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Detection of Antibodies against Striped Jack Nervous Necrosis Virus (SJNNV) from Brood Stocks of Striped Jack

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It has been indicated that parental spawners were a source of infection of a viral disease, "viral nervous necrosis", in larval striped jack *Pseudocaranx dentex*. In this study, the prevalence of antibodies against the causative virus (SJNNV) was examined among brood stocks of striped jack by indirect ELISA. Indirect ELISA using purified SJNNV, rabbit anti-SJNNV serum, and enzyme-conjugated goat anti-rabbit IgG antibody was employed for antibody detection from the plasma. The plasma IgM partially purified by ion-exchange chromatography was used for ELISA detection. The antibody to SJNNV was detected at high frequency (65%) in plasma samples collected from brood stocks reared at various facilities regardless of their sex or origin (wild or domestic). This indicates that the virus is prevalent among cultured populations of this fish species.

Various facilities in western Japan have succeeded in the seed production of striped jack *Pseudocaranx dentex* since 1978. This fish species has attracted special attention in fish farming not only because of its economical importance but also because of the expectations of a new aquaculture system known as "domesticating fishery", *i.e.* culture without net cages in the sea, taking advantage of the behavioral nature of fish remaining around the original place where they were domesticated. The Japan Sea-Farming Association (JASFA) has been collecting eggs from the reared brood stocks of striped jack since 1978; however mass mortalities from unknown causes have been encountered in larvae since 1984. Especially in the last 2 years (1989 and 1990), the larvae production of this fish species was seriously damaged due to an epizootic viral disease, and no juveniles were produced in 1990.

Our previous histopathological and electron microscopic examinations[1] indicated that the disease resembles "viral nervous necrosis (VNN)" reported in Japanese parrotfish *Oplegnathus fasciatus*.[2] A similar disease has also been reported in other marine fish such as barramundi *Lates calcarifer*,[3,4] turbot *Scophthalmus maximus*,[5] sea-bass *Dicentrarchus labrax*,[6] and red-spotted grouper *Epinephelus akaara*.[7] We purified the putative causative virus from the affected striped jack larvae by differential centrifugation and classified it as a member of the family Nodaviridae, based on its nucleic acid and coat proteins. We designated it striped jack nervous necrosis virus (SJNNV).[1] We confirmed through an infection experiment that SJNNV is the causative agent of VNN in larval striped jack (unpublished data).

We also developed an enzyme-linked immunosorbent assay (ELISA) using a rabbit antiserum against purified SJNNV particles, by which we detected the virus antigen (s) from the ovary of apparently healthy spawners as well as diseased larvae.[8] This suggests that the major inoculum source of the disease is the

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parental fish. It seems reasonable to select virus-free parental fish by examining the presence of the virus antigen(s) in their gonads; however, we could seriously damage their spawning activities by handling and biopsy. In the present study, to discover the prevalence of this infection among striped jack brood stocks, the presence of plasma antibodies against the causative virus was surveyed by indirect ELISA.

Materials and Methods

SJNNV and Rabbit Antiserum

The purified SJNNV and rabbit anti-SJNNV serum were prepared according to the procedure described previously.[1,8] Rabbit serum IgG was partially purified by 50% ammonium sulfate precipitation and the non-specific antibodies were absorbed into the blood and internal organs of normal striped jack.

Fish and Plasma Samples

An adult striped jack immunized with purified SJNNV was prepared as a positive control for the ELISA test. The virus concentration was estimated from the absorbance at 260 nm (mg/ml=absorbance at 260 nm/5).[9] Six ml of the virus suspension (3 mg/ml) was poured into a petri dish (9 cm in diameter) and irradiated with a 15W ultraviolet lamp for 30 min. The treated virus suspension was mixed with an equal volume of Freund's incomplete adjuvant and the mixture (1 ml) was injected 3 times into the trunk muscle of a 9-year-old striped jack at one week intervals. Ten days after the last injection, the fish was anesthetized with ethylene glycol monophenyl ether (400 mg/l) and bled from the aortic bulb with a heparinized syringe. The plasma was separated by centrifugation at 600×g for 10 min and was kept with 0.1% NaN_3 at 4°C. The plasma samples of 0- and 2-year-old striped jack captured in Oita and Chiba Prefectures were employed as negative controls.

