

Pathogenicity of the Nodavirus Detected from Diseased Sevenband Grouper *Epinephelus septemfasciatus*

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Viral nervous necrosis (VNN) of sevenband grouper *Epinephelus septemfasciatus* at grow-out stages, which was characterized by upside down swimming or floating behavior, has been spreading in Japan. The present study describes pathogenicity of a nodavirus found in diseased sevenband grouper. When young sevenband grouper or juvenile redspotted grouper *E. akaara* were challenged by an intramuscular injection with the filtered homogenate of infected organs (brain and eye), abnormal swimming behavior and mortality were produced, but some affected fish recovered from the disease after exhibiting the characterized behavior. Necrosis and vacuolation of the brain and retinal tissues, characteristic to VNN, were produced in the dead and abnormally-swimming fish and the viral antigens were detected in the degenerated nerve cells by FAT with an anti-SJNNV (striped jack nervous necrosis virus) serum. The infection experiment with redspotted grouper also indicated that rearing water temperature (16–28°C) influenced development of the disease: higher mortality and earlier appearance of the disease signs were observed at higher water temperatures.

Key words: nodavirus, viral nervous necrosis, VNN, sevenband grouper, *Epinephelus septemfasciatus*, pathogenicity

Sevenband grouper *Epinephelus septemfasciatus* is marked as one of new target species for marine fish culture in Japan and the production of this species has increased during the past 10 years, particularly by using wild seedlings imported from Korea. During the grow-out stages, however, a disease characterized by upside down swimming or floating behavior and inflation of the swimbladder frequently occurs in summer and early autumn and often exceeds 50% in mortalities (Fukuda *et al.*, 1996).

Fukuda *et al.* (1996) reported that the disease was characterized histopathologically by vacuolation and degeneration in the central nervous tissues of the affected sevenband grouper, and that numerous spherical, unenveloped virus particles, 28 nm in diameter, were found in the cytoplasm of the degenerated nerve cells and the virus was identified as a nodavirus by a fluorescent antibody technique (FAT) with anti-SJNNV (striped jack nervous necrosis virus; Mori *et al.*, 1992) rabbit serum and polymerase chain reaction (PCR) amplification with primers for detection of SJNNV coat protein gene. Based on these results, they suggested that the mortalities of sevenband grouper are caused by the nodavirus and higher rearing water temperature might

be a possible predisposing factor. However, pathogenicity of that sevenband grouper nodavirus was not examined in their study. Viral nervous necrosis (VNN), first named by Yoshikoshi and Inoue (1990), has been recorded in larvae and juveniles of cultured marine fish and that was the first case of VNN at adult stages, as reviewed by Munday and Nakai (1997).

The study reported here was initiated to demonstrate the pathogenicity of the virus obtained from sevenband grouper and the effects of rearing water temperature by infection experiments using two hatchery-produced marine fish species; sevenband grouper and redspotted grouper *E. akaara*. The latter species is also known to be a susceptible host of fish nodavirus (Mori *et al.*, 1991).

Materials and Methods

Experimental fish

Hatchery-reared sevenband grouper and redspotted grouper were used for the infection experiments. These fish were reared in 500 l tanks with a continuous water supply at about 15°C of water temperature at the Owase Branch of Fisheries Research Institute of Mie. Prior to experiments, the brain and retina of each 5 fish ran-

domly sampled from two fish groups were tested by a FAT procedure using an anti-SJNNV serum (Nguyen *et al.*, 1996) and a PCR procedure using a primer set designed for detection of SJNNV (Nishizawa *et al.*, 1994). All fish examined were found to be negative for the virus.

Virus preparation

Three diseased sevenband grouper (one 1-year-old and two 3-years-old) showing upside down behavior were collected from outbreaks at Mie and Wakayama prefectures. These fish were confirmed to be infected with nodavirus by the PCR test. The infected brain and eyes were pooled and then stored at -80°C until used. Just before infection experiments, the samples were thawed and homogenized with an equal volume of MEM (Nissui), and centrifuged at $1,500 \times g$ for 15 min. The supernatant was passed through a membrane filter (0.45 μm) and further diluted with MEM when required.

