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Control of VNN in Striped Jack: Selection of Spawners Based on the Detection of SJNNV Gene by Polymerase Chain Reaction (PCR)

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A preventive trial of viral nervous necrosis (VNN) in larval striped jack *Pseudocaraux dentex* was made by selecting virus-free spawners based on the detection of the causative virus (SJNNV) gene by polymerase chain reaction (PCR) method. PCR detection of SJNNV gene from fish was done by amplifying the target sequence (T4: 426 bp) of the coat protein gene (RNA2). Four spawner groups, each consisting of 12 to 16 fish, were examined for the presence of SJNNV in their gonads just before spawning at one month intervals. Their offsprings (eggs and larvae) were subjected to rearing experiments to observe the occurrence of VNN.

The SJNNV gene was detected from some spawners of these groups at the late spawnings. VNN occurred in larvae from spawner groups including SJNNV gene-positive fish, but not in larvae obtained from spawner groups which consisted of SJNNV gene-negative fish. These results suggest that the selection of striped jack spawners based on the detection of SJNNV gene from gonads by PCR just before spawning is efficacious for the prevention of vertical transmission of SJNNV in seed production.

Viral nervous necrosis (VNN) is the most devastating disease in larval stripd jack *Pseudocarnx dentex* (Arimoto *et al.*, 1994), because the disease caused extremely high mortality with vacuolation in the nerve tissues of affected fish. In VNN of striped jack, the agent was purified from diseased larvae and identified as a member of the family Nodaviridae, and the virus was designated SJNNV (striped jack nervous necrosis virus) (Mori *et al.*, 1992). It was deduced that the causative agent was transmitted vertically from spawners to their offsprings from the fact that the viral antigens were detected from spawners (ovary), fertilized eggs and hatched larvae by an enzyme-linked immunosorbent assay (ELISA) (Arimoto *et al.*, 1992). Our previous study also suggested that incidence of the disease could be reduced by using spawners with no specific antibody and giving less stress to spawners by improvement of spawning induction method (Mushiake *et al.*, 1993). However, VNN sometimes occurred in larval striped jack derived from spawners from which specific antibodies or viral antigens were not detected by ELISA. Therefore, a more sensitive method for detection of the virus is needed for the effectual selection of SJNNV-free spawners and eggs.

SJNNV contains two single-stranded, positive-sense RNA molecules of 1.01×10^6 Da (RNA1) and 0.49×10^6 Da (RNA2) and RNA2 encodes a structual protein of 42 kDa of the virus (Mori *et al.*, 1992). Recently, a polymerase chain reaction (PCR) method was developed to amplify a portion of the coat protein gene (RNA2) of SJNNV (Nishizawa *et al.*, 1994). In the present study, this method was applied to the gonad samples of striped jack spawners in a seed production site. Plasma antibodies against SJNNV in these fish were also analyzed to investigate the relation between presence of SJNNV in gonads and presence of plasma antibodies against SJNNV. Eggs and larvae obtained from every spawner group were also examined to detect the virus by PCR and ELISA, and subjected to rearing experiments to observe occurrence of VNN.

Materials and Methods

Samples of gonad and plasma from spawners

Brood stocks of striped jack used in this experiment were reared at Komame Station of Japan Sea-Farming Association (JASFA) for at least 6 years. These fish were divided into four groups (No. 1 to 4), each consisting of 12 to 16 fish (Table 1). Individual fish was driven by a tag (Pit-tag, Kawamura Inc.) into their dorsal muscle for identification.

The fish were anesthetized with ethylene-glycol monophenylether (400 mg/*l*), bled from the aortic bulb with a heparinized syringe, and then a small amount (approximately 0.1 g) of gonad samples (testis or ovary) was taken individually by a sterilized cannula. Samples of both gonad and plasma were collected from all spawners at about one month intervals during the experimental period with some exceptions. Plasmas were separated by centrifugation at 600×*g* for 10 min and kept with 0.1% NaN_3 at 4°C until used. Each gonad sample was stocked in a freezer (−80°C) for ELISA and PCR tests.

Spawning of brood stocks and collection of eggs and larvae

In this study, fish were induced to spawn by raising water temperature intermittently from 20°C to 22°C in order to minimize the stress effects for the spawners (Mushiake *et al.*, 1993). Eggs were collected everyday from evening (17:00) to the next morning (9:00) as long as fish continued to lay eggs during that spawning season (Dec., 1992–May, 1993). Floating eggs collected from each lot were kept in a net placed in a tank (10 k*l*) and hatched larvae were reared without feeding at 22°C. A batch of eggs (ca. 0.2 g) and a pooled sample of larvae (ca. 50 fish) collected everyday from each group for consecutive 10 days (1-day to 10-day old) were kept at −80°C until analysis. Hatched larvae could not survive more than 10 days after hatching under the given condition of rearing. After SJNNV was detected by PCR from gonads of No. 3 and No. 4 spawner groups at the last sampling, SJNNV-positive fish were excluded from those spawning groups and an additional spawning trial was conducted in each group by using only SJNNV-negative spawners.