The test plasmas were collected from brood stocks, either wild (captured and reared) or domestic (reared from larval stage), reared for 4 to 11 years (6 to 13 years old, $n=179$ in total) at Komame (Kochi Prefecture) and Goto (Nagasaki Prefecture) Stations of the JASFA. Fish were bled just before spawning in January or February 1991. Some ($n=130$) of them were bled again in July 1991. In addition to these samples, brood stocks (2 to 13 years old, $n=93$ in total) which were captured in Shizuoka, Chiba, Ishikawa, Kagoshima Prefectures, or Tokyo and reared for 1 to 10 years were also examined from October to December 1990 and in January 1991 (Table 1).

Partial Purification of Plasma Immunoglobulin

The IgM fraction was separated from the immune plasma of striped jack by high-performance liquid chromatography (HPLC) using a Mono Q column (Pharmacia) equilibrated with 10 mM phosphate buffered saline (PBS, 0.15 M NaCl, pH 7.2). After elusion of the unadsorbed fraction, adsorbed proteins were eluted with 0.15–1.0 M NaCl gradient PBS at a speed of 0.5 ml/min. One μl of each fraction was mixed with 1 μl of purified virus suspension (2 mg/ml) at

Table 1. Detection of plasma antibodies against SJNNV from striped jack brood stocks by indirect ELISA

Rearing place	Origin	Age (year)	Rearing period (year)	No. of fish		Detection rate (%)
				Antibody positive	Antibody negative	
Kochi Pref.	W*1	13	11	31 (0.29–1.02)*3	3 (0.00–0.04)	91.2
(Komame Station)	W	6	4	17 (0.24–0.41)	4 (0.01–0.03)	81.0
	D*2	10	10	18 (0.24–0.35)	7 (0.01–0.03)	72.0
	D	7	7	35 (0.44–0.58)	14 (0.00–0.09)	71.4
Nagasaki Pref.	D	10	10	26 (0.26–0.41)	4 (0.00)	86.7
(Goto Station)	D	7	7	14 (0.30–0.34)	6 (0.00–0.07)	70.0
Shizuoka Pref.	W	7–13	6	14 (0.28–0.32)	2 (0.00–0.03)	87.5
Chiba Pref.	W	2	2	6 (0.20–0.23)	3 (0.05–0.07)	66.7
Ishikawa Pref.	W	13	10	1 (0.43)	2 (0.00)	33.3
Kagoshima Pref.	W	3	1	12 (0.39–0.44)	43 (0.00–0.07)	21.8
Tokyo	W	2–10	2–5	3 (0.33–0.40)	7 (0.00–0.04)	30.0

*1 Wild (captured and reared).
*2 Domestic (reared from larval stage).
*3 ELISA absorbance at 405 nm.

room temperature for 30 min in a humid chamber. The protein peak showing positive agglutination was used as the IgM fraction. An Ouchterlony immunodiffusion (1% agarose gel in PBS) was also made between agglutination-positive fraction and purified SJNNV or tissue homogenate of normal striped jack. A fraction eluted at the same concentration of NaCl where IgM fraction was eluted from the immune sample was collected from the plasma of non-immune control striped jack.

A rapid and simple method was developed in order to purify IgM from numerous plasma samples. Immune and non-immune plasmas were submitted to purification by a DEAE-Sephadex A50 (Pharmacia). Plasma (100 μl) was mixed with 900 μl of PBS in an eppendorf tube. DEAE-Sephadex (200 μl) equilibrated with PBS was added in the tube and mixed by turning it upside down, after which the tube was stood at room temperature for 30 min. After centrifuging at 500 rpm for 30 s, sedimented Sephadex particles were washed 3 times with 1 ml of PBS. One ml of PBS containing 1 M NaCl was added to the particles and the supernatant obtained from centrifugation at 10,000 rpm for 10 s was employed for indirect ELISA.

