodavirus associated with pathological changes in adult spotted coralgroupers (*Plectropomus maculatus*) in Thailand with viral nervous necrosis

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ABSTRACT

The present study characterized viral nervous necrosis in sea cage-reared adult spotted coralgroupers (*Plectropomus maculatus*). Histopathological study showed extensive vacuolation and neuronal necrosis of the olfactory bulb and the optic lobe of the forebrain and the inner and outer nuclear layer of retina. Mild necrosis was observed in the spinal cord. Homogeneous intranuclear inclusion bodies were noted in the hyperplastic and hypertrophic glandular epithelial cells of the swim bladder suggesting viral etiology. Etiological diagnosis of VNN was confirmed by RT-PCR, immunohistochemistry and *in situ* hybridization. The immunohistochemistry and *in situ* hybridization gave strongly positive staining in the same area of the infected cells of the brain, spinal cord and retina correlating with histopathological changes. No positive reaction was detectable in the affected gas glandular epithelium and other organs, confirming the consistent neurotropism of this nodavirus. Nodavirus was mainly detected in the olfactory bulb of the brain. The result suggests nasal transmission was the major route of infection.

1. Introduction

Grouper culture is a rapid growing and economically important industry in marine aquaculture worldwide including Thailand. However, the high mortality associated with piscine nodavirus infection has been considered as a main cause of severe economic loss in grouper aquaculture.

Viral nervous necrosis (VNN), viral encephalopathy and retinopathy, is one of the most contagious diseases reported in many marine fish species (Arimoto et al., 1993; Boonyaratpalin et al., 1996; OIE, 2003). Nodavirus infection has been reported in larvae and juvenile groupers in Thailand (Boonyaratpalin et al., 1996; Roongkamnertwongsa et al., 2006) but not in adult.

The causative agent has been characterized as a small, with a diameter of 25–30 nm, non-enveloped, bi-segmented, single-stranded, positive-sense RNA virus. This virus belongs to the genus Betanodavirus, family Nodaviridae (Mori et al., 1991). To date, Betanodaviruses have been classified into four genotypes (Nishizawa et al., 1997): SJNNV (striped jack nervous necrosis virus), TPNNV (tiger puffer nervous necrosis virus), RGNNV (red-spotted grouper nervous necrosis virus) and BFNNV (barfin flounder nervous necrosis virus) types. Over 26 marine fish species from 16 families have been reported (Munday et al., 2002). The VNN clinical signs in larvae and juveniles are characterized by a variety of neurological abnormalities, such as erratic swimming behavior (spiral, whirling or belly-up at rest), a darkening of the body, an enlarged swim bladder and massive mortality (Munday et al., 2002). Nodavirus distribution is observed in many tissues such as brain, retina, gill, muscle, liver, pyloric gland, stomach, intestine and blood cells in affected larvae and juvenile fish (Mladineo, 2003), whereas there are a few reports of VNN characteristics in adult fish (Fukuda et al., 1996; Johansen et al., 2004; Tanaka et al., 2004). Several investigators stated that genotypic variants of Betanodaviruses are different in their pathogenicity and host specificity (Nishizawa et al., 1997; Iwamoto et al., 2000; Mori et al., 2003) and also that the pathogenesis of each nodavirus genotypic variant have not been clearly elucidated. The present study aims to determine nodavirus infection in adult spotted coralgroupers (*Plectropomus maculatus*) in Thailand and to characterize the distribution pattern of viral antigens and the specific Betanodavirus-target cells using immunohistochemistry, chromogenic *in situ* hybridization (CISH).

2. Materials and methods

Ten moribund sea cage-reared adult spotted coralgroupers (*P. maculatus*) (10–20 cm in length) from Phangnga (Thailand) were submitted for examination. The clinical signs were lethargy, dark

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coloration of skin and abnormal swimming behavior. Mortality of 30% had been recorded. All fish were euthanized with cloved oil before diagnostic examination.

2.1. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Samples from brain, eye, spinal cord and swim bladder from three affected fish were homogenized. Total RNA was then extracted using Trizol® Reagent (Invitrogen, Carlsbad, USA). First strand cDNA was synthesized using an oligo d(T) and AMV reverse transcriptase-first strand cDNA synthesis kit (Life Science Technologies, USA). Degenerative oligonucleotide primers were designed based on the amino acid alignments of the coated proteins of four different nodavirus genotypes; SJNNV, RGNNV, BFNNV, TPNNV (Nishizawa et al., 1997);

VNN-F1 : 5′ GGATTTGGACGTGCGACCAA3′
VNN-R1 : 5′ CTGAATKTCRAACTCCAGTG3′

One microlitre of reverse transcribed cDNA was used as a template in the polymerase chain reaction amplification. The PCR reaction was conducted with an initial denaturation step at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, and the final extension step at 72 °C for 5 min. The PCR product was visualized by using 2% agarose gel electrophoresis stained with ethidium bromide. Materials from experimental SJNNV infected sevenband groupers (*Epinephilus septemfasciatus* Thunburg) and from healthy uninfected spotted coralgroupers were served as positive and negative control.