Detection of plasma antibody against SJNNV

Plasma IgM was partially purified by DEAE-Sephadex (Pharmacia) and then employed for the detection of antibodies against SJNNV by an indirect ELISA (Mushiake *et al.*, 1992). A plasma sample of 0-year-old wild striped jack captured in Oita Prefecture was employed as the negative control. The paritially purified IgM, purified SJNNV (100 ng), rabbit anti-SJNNV serum (diluted 1:2,000), and alkaline phosphatase conjugated goat anti-rabbit IgG (Bio-Rad, diluted 1:3,000) were used in a series of ELISA reaction, and absorbance was read at 405 nm using an ELISA microplate reader (Tosoh). The result was judged as positive when the absorbance (A_{405}) in the ELISA test was higher than 0.10 after deducing the value of the negative control analyzed simultaneously.

Table 1. Brood stocks of striped jack used for spawning in this study

Group of spawners	Origin*	Age in 1993 (year)	Number of fish (Male:Female)
1	D	12	13 (7: 6)
2	D·W	8, 9	16 (6:10)
3	W	15	12 (6: 6)
4	W	15	12 (6: 6)

* D: domestic (reared from larval stage), W: wild (captured and reared).

Detection of SJNNV by ELISA and PCR

Detection of antigens and RNA2 gene of SJNNV was conducted from gonads, eggs and larvae by indirect ELISA and PCR amplification, respectively.

In ELISA tests, 0.1 g of a sample was homogenized with 0.9 m*l* of 50 mM carbonate-bicarbonate buffer (pH 9.6) and centrifuged at 10,000×*g* for 5 min. The supernatants were examined for the presence of SJNNV antigens by the indirect ELISA using a rabbit anti-SJNNV serum as described previously (Arimoto *et al.*, 1992). When ELISA value (A_{405}) was higher than 0.10, the result was judged as positive.

In PCR tests, each sample (0.1 g) was homogenized with 0.5 m*l* of distilled water treated with 0.1% DEPC (diethyl pyrocarbonate, Sigma) and centrifuged at 10,000×*g* for 10 min. The resultant supernatant was mixed with 0.04 m*l* of proteinase K (1 mg/m*l*) and 0.04 m*l* of 1% SDS (sodium dodecyl sulfate), and incubated at 37°C for 30 min. After centrifugation at 10,000×*g* for 5 min, total nucleic acids were extracted through phenol-chloroform

method. The detection of SJNNV RNA2 gene by PCR was carried out according to the procedure described previously (Nishizawa *et al.*, 1994). Among 5 target regions in the open reading frame of the RNA2 used by Nishizawa *et al.* (1994), T4 (426 bp) region was selected as a target sequence for PCR amplification in this experiment. Briefly, complementry DNA was synthesized from extracted RNAs by using M-MLV reverse transcriptase (Takara) and a reverse primer (R3: 5′-CGAGTCAACACGGGTGAAGA-3′) under conditions at 42°C for 30 min and 99°C for 10 min. After addition of a forward primer (F2: 5′-CGTGTCAGTCATGTGTCGCT-3′) and Taq DNA polymerase (Takara) to the mixture, each cycle of amplification was repeated 25 times under conditions at 95°C (40 s), 55°C (40 s), and 72°C (40 s) by using a DNA thermal cycler (PC-700, Astec). Amplified DNA was analyzed by agarose gel electrophoresis using 2% agarose (Agarose ME, Nacalai Tesque).

Results

Results of detection of SJNNV (exactly, coat protein gene) in gonads by PCR and plasma antibodies against SJNNV in spawners by ELISA are shown in Table 2 and detection of SJNNV in eggs and larvae at various developmental stages is shown in Fig. 1. Neither SJNNV in the gonads nor antibodies in the plasma were detected from any spawner groups before spawning (Dec., 1992–Feb., 1993). In Group 1, fish spawned 7 times until Feb. 8 and SJNNV was not detected from their eggs and hatched larvae during that period. The virus was detected from gonads of 2 spawners at the last sampling (Mar. 16, 1993), however, they did not spawn after that. In Group 2, the virus had never been detected from spawners or their offsprings (21 spawnings) throughout the experiment (Table 2, Fig. 1).