Indirect ELISA for Antibody Detection

The partially purified IgM or native plasma of striped jack was serially 2-fold diluted with a 50 mM carbonate-bicarbonate buffer (pH 9.6) and 200 μl of the diluted sample were added in a 96-well microtiter plate (Terumo). After incubation at 25°C for 2 h, plates were washed 5 times with PBS containing 0.05% Tween 20 (PBST). Purified SJNNV (100 ng) was added to each well and incubated at 25°C for 2 h. After 5 washes with PBST, 200 μl of a rabbit anti-SJNNV serum diluted 1: 2,000 with PBST containing 25% of a blocking solution (Block Ace, Japan Chemical) was added and incubated at 37°C for 2 h. The plates were washed 5 times with PBST. Then 200 μl of goat anti-rabbit IgG (Bio-Rad) diluted 1: 3,000 with PBST were added and the plates were incubated at 37°C for 2 h. Following the final washing, 200 μl of *p*-nitrophenyl phosphate disodium salt (1 mg/ml) in diethanolamine solution (pH 9.8) were added and the plates were incubated for 60 min at room temperature. The absorbance of each well was read at 405 nm using an ELISA microplate reader (Tosoh).

Indirect ELISA for Antigen Detection

Twenty brood stocks (12 females and 8 males), which had been reared at Komame Station for 10 years from wild juveniles, were killed for SJNNV detection from the gonads and brains. The tests were carried out in October, 2 months before the usual spawning season, in 1990. After the fish had been bled for plasma antibody detection, 0.2 g of the ovary or testis was homogenized with 0.8 ml of a 50 mM carbonate-bicarbonate buffer and centrifuged at 15,000 rpm for 5 min. The supernatants were examined for the presence of SJNNV antigens by the indirect ELISA test as described previously.[8]

Results

Purification of Plasma IgM by HPLC

Plasma of the immunized fish was fractionated by the Mono Q column and the 3rd peak (fraction no. 7) agglutinated with purified SJNNV (Fig. 1). In Ouchterlony double-diffusion analysis, a single precipitation line was formed between the IgM fraction and the purified SJNNV (Fig. 2).

ELISA of Immune Plasma

Figure 3 shows the results of ELISA for HPLC-purified IgM of immune- and non-immune plasmas. Immune plasma IgM was highly reactive against SJNNV antigen and the absorbance was dependent on the dilution rate from 80 to 10,240. By contrast, non-immune control plasma did not show a significant value of absorbance at any dilution. As shown in Fig. 4, the Sephadex A50-treated immune plasma showed a clear dose-dependency at dilutions from 160 to 10,240. In non-treated plasma, a dose-dependency was observed between dilutions from 160 to 5,120 with lower absorbance than in the treated plasma.

Judging from these results, the Sephadex-treated plasma can be used for ELISA instead of HPLC-purified IgM, and thus this pretreatment of plasma was employed for the following ELISA. The Sephadex-treated plasma was 30-fold diluted and the antibody was judged as positive when the absorbance in the ELISA test was higher than A405=0.1 after reducing the value of the negative control analyzed simultaneously.