2.2. Histopathology

On necropsy no gross lesions were observed except swim bladder enlargement. Brains, spinal cords, eyes, swim bladders, livers, kidneys, intestines, hearts from seven fish were fixed in 10% formalin and routinely embedded in paraffin for histopathology, immunohistochemistry and *in situ* hybridization.

2.3. Immunohistochemistry

Serial tissue sections on silane coated slides were immunohistochemically stained according to the following procedure. After rehydration through serial diluted ethanols, slides were treated with 3% hydrogen peroxide in concentrated methanol for 20 min. for blocking non-specific endogenous peroxidase reaction, washed in three changes of phosphate buffered saline (PBS) and incubated with 1% bovine serum albumin in PBS for 30 min. Sections were then incubated at 4 °C overnight with a rabbit polyclonal antibody against Striped jack nervous necrosis (SJNNV) diluted 1:1000 in PBS. Then rinsed in three changes of PBS and incubated with secondary antibody, universal immuno-enzyme polymer, using a Histofine MAX PO kit (Nichirei, Tokyo, Japan) for 30 min at room temperature. The tissue slides were thoroughly rinsed in PBS and incubated with diaminobenzidine (DAB) solution. The slides were counterstained with hematoxylin and eosin (H&E), rinsed in serial graded ethanols and xylene and mounted.

2.4. Chromogenic in situ hybridization (CISH)

About 198 bp RT-PCR products from the nodavirus coat protein gene labeled with digoxigenin (DIG) were used as the probe. The sections were deparaffined and rehydrated in a series of graded ethanol, followed by digestion with proteinase K (100 μg/mL) and then fixed in 4% paraformaldehyde. The sections were incubated at 85 °C for 3 min and allowed to hybridize at 50 °C overnight in a hybridization buffer containing 50% formamide, 1 μg/mL DIG-labeled probe, 10% dextran sulphate, Denharht's solution, Tris HCl buffer (pH 7.5), 0.25% SDS and yeast tRNA. Color detection was performed using an alkaline phosphatase-conjugated anti-DIG antibody and substrate-solution (BCIP/NBT). After the development of color due to brownish-purple precipitation, sections were counter-stained with hematoxylin for 10 s. and mounted. Tissues from experimental SJNNV-infected sevenband groupers (*E. septemfasciatus* Thunburg) and healthy uninfected sevenband and spotted coralgroupers were used as positive and negative controls, respectively.

3. Results

3.1. RT-PCR

A 198-bp DNA product compatible with nodavirus was mostly detected in samples from brain, eye and spinal cord from all three fish, whereas no product was observed from the swim bladder from all affected fish (Fig. 2). The RT-PCR product was not detected from one of the spinal cords examined.

3.2. Histopathology

The histopathology revealed extensive vacuolisation and neuronal degeneration of the olfactory bulb and the optic lobe of the forebrain in all affected fish (Fig. 1A). Perivascular cuffing was moderately noted in the olfactory bulb. Many small nerved cells, especially in granular cell layer, showed severe vacuolar degeneration and necrosis. Mild neuronal necrosis was also observed in the granular and Purkinje cell layers of cerebellum. Vacuolar degeneration was severely detectable in dendritic cells extending into the outer molecular layer of cerebellum (4/7). In the hindbrain, the lesions were shown by vacuolar degeneration in the cytoplasm of the affected megalo cells and neuropils along the medial longitudinal fascicularis of the medulla oblongata (4/7). Gliosis and neuronophagia characterized by accumulation of microglias around degenerated or necrotic neurons were occasionally seen. In the spinal cord, some fish (3/7) displayed slight vacuolar lesions in the dendrite and the cytoplasm of megalocells in the spinal cord. The retina of some infected fish showed marked histopathological changes in the outer nuclear layer, inner nuclear layer and ganglion cell layer (4/7). Massive necrosis of small round cells was noted in inner nuclear layer and ganglion cell layer (2/7). Numerous large vacuolar spaces formed by fragmentation and degeneration of infected cells were also noteworthy (5/7) (Fig. 1B). No viral inclusion body was observed in the neuronal cells of the brain and the retina. In the swim bladder, the marked hyperplasia and necrotic changes observed in the gas glandular epithelium were the most striking lesions. The hyperplastic and hypertrophic glandular epithelial cells of the swim bladder showed large, dense, pyknotic and bizarre nuclei or some of the cells were binucleated (Fig. 1C). Homogenous pale pink intranuclear inclusion bodies were occasionally seen in hyperplastic gas glandular epithelium (3/7) (Fig. 1D). Multiple macro-vacuolar lesions were also seen in the cytoplasm of affected cells. The gas gland rete mirabile (capillary networks) showed marked congestion with multiple yellowish pigments of hemosiderin. Extensive mucous secretion was also observed in hyper-inflated swim bladder. No remarkable lesions were seen in other organs.

3.3. Immunohistochemistry

Strong immunostaining was mostly seen in the cytoplasm of infected cells in brain, retina and spinal cord correlating with histopathological change. In the retina, the positive reaction was mainly