In Groups 3 and 4, SJNNV was detected from spawner's gonads at each last sampling (Mar. 22 in Group 3, May 14 in Group 4) by PCR as shown in

Table 2. Detection of SJNNV gene from gonads of spawners, eggs and larvae of striped jack by PCR and parental plasma antibodies against SJNNV by ELISA

Group of spawners	Sampling date	SJNNV in gonad		Plasma antibody		Round of spawning	SJNNV in	
		Male	Female	Male	Female		eggs	larvae
1	Dec. 1, 1992	0/7*1	0/6	0/7*2	0/6	0		
	Jan. 14, 1993	0/7	0/6	0/7	0/6	0		
	Feb. 8	ND*3	ND	ND	ND	7th*4	−	−
	Mar. 16	1/7	1/6	1/7	1/6	NS*5		
2	Dec. 8, 1992	0/6	0/10	0/6	0/10	0		
	Jan. 29, 1993	0/6	0/10	0/6	0/10	0		
	Mar. 1	0/6	0/10	1/6	0/10	12th	−	−
	Apr. 13	0/6	0/10	3/6	0/10	21st	−	−
3	Dec. 2, 1992	0/6	0/6	0/6	0/6	0		
	Jan. 19, 1993	0/6	0/6	0/6	0/6	0		
	Feb. 18	0/6	0/6	0/6	0/6	7th	−	−
	Mar. 22	2/6	0/6	2/6	1/6	18th	−	+
	Additional	0/4	0/6	0/4	1/6	19th	−	−
4	Dec. 5, 1992	0/6	0/6	0/6	0/6	0		
	Feb. 27, 1993	0/6	0/6	0/6	0/6	1st	−	−
	Apr. 12	0/6	0/6	0/6	1/6	21st	−	−
	Apr. 24	0/6	0/6	0/6	1/6	26th	−	−
	May 14	0/6	4/6	1/6	4/6	40th	−	+
	Additional	0/6	0/2	1/6	0/2	41st	−	−

*1 Number of fish SJNNV-positive/examined.
*2 Number of fish antibody-positive/examined.
*3 ND: Not done.
*4 The given round of spawning occurred following the sampling shown on the left.
*5 NS: Not spawned.

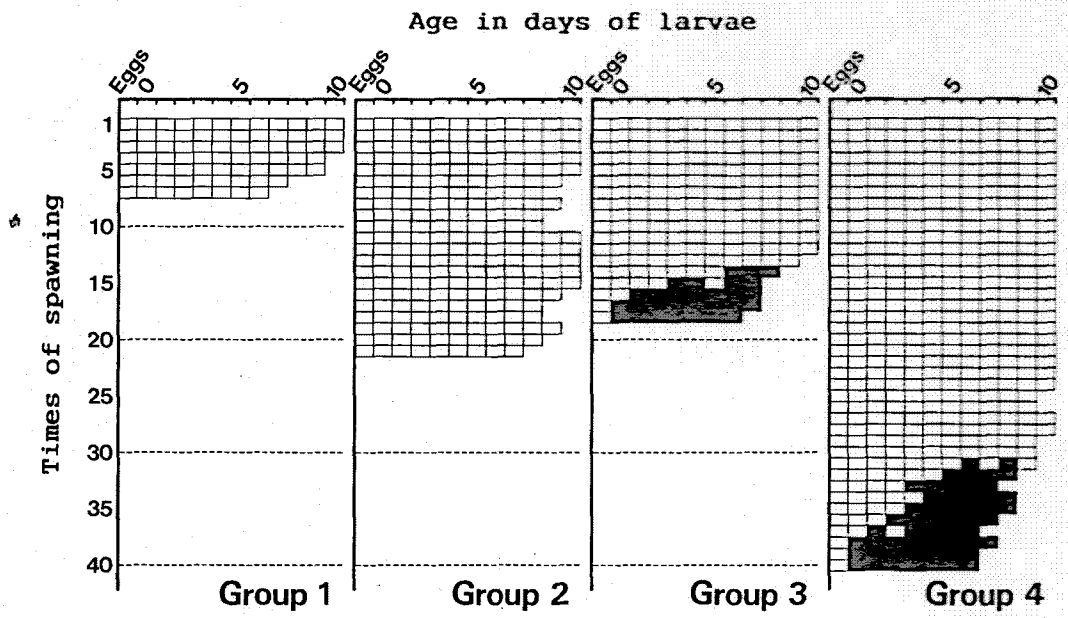

Fig. 1. Detection of SJNNV gene from eggs and larvae obtained from striped jack brood stocks (Groups 1~4) by PCR.
□ SJNNV gene negative, ■ SJNNV gene positive.