Infection experiments

Fish were transferred to 15 l tanks and the tank waters were adjusted to each required water temperature by an increase of 2°C a day. Fish were acclimated for one week before experiments were commenced. Eight sevenband grouper (33 g in average body weight) in the experimental group were intramuscularly injected with 0.04 ml of serial 10-fold dilutions (10^{-1} – 10^{-6}) of the filtrate and eight control fish received intramuscular injections of MEM. After injection, fish were observed in 15 l sea water tanks at 28°C ($\pm 0.5^{\circ}\text{C}$) for 20 days. Redspotted grouper (7 g) were divided into 4 groups, each consisting of 9 or 10 fish, and challenged by intramuscular injection with 0.04 ml of 5-fold dilution of the filtrate (Group 1, duplicated) or by a bath method where fish were exposed to 500 ml of sea water containing 1 ml of the filtrate for 1 h (Group 2). Group 3 (injection method, duplicated) and Group 4 (bath method) sham-challenged with MEM instead of the filtrate were served as controls. Fish were then transferred to 15 l tanks at 27 – 28°C and observed for 19 days (Groups 1 and 3) or 15 days (Groups 2 and 4). Each one of duplicated tanks of Groups 1 and 3 was used to sample abnormal swimming fish ($n = 5$) and normal swimming fish ($n = 5$), respectively, 10 days after challenge. Fish were fed commercially prepared pellet once a day. Dead fish, fish showing signs of disease, and survivors at the termination of experiments were examined histopathologically and microbiologically.

In a separate infection experiment to examine the ef-

fect of water temperature, redspotted grouper (13 g) were divided into 6 groups, each containing 7 fish, and transferred to 15 l tanks where water temperature was raised by 1 – 3°C a day to 16°C , 20°C , 24°C , and 28°C ($\pm 0.5^{\circ}\text{C}$) and then maintained at each temperature for 5 days. In the fifth group (designated as the 24 – 28°C group), tank water temperature was manipulated to 24°C and 28°C on alternate days to examine effect of fluctuation of water temperature on infection. Fish were injected intramuscularly with 0.04 ml of the virus filtrate. The sixth group was sham-challenged with MEM at 28°C . After challenge, fish were observed for 50 days at each water temperature. Dead fish and survivors at the termination of experiments were examined as described above.

Histopathological and microbiological examinations

The brain and eyes of fish were fixed in 10% buffered formalin and embedded in paraffin wax. In redspotted grouper, the gills and internal organs were also examined. The sections were stained with haematoxylin-eosin (H & E) and observed under a light microscope. An indirect FAT method was used for detection of the nodavirus. Paraffin sections of the brain and eyes were immuno-stained with the anti-SJNNV serum and FITC-conjugated swine Ig to rabbit Ig (Dako) according to the procedure of Nguyen *et al.* (1996). Dead fish were also examined for the presence of ectoparasites on the gills, and bacterial isolation from the kidneys was attempted on brain heart infusion agar (Nissui, 2% NaCl) at 25°C for 5 days.

Results

Pathogenicity of the virus in sevenband grouper

The results of experimental infection with young sevenband grouper were shown in Table 1. The abnormal swimming included loss of balance of fish around water surface or tank bottom. Inflation of swimbladder was observed only in fish floating around water surface with loss of balance. Fish inoculated with 10^{-1} and 10^{-2} dilutions of the filtrate evidenced abnormal swimming at 3 days post-inoculation (p.i.) and all died by 6 days p.i. Fish inoculated with the 10^{-3} dilution also showed abnormal swimming and high mortality by 12 days p.i. but the incidence of these abnormalities in fish with the 10^{-4} and 10^{-5} dilutions was low. One of the fish in 10^{-5} challenge group exhibited abnormal swimming at 8 days p.i. but recovered at 16 days p.i. No apparent abnormalities were observed in the 10^{-6} dilution and control groups.

