Detection of Striped Jack Nervous Necrosis Virus (SJNNV) by Enzyme-Linked Immunosorbent Assay (ELISA)

Misao Arimoto^{*1}, Keiichi Mushiake^{*2}, Younosuke Mizuta^{*1},
Toshihiro Nakai^{*3}, Kiyokuni Muroga^{*3}
and Iwao Furusawa^{*4}

^{*1}*Goto Station of Japan Sea-Farming Association, Tamanoura, Nagasaki
853–05, Japan*

^{*2}*Komame Station of Japan Sea-Farming Association, Komame, Kochi
788–03, Japan*

^{*3}*Faculty of Applied Biological Science, Hiroshima University,
Higashi-Hiroshima 724, Japan*

^{*4}*Faculty of Agriculture, Kyoto University, Kyoto 606, Japan*

(Received June 29, 1992)

The present paper describes the detection of striped jack nervous necrosis virus (SJNNV), the causative agent of viral nervous necrosis, in eggs, larvae, and brood stocks of hatchery-reared and captured striped jack *Pseudocaranx dentex* by indirect ELISA. An appropriate condition for the ELISA was determined using purified SJNNV and the detection limit of virus antigens was approximately 5 ng/well. All samples of larvae supposed to be affected with the disease were proved positive for the presence of SJNNV antigens in this ELISA test. The viral antigens were also detected in some samples of fertilized eggs and ovaries of spawners but not in the testis or brain. Thus, it was indicated that spawners were an inoculum source of this viral disease in striped jack.

In recent years, a viral disease similar to “viral nervous necrosis” of Japanese parrotfish *Opiegnathus fasciatus* (Yoshikoshi and Inoue, 1990) or “encephalomyelitis” of turbot *Scophthalmus maximus* (Bloch *et al.*, 1991) has been reported in hatchery-reared larvae and juveniles of barramundi *Lates calcarifer*, sea bass *Dicentrarchus labrax*, redspotted grouper *Epinephelus akaara*, and striped jack *Pseudocaranx dentex* in several countries (Glazebrook *et al.*, 1990; Renault *et al.*, 1991; Breuil *et al.*, 1991; Mori *et al.*, 1991, 1992). Although Koch’s postulates on viral etiology were not fulfilled in these disease cases except for redspotted grouper (Mori *et al.*, 1991) and the putative causative viruses have not been isolated in fish cell lines, all of them seem to have a similar viral etiology. The disease occurs mainly at larval stages of these fish species affecting the central nervous system, and numerous virus particles, non-enveloped and spherical (25 to 34 nm in diameter), are found in the cytoplasm of affected nerve cells.

Some papers indicated that the putative causative virus could be classified as picornavirus from its size, replication site, and nucleic acid type (Glazebrook *et al.*, 1990; Renault *et al.*, 1991; Bloch *et al.*, 1991). Our previous study revealed that virus purified from diseased striped jack larvae by differential centrifugations belongs to the family Nodaviridae based on its properties of nucleic acids and structural proteins (Mori *et al.*, 1992). The virus was tentatively named striped jack nervous necrosis virus (SJNNV). Lately SJNNV was confirmed to be able to cause the disease in larval striped jack through infection experiments (Arimoto, Mori, Nakai, Muroga and Furusawa, unpublished data).

Because of no available cell cultures for the virus, current diagnosis of this disease depends on histopathological and electron microscopic examinations. This study was conducted to develop an enzyme-linked immunosorbent assay (ELISA), a more rapid and reliable immunological method, for detection of the virus in eggs, larvae, and tissues of the spawners of cultured striped jack.

^{*3} To whom correspondence should be directed

Materials and Methods

Fish Samples

A large quantity of dead and moribund striped jack larvae (3-day-old, 3.5 mm in total length) were collected during mass mortalities at Goto Station (Nagasaki Prefecture) of the Japan Sea-Farming Association (JASFA) in 1991 and used for virus purification. For ELISA tests, several batches of diseased or normal larvae (1- to 5-day-old, 3.3 mm to 3.8 mm) sampled at the station from 1990 to 1991 were examined. These diseased fish were diagnosed to be affected with viral nervous necrosis by histopathological and electron microscopic examinations according to Mori *et al.* (1992). In a separate series of seed production in 1991, fertilized eggs and hatched larvae at different developmental stages were also employed for ELISA. Tissue samples (brain, ovary, and testis) were taken from 54 males and females of brood stocks reared at hatcheries or of feral fish just captured. These samples were stored at -80°C until use.