Table 2. SJNNV was detected from larvae of 18th spawning (Mar. 22) in Group 3 and 40th spawning (May 14) in Group 4 while the virus gene was not detected from eggs at any spawnings in Group 3 or 4 (Fig. 1) although the reason why SJNNV gene was not detected from eggs by PCR remains unknown. Agarose gel electrophoresis profiles of T4 region of SJNNV RNA2 using representative samples (Group 4) are shown in Fig. 2. In an additional spawning experiment made by using only SJNNV-negative spawners in Group 3 (19th spawning, Apr. 3) and Group 4 (41st spawning, May 17), VNN did not occur in larvae obtained from these spawnings (Table 2).

As shown in Fig. 1, SJNNV was also detected from larvae at 14th to 18th spawnings in Group 3 and 31st to 40th spawnings in Grop 4. The SJNNV was detectable at earlier developmental stages of larvae with the increase of spawning times. Although both ELISA and PCR were available to detect SJNNV from these larvae, the latter method was able to detect the virus in earlier stages of the infection than the former method (Table 3).

Detection rate of plasma antibodies increased as spawnings were repeated in any groups. The fish

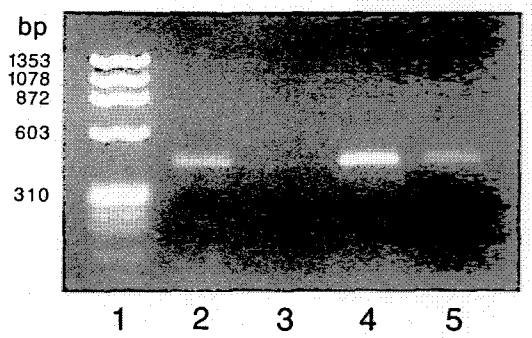

Fig. 2. Agarose gel electrophoresis profiles of T4 region of SJNNV RNA2. Lanes: 1, DNA ladder; 2, gonad of spawner (Group 4); 3, eggs obtained from those spawners; 4, larvae from those eggs; 5, purified SJNNV.

from which the virus was detected always turned positive for the presence of antibodies in the plasma; however the converse was not true, that is, there were some spawners from which antibodies were detected but the virus was not detected from gonads.

Table 3. Detection of SJNNV from eggs and larvae by ELISA and PCR

Group of spawners	Date of spawning	Detection method	SJNNV in eggs and larvae							
			Age in days of larvae							
			Eggs	0	1	2	3	4	5	6
3	Mar. 27	ELISA	−	−	−	−	−	+	+	+
		PCR	−	+	+	+	+	+	+	+
4	May 14	ELISA	−	−	−	−	−	+	+	+
		PCR	−	+	+	+	+	+	+	+

Discussion

Nishizawa *et al.* (1994) developed the basic procedure for PCR amplification of portions of SJNNV coat protein gene (RNA2) as a sensitive method to detect the virus. In the present study, this PCR method proved to be useful for the detection of the virus gene from gonads, eggs, and larvae of striped jack.

The present study showed that the presence of SJNNV gene detected by PCR from gonads of striped jack brood stocks well corresponded with the occurrence of VNN in their offsprings. Moreover, even in the cases where VNN occurred successively in larvae produced from specified spawner groups (Groups 3 and 4) at the later spawning period as shown in Fig. 1, the elimination of SJNNV gene-positive fish from spawning groups proved to be able to prevent VNN outbreaks in larvae. These results clearly indicate that the selection of spawners based on the detection of SJNNV gene from gonads by PCR just before spawning is effective for the prevention of VNN occurrence in seed production of this fish species.

It is a matter of course that the PCR method can detect a portion of SJNNV gene (T4) and a positive result of PCR test does not always mean the presence of the virus with infectivity. However, the above results seem to indicate that a positive PCR result can be interpreted as positive for the presence of the infectious virus in the present cases.

In the present experiment, it was confirmed again that the multiplication of SJNNV in brood stocks will occur in the spawners which experienced repeated spawnings in the same season. From this result, the number of spawnings of one spawner was considered to be limited to less than 10 times in one season to prevent the vertical transmission of SJNNV from spawners to their offsprings.

Plasma antibodies against SJNNV became detectable from spawners at the 2nd and 3rd examinations (Mar. 1, Apr. 13) in Group 2 and at the 3rd and 4th examinations (Apr. 12, Apr. 24) in Group 4, although SJNNV was not detected from gonads of these fish. This result suggests that SJNNV will multiply in some other organs than the gonad in spawners. It is interesting to determine the organ(s) where SJNNV multiplies in adult fish when they are stressed.

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