ELISA of Test Plasma

Plasma antibodies against SJNNV were detected

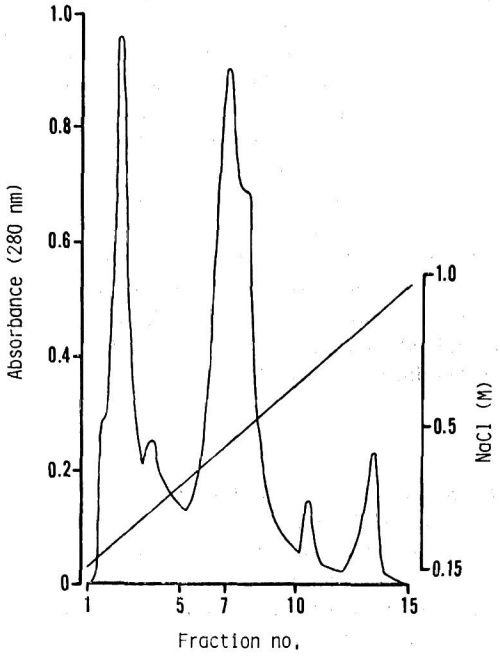

Fig. 1. Elution profile of anti-SJNNV striped jack serum by HPLC. Purified SJNNV agglutinated with fraction no. 7.

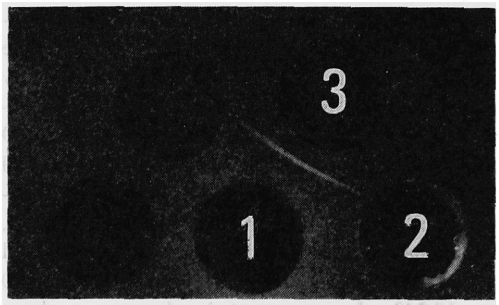

Fig. 2. Ouchterlony double-diffusion analysis of partially purified striped jack IgM. Wells: 1 (partially purified IgM), 2 (tissue homogenate of normal striped jack), and 3 (purified SJNNV).

in 177 of 272 (65%) brood stocks of striped jack (Table 1). A405 values of antibody-positive and -negative plasmas ranged from 0.20–1.02 and 0.00–0.09, respectively. The detection rate has no connection with the initial source, either wild or domestic, of the fish. It was not related to the sex, either, when 130 fish reared at Komame and Goto Stations were examined for individual sex just before spawning (Fig. 5). The second trial (July, 1991) of antibody detection on spawning fish (n=130) reared at Komame and Goto Stations exhibited a marked decrease in the total

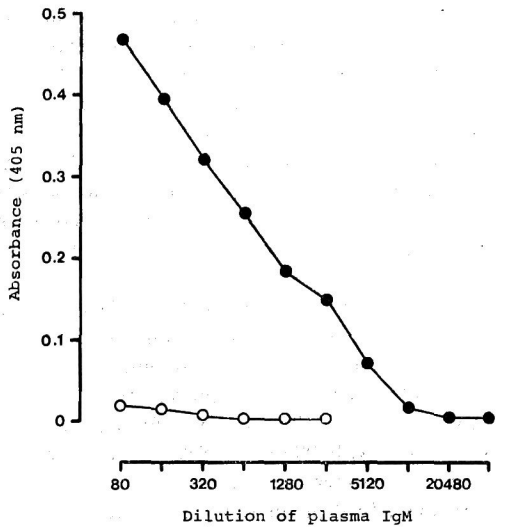

Fig. 3. Comparison in indirect ELISA between immune and non-immune plasma IgM partially purified by HPLC.
●, Immune plasma; ○, non-immune plasma.

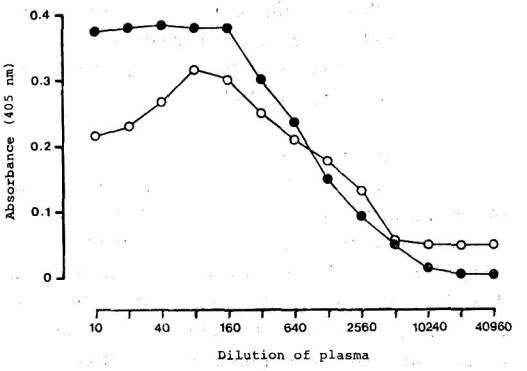

Fig. 4. Indirect ELISA of striped jack immune plasma treated by Sephadex A50.
●, Treated plasma; ○, non-treated plasma.