Virus Purification

The virus was purified from about 100 g of diseased larvae as described previously (Mori *et al.*, 1992). Shortly, the larvae were homogenized with buffer and the virus was collected by differential centrifugations. After purified by further centrifugations with 10–40% sucrose and 30–40% CsCl gradients, the virus was adjusted to approximately 1 mg/ml in 10 mM phosphate buffered saline (PBS, pH 7.0). The virus concentration was estimated from the absorbance at 260 nm ($\text{mg/ml} = \text{absorbance at } 260 \text{ nm}/5$) (Ehara, 1987).

Antiserum for ELISA

Purified virus mixed with Freund's incomplete or complete adjuvants (Difco) at 1:1 was injected 4 times weekly in the muscle of rabbits. Ten days after the last injection, rabbits were bled and the serum was separated by centrifugation after clotting. Serum IgG was partially purified by 50% ammonium sulfate precipitation and dialyzed against 10 mM PBS. In order to remove non-specific antibodies, serum IgG was absorbed with acetone-dried tissues (blood and viscera) of normal striped jack. The antibodies were ana-

lyzed by Ouchterlony immunodiffusion (1% agarose gel) with purified SJNNV and tissue homogenate of normal striped jack using a plant RNA virus (brome mosaic virus) as a negative control.

Indirect ELISA

One gram or 0.1 g of fish samples (eggs, larvae, tissues) were homogenized with 9 volumes of 50 mM carbonate-bicarbonate buffer (pH 9.6) and centrifuged at $10,000 \times g$ for 25 min. Serially 2-fold diluted supernatant or purified SJNNV (0.2 ml) was added in microtiter wells (Corning) and incubated at 25°C for 2 h or at 4°C overnight. After 5 washings with PBS containing 0.05% Tween 20 (PBST) and blocking with 2% bovine serum albumin, wells were filled with 0.2 ml of diluted rabbit antibody and incubated at 37°C for 2 h. Following washes with PBST, 0.2 ml of alkaline phosphatase conjugated anti-rabbit IgG goat antibody (Bio-Rad) diluted 1:3,000 was added to each well, incubated at 37°C for 2 h, and washed. Wells were reacted with *p*-nitro-phenyl phosphate disodium salt solution (1 mg/ml) in diethanolamine (pH 9.8) at room temperature for 40 min. The absorbance was measured at 405 nm using a microtiter plate reader (Bio-Rad Model 450).

Results

Rabbit Antibody

In Ouchterlony double-diffusion analysis, a single precipitin line was formed between rabbit antibody and purified SJNNV, but not against tissue homogenates of normal striped jack or brome mosaic virus (Fig. 1).

ELISA for Purified Virus

ELISA using purified virus (25–100 ng/ml) showed absorbance higher than 0.1 with up to 12,800- or 25,600-dilutions of rabbit antibody (Fig. 2). Since there were no marked reflection of dilution on the absorbance at dilutions lower than 800 or 1,600, the rabbit antibody was diluted to 2,000-fold in the subsequent ELISA. In this ELISA condition with purified SJNNV, the detection limit of the virus antigens was 25 ng/ml or 5 ng/well (Fig. 3).

Fig. 1. Ouchterlony double-diffusion analysis of rabbit anti-SJNNV serum.

A, Anti-SJNNV serum; B, Tissue homogenate of normal striped jack; C, Purified SJNNV; D, Brome mosaic virus.

Fig. 2. Dilution of rabbit antibody (first antibody) in indirect ELISA with three concentrations of antigen (purified SJNNV)

Purified SJNNV: ●, 100 ng/ml; ○, 50 ng/ml; ▲, 25 ng/ml.

ELISA for Larvae and Eggs

Fig. 4 shows typical ELISA profiles with normal and diseased striped jack larvae. ELISA values for normal larvae were lower than 0.1 in every sample. By contrast, significantly higher absorbance was recorded in all samples of diseased larvae examined. In the two samples shown in Fig. 4, a dose-dependency was found in dilutions of sample from 32 to 1,024 (A) or 1,024 to 16,384 (B). It was possible to detect the virus antigens even in individual affected larva.

SJNNV antigens were detected in a batch of eggs at their germ ring and eyed stages with the absorbance (the amount of antigens) increasing

Fig. 3. Dilution of purified SJNNV in indirect ELISA.

Rabbit antibody: $\times 2,000$.

Fig. 4. Detection of SJNNV antigens by ELISA from striped jack larvae.

●, Diseased (A); ○, Diseased (B); ▲, Normal.

with age in days (Fig. 5). Viral nervous necrosis occurred in the larval stage and all larvae were dead within 3 days after hatching. In the other batch of eggs from which the viral antigens were not detected, larvae were not affected with the disease.

ELISA for Spawner's Tissues

Changes in ELISA absorbance with dilution of tissue samples (gonads and brain) of a female

Fig. 5. Detection of SJNNV antigens by ELISA from fertilized eggs and larvae of striped jack.