Table 1. Experimental infection of the virus in young sevenband grouper

Dilution factor of the filtrate* ¹	No. of fish with abnormal swimming/ examined	No. of fish dead/examined	No. of fish FAT-positive/examined in	
			Dead fish	Survivors* ²
10 ⁻¹	8/8	8/8	8/8	—
10 ⁻²	8/8	8/8	8/8	—
10 ⁻³	7/8	6/8	6/6	2/2
10 ⁻⁴	1/8	2/8	2/2	3/6
10 ⁻⁵	1/8	0/8	—	0/8
10 ⁻⁶	0/8	0/8	—	0/8
Control	0/8	0/8	—	0/8

*¹ Fish were injected intramuscularly with 0.04 ml of the virus-filtrate or MEM (control) and kept at 28°C.

*² Sampled at 20 days p.i.

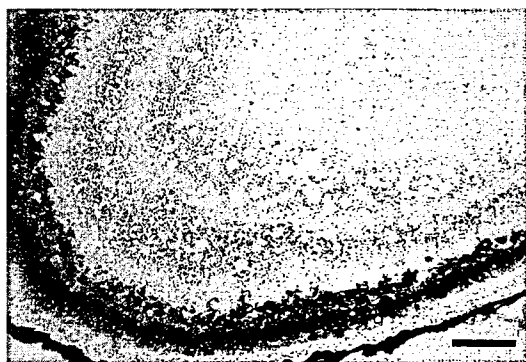


Fig. 1. Light micrograph showing extensive vacuolation in the retina of an experimentally infected sevenband grouper (H & E). Bar: 100 μ m

Histopathologically the degeneration (necrosis and vacuolation) of brain and retinal tissues, similar to that of the naturally infected fish, was observed in the experimental group (Fig. 1). The brain and/or retina of all dead fish were positive in the FAT test using anti-SJNNV serum (Table 1). Positive FAT reaction were also detected in 5 of the 8 survivors in the 10⁻³ and 10⁻⁴ challenge groups, but all the 16 survivors in the 10⁻⁵ and 10⁻⁶ challenge groups were negative in FAT test when they were examined at 20 days p.i. No parasites were observed on the gills and no dominant bacteria were isolated from the kidney of dead fish.

Pathogenicity of the virus in redspotted grouper

The pathogenicity of the virus in juvenile redspotted grouper was also demonstrated in both the inoculation methods (Table 2). In Group 1, inoculated with the fil-

trate by intramuscular injection, poor feeding performance was observed at 2 days p.i. and 8 out of 10 fish lay on the bottom or floated on the surface at 4 to 9 days p.i. Only 2 of these fish died at 7 and 9 days p.i. and the other fish recovered from abnormal swimming and poor feeding performance at 11 and 16 days p.i., respectively. In Group 2, bath-challenged with the filtrate, poor feeding performance was observed from 2 days p.i. and 3 out of 9 fish lay on the bottom at 5 to 6 days p.i. However, fish recovered from these disease conditions at 8 days p.i. and all survived until the termination of experiment (15 days p.i.). No abnormality was observed in their control groups.

Necrosis and vacuolation, as in experimentally infected sevenband grouper, were observed in the brain and retinal tissues of dead and abnormally-swimming fish. In addition to the major changes in the nervous tissues, infiltration of lymphocyte-like cells was observed in the pancreas of affected fish. As shown in Table 2, specific fluorescence in the retinal and/or brain tissues was observed in both dead fish of the intramuscularly challenged group (Group 1). The positive FAT reaction was also observed in all the abnormally-swimming fish which were sacrificed at 10 days p.i. (Group 1), in all Group 1 survivors at 19 days p.i., and in 8 out of 9 Group 2 survivors at 15 days p.i., though the detection rate was always higher in the retina than in the brain. No specific fluorescence was observed in both brains and retinas of all fish of control groups. No parasites or dominant bacteria were detected on the gills and from the kidney of dead fish.

