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NOTE

Selection of brood stock candidates of barfin flounder using an ELISA system with recombinant protein of barfin flounder nervous necrosis virus

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ABSTRACT: Barfin flounder nervous necrosis virus (BFNNV), the causative agent of viral nervous necrosis (VNN) of barfin flounder, is vertically transmitted from spawners to larvae. In the present study, an ELISA with a recombinant protein of BFNNV was performed for the detection of antibodies against BFNNV and applied for the selection of brood fish in order to prevent viral vertical transmissions. Brood stocks were divided into 4 groups based on ELISA antibody titers (≤10, 20, 40 and >40), and the BFNNV status of the brood stocks was determined by PCR. BFNNV was detected from the brood fish in the group with an antibody titer of >40 but not from those with titers ≤10, 20 and 40. The offspring obtained from PCR-negative brood fish pairs in each group of ELISA antibody titers were subsequently reared for observation of VNN occurrence. VNN occurred in juveniles from 2 of 9 pairs of spawners with an antibody titer ≥40, but did not occur in spawners with an antibody titer of ≤10. Therefore, it was concluded that selection of brood fish using both the PCR test and ELISA antibody titers could help prevent vertical transmission of BFNNV in larval production of barfin flounder.

KEY WORDS: Viral nervous necrosis (VNN) · Barfin flounder nervous necrosis virus (BFNNV) · Selection of brood fish · Recombinant protein · ELISA antibody titer

In coastal areas of the northern part of Japan, the population of barfin flounder *Verasper moseri* has been decreasing. Aquaculture and sea farming of barfin flounder are very important and have expanded because barfin flounder grows rapidly at low temperatures and is a relatively high-price commodity. The Akkeshi Station of the Japan Sea-Farming Association (JASFA) has established larval production and rearing methods for barfin flounder. In 1993, mass mortalities occurred in barfin flounder juveniles. The affected fish, which demonstrated abnormal swimming behavior and had eye hemorrhages, were diagnosed as viral nervous necrosis (VNN)-positive by a PCR test and histological and electron microscopical observations (Watanabe et al. 1999). Since the first VNN case was reported in Japanese parrotfish *Oplegnathus fasciatus* (Yoshikoshi & Inoue 1990), VNN and similar diseases have been observed worldwide in 22 fish species (Muroga et al. 1998). The causative agent of VNN in barfin flounder, barfin flounder nervous necrosis virus (BFNNV), is a member of fish nodaviruses but genetically distinguishable from a type species of fish nodavirus, striped jack *Pseudocaranx dentex* nervous necrosis virus (SJNNV) (Mori et al. 1992, Nishizawa et al. 1995, 1999).

In larval production of striped jack, the selection of spawner based on PCR detection of SJNNV just before spawning is useful in preventing vertical virus transmission (Mushiake et al. 1994). SJNNV was often detected from PCR-negative spawners after repeated experimental spawning and resulted in the occurrence of VNN in their offspring (Mushiake et al. 1993). Thus, larval production in striped jack should be performed with offspring from PCR-negative brood fish which have spawned fewer than 10 times (Mori et al. 1998). In the case of barfin flounder, it was hard to prevent vertical transmission of BFNNV because VNN occurred in larvae around 60 or more days old even though PCR-negative spawners with little spawning experience had been used (Watanabe et al. 1999). This is in contrast to striped jack, and we have concluded that an additional method is therefore needed for the selection of barfin flounder spawners. In the present study, an

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ELISA system using a recombinant coat protein of BFNNV was established to detect antibodies against BFNNV and applied to select spawners based on ELISA antibody titers in order to prevent vertical transmission of BFNNV.