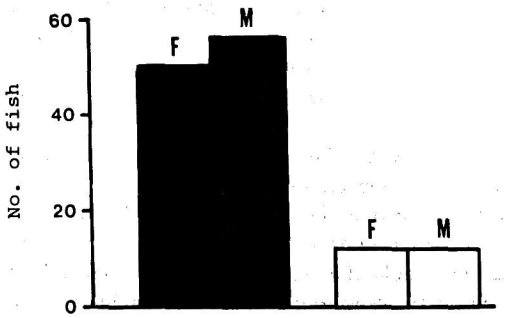

Fig. 5. Detection rate of plasma antibodies in female and male striped jack.
F, female (n=62); M, male (n=68); ■, antibody-positive; □, antibody-negative.

Table 2. Detection of antibodies in plasma and SJNNV antigens in gonads of striped jack

Fish no.	Age (year)	Sex	Antibody in plasma (A405)	SJNNV antigen in gonad (A405)
1	12	F	+ (0.13)	+ (0.27)
2	12	F	+ (0.19)	+ (0.41)
3	12	F	+ (0.18)	+ (0.39)
4	13	F	+ (0.24)	+ (0.16)
5	13	F	+ (0.21)	+ (0.17)
6	13	F	+ (0.21)	+ (0.22)
7	12	F	+ (0.51)	− (0.06)
8	12	F	+ (0.17)	− (0.06)
9	12	F	+ (0.21)	− (0.05)
10	12	F	+ (0.19)	− (0.06)
11	12	F	− (0.06)	+ (0.22)
12	12	F	− (0.09)	+ (0.27)
13	12	M	+ (0.16)	− (0.01)
14	12	M	+ (0.14)	− (0.05)
15	12	M	+ (0.20)	− (0.02)
16	12	M	+ (0.15)	− (0.02)
17	13	M	+ (0.22)	− (0.01)
18	12	M	− (0.01)	− (0.02)
19	12	M	− (0.08)	− (0.03)
20	13	M	− (0.03)	− (0.03)

F, female; M, male.

number of antibody-positive fish, from 67.7 (January) to 25.4% (July), but some negative fish became positive in the second test.

Antigen Detection from the Tissues

Table 2 shows the results of antibody and antigen detections from 20 fish. We only detected SJNNV antigen(s) from the ovaries, while there was no relationship between the presence of antigen in the ovary and that of antibodies in the plasma.

Discussion

In the first stage of this study, we tried to detect antibodies to SJNNV from brood stocks of striped jack by indirect ELISA using native plasma, but no clear dose-dependency was observed (Fig. 4), probably due to the presence of disturbing substances in the plasma. This problem was solved by using plasma IgM partially purified by HPLC, but this treatment is too time-consuming for application to numerous samples. Therefore, we applied a simple and rapid method for purifying plasma IgM by a DEAE-Sephadex A50, which proved effective for the purpose. Only a small volume of plasma (0.1 m*l*) was needed for this method and monthly successive bleedings of a maximum 0.5 m*l* per fish (average body weight 4.5 kg) at a time did not affect the spawning activities.

Antibodies against SJNNV were detected at a high frequency from reared brood stocks of striped jack, irrespective of the sex or origin (wild or domestic) (Table 1). This seems to indicate a high prevalence of SJNNV in reared populations of striped jack. The detection rates were relatively high in older fish (more than 6 years old) or in fish reared for a longer period (longer than 6 years) in artificial conditions except for the samples at Ishikawa, which possibly suggests that SJNNV could be transmitted among adult striped jack. Hormone injection, rearing in warmed water, and repeated spawning in a relatively short period are thought to be so stressful to spawners that the lower resistance of the fish would support viral multiplication.

There was no correlation between the presence of virus antigen in the ovary and the presence of antibody in the plasma; we detected the virus antigen(s) from the ovary of both plasma antibody-positive and -negative fish (Table 2). Therefore, a single examination of plasma antibody just before spawning was found to be useless in selecting virus-free spawners.

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