●, VNN occurred in the larval stage; ○, VNN did not occur.

Fig. 6. Detection of SJNNV antigens by ELISA from tissues of a female and a male striped jack.

●, Ovary; ○, Testis; ▲, Brain.

Table 1. Detection rate of SJNNV antigens from tissues of striped jack brood stocks

Tissue	No. of samples	No. of samples positive in ELISA	Detection rate (%)
Brain	54	0	0
Ovary	31	20	65
Testis	14	0	0

and a male striped jack were shown in Fig. 6. The values higher than 0.2 were obtained in the ovary sample with up to 128 of dilutions, but the values for the testis and brain were lower than 0.1 in any dilutions.

Samples of other parental fish were tested at a 32-fold dilution and the absorbance higher than 0.2 was judged as SJNNV-positive. As shown in Table 1, the antigens were detected from the ovaries from 20 out of 31 spawners, but from any samples of testis or brain.

Discussion

In the JASFA, seed production of striped jack has suffered considerable losses from viral nervous necrosis during the last 3 years. Particularly in 1990, the disease occurred in all cases of 51 production trials and no juveniles were produced.

The purified SJNNV was strongly immunogenic and the virus specific antibodies at high titers were raised in rabbit by intramuscular injections. When the purified virus was used in the present indirect ELISA, the detection limit of the antigens was approximately 5 ng/well. In the SJNNV detection in diseased striped jack larvae, which were positively diagnosed for viral nervous necrosis by histopathological and electron microscopic examinations, all samples examined exhibited high values in ELISA. Thus, this ELISA has proved to be very useful as a rapid and specific diagnostic method for the disease. In future studies, serological comparisons between SJNNV and SJNNV-like agents will be possible by using the present rabbit antibodies.

Glazebrook *et al.* (1991) experimentally demonstrated the possibility of horizontal transmission of the virus from the diseased barramundi larvae to healthy ones. Vertical transmission, another transmission mode of the causative virus, has been estimated in striped jack as well as in sea bass (Breuil *et al.*, 1991) because of the occurrence of the disease in early larval stages. SJNNV antigens were detected in the ovaries of spawners at high frequency (65%) and fertilized eggs, though it is not clear whether the virus particles are present on the surface or inside the eggs. This indicates that the spawner is an important virus reservoir and virus particles propagated in the ovaries are shed with eggs in the environmental water.

Therefore, the present result shows that the disease could be controlled by selecting virus-free spawners.

Acknowledgement

We thank Dr. T. Okuno and members at the Laboratory of Plant Pathology of Kyoto University for technical supports and criticism.

References

- Bloch, B., K. Gravningen and J. L. Larsen (1991): Encephalomyelitis among turbot associated with a picornavirus-like agent. *Dis. Aquat. Org.*, **10**, 65–70.
- Breuil, G., J. R. Bonami, J. F. Pepin and Y. Pichot (1991): Viral infection (picorna-like virus) associated with mass mortalities in hatchery-reared sea-bass (*Dicentrarchus labrax*) larvae and juveniles. *Aquaculture*, **97**, 109–116.
- Ehara, Y. (1987): Virus purification. In “Manual for experiments in biotechnology” (ed. by F. Yamauchi, K. Hinata, S. Mizuno, and M. Katsuno). Sankyo Shuppan, Tokyo, pp. 209–212. (In Japanese.)
- Glazebrook, J. S., M. P. Heasman and S. W. de Beer (1990): Picorna-like viral particles associated with mass mortalities in larval barramundi, *Lates calcarifer* (Bloch). *J. Fish Dis.*, **13**, 245–249.
- Mori, K., T. Nakai, K. Muroga, M. Arimoto, K. Mushiake and I. Furusawa (1992): Properties of a new virus belonging to Nodaviridae found in larval striped jack (*Pseudocaranx dentex*) with nervous necrosis. *Virology*, **187**, 368–371.
- Mori, K., T. Nakai, M. Nagahara, K. Muroga, T. Mekuchi and T. Kanno (1991): A viral disease in hatchery-reared larvae and juveniles of redspotted grouper. *Gyobyo Kenkyu (Fish Pathol.)*, **26**, 209–210.
- Renault, T., Ph. Haffner, F. Baudin Laurencin, G. Breuil and J. R. Bonami (1991): Mass mortalities in hatchery-reared sea bass (*Lates calcarifer*) larvae associated with the presence in the brain and retina of virus-like particles. *Bull. Eur. Ass. Fish Pathol.*, **11**, 68–73.
- Yoshikoshi, K. and K. Inoue (1990): Viral nervous necrosis in hatchery-reared larvae and juveniles of Japanese parrotfish, *Oplegnathus fasciatus* (Temminck & Schlegel). *J. Fish Dis.*, **13**, 69–77.