Materials and methods. ***Antigen for ELISA:*** A recombinant coat protein of BFNNV was obtained from a culture broth of *Escherichia coli* strain EX-B203. The T2 region of the BFNNV coat protein gene was amplified from total nucleic acids of infected barfin flounder juveniles by PCR under the same conditions used for fish nodaviruses (Nishizawa et al. 1994). The forward primer, F1-exp, which contained the *Nde*I site (5'-aaacatatagGGATTTGGACGTGCGACCAA-3'), and the reverse primer, R3-exp, which contained the *Hin*dIII site (5'-TCTTCACCCGTGTTGACTCGtgaagcttagc-3'), were employed for the PCR amplification. The PCR products were digested with *Nde*I and *Hin*dIII and were ligated into the *Nde*I -*Hin*dIII site of the pET-25b (+) expression vector (Novagen) to transform the *E. coli* strain BL21 (DH3) according to the instructions of the manufacturer. The transformed *E. coli* EX-B203 was cultured at 37°C until optical density (OD_{600}) reached 0.4 to 0.6 in Luria-Bertani (LB) broth (1% Bacto tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5) containing 50 mg ml^{-1} ampicillin. Expression products from the T2 region of BFNNV in the plasmid were induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) to *E. coli* cells. Following incubation at 37°C for 2.5 h, the cells were washed and resuspended in 50 mM Tris-HCl (pH 8.0) and 2 mM EDTA. After the addition of 100 mg ml^{-1} lysozyme and 0.1% Triton X-100, the cell suspension was incubated at 30°C for 15 min, ultrasonicated, and centrifuged at low speed (300 × *g*) for 15 min. The pellet and supernatant were harvested as insoluble and soluble induced fractions, respectively. The obtained fractions were analyzed by 12% polyacrylamide gel electrophoresis (PAGE) under the reducing conditions of Laemmli (1970), and proteins in a gel were electroblotted to the nitrocellulose membrane using the procedure of Towbin et al. (1979). Proteins on the nitrocellulose membrane were detected using rabbit serum against SJNNV, horseradish peroxidase (HRP)-conjugated antibody against rabbit immunogloblin and the HRP development kit (BioRad).

ELISA system for detection of fish antibody: ELISA plates (Greiner) were coated overnight at 4°C by adding 50 µl $well^{-1}$ (10 µg ml^{-1}) of purified SJNNV particles (kindly provided by Dr M. Arimoto, Kamiura Station, JASFA) or recombinant coat protein of BFNNV (in carbonate-bicarbonate buffer, pH 9.6). The plate was washed 3 times with PBS containing 0.05% Tween 20 (PBST) and blocked with 2% skim-milk in PBST at 37°C for 1 h. After washing in PBST 3 times, diluted fish sera were added and incubated at 37°C for 2 h. The plate was washed 3 times with PBST and then rabbit serum against barfin flounder IgM was added and incubated at 37°C for 1 h. The fish IgM binding to the antigen was detected with a HRP-conjugated antibody against rabbit immunogloblin (DAKO) and *o*-phenylenediamine. The ELISA absorbance was measured by a spectrophotometer at OD_{492}. ELISA antibody titer is defined as the maximum dilution of serum in which the absorbance values reach baseline absorbance (Yoshimizu et al. 1998). Sera from brooders were diluted from 1:10 to 1:2560 to determine ELISA antibody titers and baseline absorbance. The baseline absorbance was approximately 0.01 in our system.

Grouping of brood fish and larval production: Individually identified brooders (131 males and 66 females) distinguished by Pit Tags (diameter: 2 mm × 11 mm, Kawamura-tsusho) were divided into 4 groups based on ELISA antibody titer against BFNNV: the titers ≤10, 20, 40 and >40. ELISA was performed 3 mo before spawning. The BFNNV status of all brood fish was determined with a PCR test using their sperm and ovarian fluid. The PCR-negative sperm and ovarian fluid from some pairs of brooders which had had few spawning experiences in each ELISA group were used for artificial fertilizations. The fertilized eggs were disinfected with ozonated seawater (0.5 mg l^{-1}, 5 min) in the morula stage (Watanabe & Yoshimizu 1998). Some larvae obtained from each brooder pair were subjected to PCR detection of BFNNV at 5 d intervals for more than 60 d rearing. The PCR detection of BFNNV was performed as described previously (Nishizawa et al. 1994).