Effect of water temperature on infection

The results of infection experiment with redspotted

Table 2. Experimental infection of the virus in juvenile redspotted grouper

Challenge* ¹ method	No. of fish with abnormal swimming/ examined	No. of fish dead/examined	No. of fish FAT-positive/examined in		
			Dead fish	Survivors* ²	Sacrificed fish* ³
Injection (Group 1)	8/10	2/10	2/2	8/8	5/5* ⁴
Control (Group 3)	0/10	0/10	—	0/5	0/5* ⁵
Bath (Group 2)	3/9	0/9	—	8/9	—
Control (Group 4)	0/10	0/10	—	0/5	—

*¹ Fish were challenged with the virus-filtrate by intramuscular injection or bath method and kept at 27–28°C. Control fish were sham-challenged with MEM.

*² Sampled at 19 days p.i. (Injection) or 15 days p.i. (Bath)

*³ Sampled at 10 days p.i.

*⁴ Fish with abnormal swimming.

*⁵ Fish with normal swimming.

Table 3. Experimental infection of the virus in redspotted grouper juveniles at different water temperatures

Water* ¹ temperature	No. of fish with abnormal swimming/ examined	No. of fish dead/examined	No. of fish FAT-positive/examined in	
			Dead fish	Survivors* ²
28°C	7/7	6/7	6/6	0/1
24–28°C	7/7	4/7	4/4	1/3
24°C	7/7	2/7	2/2	0/5
20°C	5/7	1/7* ³	1/1	0/4
16°C	4/7	1/7	1/1	6/6
28°C (Control)	0/7	0/7	—	0/7

*¹ Fish were injected intramuscularly with the virus-filtrate or MEM (control) and kept at each water temperature (See Materials and Methods).

*² Sampled at 50 days p.i.

*³ Except two fish which died by cannibalism.

grouper juveniles at different water temperatures are shown in Table 3 and Fig. 2. The appearance of abnormal swimming and mortality of fish were strongly influenced by water temperature. In the 28°C group, all 7 fish exhibited abnormal swimming at 4 to 9 days p.i. and 6 fish died at 6 to 10 days p.i. Similarly, high incidence of abnormal swimming was also recognized in the 24–28°C and 24°C groups, though the mortality rates were lower than that in the 28°C group. In the 20°C and 16°C groups, the onset of abnormal swimming was delayed, leading to low mortalities. Most of fish survived at 20°C to 28°C recovered from abnormal swimming behavior, however fish in the 16°C group did not (Fig. 2). Throughout the observation period of 50

days, no abnormalities were observed in the control group at 28°C. As shown in Table 3, positive FAT reaction was observed in the retinal and/or brain tissues of all dead fish and also 4 out of 6 survivors showing abnormal behavior when examined (50 days p.i.). Degeneration of nervous tissues was seen in these FAT-positive sections. However, 8 survivors that recovered from abnormal swimming behavior were all negative for FAT. All fish in the control group were found to be negative.

Discussion

The present infection experiments confirmed that the filtered homogenate of organs infected with the nodavirus