Results and discussion. The results of SDS-PAGE and Western blot analyses of soluble and insoluble fractions of *Escherichia coli* EX-B203 are shown in Fig. 1. There was more expressed protein from the T2 region of BFNNV in the insoluble fraction than in the soluble one. The expressed protein immunostained with antiserum against SJNNV was approximately 32 kDa and was consistent with the estimated size based on the amino acid sequence encoded in the T2 region of BFNNV (Nishizawa et al. 1995). Approximately 700 mg of the recombinant protein from the BFNNV T2 region was obtained by a 1 l culture broth of *E. coli* EX-B203. To compare the antigenicity between the recombinant protein of BFNNV and the native coat protein of SJNNV, 40 times diluted sera from 7 antibody-positive barfin flounders were used for ELISA. There was not much difference in ELISA absorbance values between the 2 capture antigens, and it was confirmed again that BFNNV and SJNNV coat proteins shared common antigens (Table 1). BFNNV and SJNNV are serologically distinguishable from each other by monoclonal antibodies against

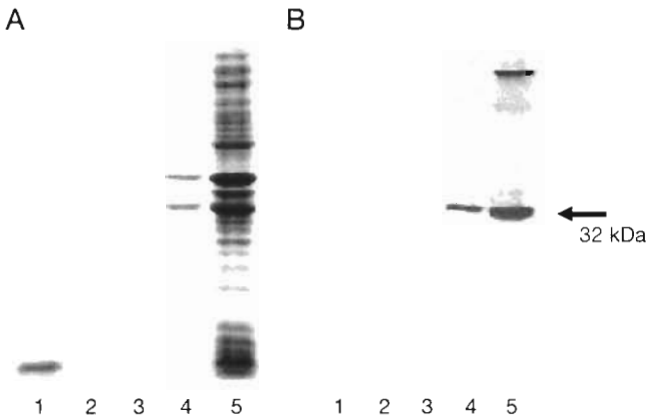

Fig. 1 SDS-PAGE and Western blot analyses of *in vitro* expression products from culture of *Escherichia coli* EX-B203 containing the T2 region of the BFNNV coat protein gene. (A) 12% polyacrylamide gel stained by Coomassie brilliant blue. (B) Proteins separated by SDS-PAGE were transferred to nitrocellulose membrane and immunostained with antiserum against SJNNV. Lane 1: soluble protein fraction; lane 2: supernatant of a single washing; lane 3: supernatant of a double washing; lane 4: 1/10 concentration of insoluble fraction; lane 5: insoluble fraction. A recombinant protein of BFNNV with approximately 32 kDa was expressed in the insoluble fraction of the *E. coli* EX-B203 culture

SJNNV (Nishizawa et al. 1999). It is possible to culture both viruses with the SSN-1 cell line (Watanabe & Yoshimizu 1999), but purification of a capture antigen from the viral culture system is more costly and more complicated than purification from the recombinant protein system. Thus, it is better to use a recombinant protein of BFNNV as a capture antigen for the ELISA system to detect antibodies against BFNNV in barfin flounder sera.

After division of brooders into 4 groups based on the ELISA antibody titer, the BFNNV status of the flounder was determined using a PCR test. No brooders were PCR-positive in the groups with an ELISA antibody titer of ≤10, 20 and 40, but BFNNV was detected by PCR from 11 of 18 brood fish in the group with an antibody titer of >40 (Table 2). Approximately 40 000 larvae were obtained from 6, 7 and 2 pairs of brooders in the groups with an ELISA antibody titer of ≤10, 40 and >40, respectively, but there were not enough larvae from any fish pairs in the group with an antibody titer of 20 to study VNN outbreaks. These larvae from different pairs were separately reared for more than 60 d for observation of VNN occurrence. Outbreaks of VNN were observed in juveniles from 1 of 2 pairs of spawners in the group with an ELISA antibody titer of >40 and in juveniles from 1 of 7 pairs with an antibody titer of 40, but no VNN was found in 6 pairs of juveniles with an antibody titer ≤10 (Table 2). Unfortunately, it was not possible to test whether or not VNN occurred in larvae from spawners with an antibody titer of 20. Additionally, BFNNV was never detected by PCR from any juveniles without VNN. These results indicate that a small amount of BFNNV, below the PCR detection limit, could have existed in the sperm and ovarian fluid obtained from the spawners with an antibody titer of ≥40, and resulted in an occurrence of VNN in their juveniles. Similar results were also reported in a case of striped jack larvae; that is, a very small amount of SJNNV in spawners escaped PCR detection and was able to produce infection in their offspring (Nishizawa et al. 1996). Thus, we assume that there is still a slight possibility of VNN occurrence in larvae and juveniles due to vertical viral transmission from brooders even if they have been selected by PCR. A negative PCR assay does not guarantee that a fish is not infected with viruses. Conversely, a positive PCR assay and detection of antibody only indicate that the fish were infected with virus at some point, not that there is necessarily an active infection. However, we conclude

Table 1 Comparisons of capture antigens, purified SJNNV particles and a recombinant protein of BFNNV, in an ELISA system for detection of antibodies against BFNNV in barfin flounder

Fish no.	ELISA absorbance (492 nm) × 1000			
	Purified SJNNV		BFNNV coat protein	
	Well 1	Well 2	Well 1	Well 2
236	168	105	177	169
305	42	54	48	52
310	306	170	118	125
764	205	198	133	125
821	66	62	66	70
5120	61	67	39	38
5775	186	316	224	229

Table 2. Results of PCR test on barfin flounder brood fish in groups based on ELISA antibody titer and outbreaks of VNN in their offspring in larval production

ELISA antibody titer	No. of fish		PCR examination[a]	Outbreaks of VNN in larval production[b]
	Male	Female		
1:≤10	23	31	0/54	0/6
1:20	8	8	0/16	–[c]
1:40	19	13	0/32	1/7
1:>40	81	14	11/18	1/2
Total	131	66		

[a]Sperm and ovarian fluid were investigated. No. of positive samples/no. of samples examined
[b]No. of production events in which VNN occurred/no. of production events conducted
[c]Not enough larvae were obtained

from the present results that the risk of viral vertical transmission is greatly decreased by brood fish selection based on both PCR assay and antibody detection.

In general, the detection of fish antibodies against fish pathogens has not been accepted as a routine detection method for assessing the viral and bacterial status in fish populations (Thoesen 1994, OIE 1997). However, there are many reports of the detection of antibodies against fish viruses, such as the Egtved virus (Olesen et al. 1991), spring viremia of carp virus (Dixon et al. 1994), infectious pancreatic necrosis virus (Dixon & De Groot 1996) and lymphocystis virus (Lorenzen & Dixon 1991, Dixon et al. 1996, Nishida et al. 1998). In a case of VNN on striped jack, a single examination of plasma antibody just before spawning was found to be useless for the selection of virus-free spawners because there was no correlation between the presence of antigen in the ovary and the presence of antibody in plasma (Mushiake et al. 1992). Conversely, an ELISA system to detect antibody against nodavirus of sea bass *Dicentrarchus labrax* was useful for screening spawners for the purpose of preventing vertical virus transmission (Breuil & Romenstand 1999). These different results may be due to differences in fish species and/or type of causative viruses. At least in the case of barfin flounder, VNN has not occurred in larvae and juveniles obtained from spawners with an ELISA antibody titer of ≤10. The annual production of barfin flounder juveniles at the Akkeshi Station of JASFA was less than 10 000 after VNN occurred. However it recovered to more than 100 000 juveniles after PCR and ELISA antibody titer assays were employed for brood fish selection. Therefore, we believe that selection of brood fish using both assays could more effectively prevent vertical transmission of BFNNV in the larval production of barfin flounder.

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