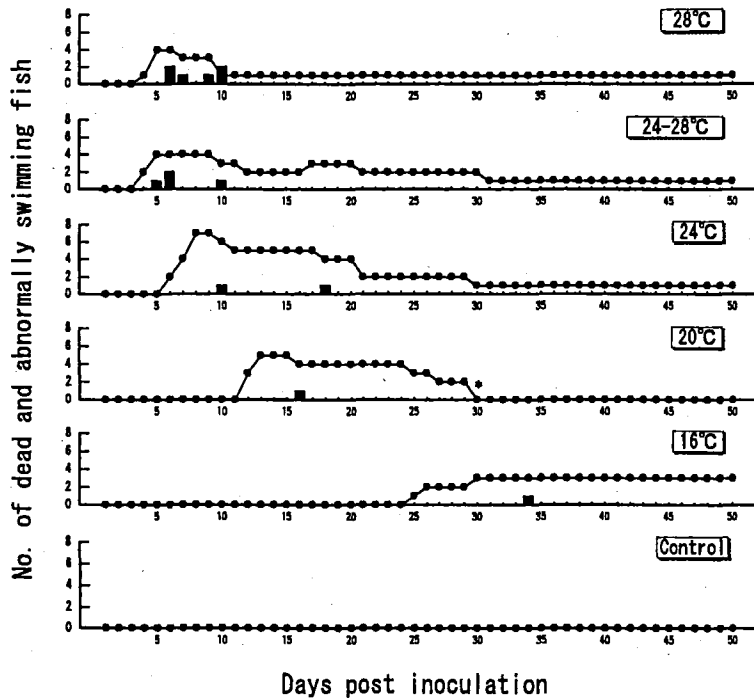


Fig. 2. Appearance of dead and abnormal swimming fish in redspotted grouper challenged with the virus at different water temperatures. ■: Number of dead fish, —●—: Cumulative number of fish showing abnormal swimming (dead and recovered fish were counted out) *Death by cannibalism

produced the disease similar to that of naturally infected sevenband grouper, accompanying abnormal swimming behavior and mortality. Necrosis and vacuolation of the brain and retinal tissues characteristic to VNN (Munday and Nakai, 1997) were found in the dead and abnormally-swimming fish and the viral antigens were detected in the degenerated nervous tissues by FAT with the anti-SJNNV serum, demonstrating that the nodavirus caused VNN disease in young sevenband grouper. The high FAT-positive results of bath challenge in redspotted grouper (Table 2), though mortalities were not produced, indicate a possible water-borne transmission of the virus, seemingly causing spread of the disease among cultured fish population. In addition to VNN in this sevenband grouper, Breton *et al.* (1997) recently reported VNN in cage-cultured sea bass *Dicentrarchus labrax*, ranging 10 to 580 g in body weight, though infection experiment to fulfill Koch's postulates was not carried out in their report.

Natural infections of nodavirus in marine fishes occur in a wide range of rearing water temperature, for example 28-30°C in juvenile brownspotted grouper *E.*

malabaricus (Danayadol *et al.*, 1995), 20-26°C in striped jack larvae (Arimoto *et al.*, 1994) and 4-15°C in barfin flounder *Verasper moseri* juveniles (Japan Sea-Farming Association, 1995)*. Arimoto *et al.* (1994) reported in an experiment using striped jack larvae naturally infected with SJNNV that the earliest mortality was observed at 24°C of water temperature in a test range of 18-27°C. The VNN outbreaks in sevenband grouper occur from July to October, water temperature ranging 25-28°C, and Fukuda *et al.* (1996) suggested higher water temperature as a possible predisposing factor of the disease. In the present study, the pathogenicity of the virus was found to be associated with rearing water temperature and the highest pathogenicity was displayed at 28°C. The virulence of the virus was reduced with the decrease of water temperature, though fish was susceptible to the virus even at 16°C. This supports the fact that a similar abnormality was observed in January (about 16°C water temperature) among a cultured popu-

* Japan Sea-Farming Association (1995): Diseases of hatchery reared larvae and juveniles, Kyokai kenkyu Shiryo No. 66 (in Japanese)

lation of sevenband grouper which had experienced heavy mortalities in the previous August to September in Mie prefecture. As the disease often becomes more severe when there is daily fluctuation of water temperature in addition to high water temperature (Dr. Y. Fukuda personal communication), this fluctuation may affect the defense mechanisms of fish against the virus. However, the effect of fluctuation of water temperature on the susceptibility was not clear in our infection experiment (Table 3).

Interestingly, some fish survived with abnormal swimming for a long time or recovered, and the virus antigens disappeared from brain and retina of all of these recovered fish (Table 3). Fukuda *et al.* (1996) reported that the virus was not detected from some naturally affected fish and suggested that these fish were in the convalescent stage. As there exists age or adult resistance of fish in VNN (Arimoto *et al.*, 1994; Munday and Nakai, 1997), the infection using sevenband and redspotted groupers will be useful to elucidate the resistance mechanisms of fish against the nodaviruses. Another interesting finding was that the virus antigen was detected frequently in survivors (50 days p.i.) at 16°C, indicating long lasting survival or slow multiplication in the host at low water temperature. These findings may help to identify the source and etiology of the virus in cultured groupers and to control the disease.

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