



## A COMPARATIVE STUDY ON BRAIN-TROPISM OF BETANODAVIRUSES IN YOUNG MARINE FISH: A WAY TO PERFORM HOST SPECIFICITY

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**Abstract:** Investigation on the brain-tropism was carried out through direct injection of the virus into the vitreous body of the eye using five marine fish species: kelp grouper, striped jack, Japanese flounder, threeline grunt and yellow tail. When the brains of intravitreally-injected fish ( $10^6$  TCID<sub>50</sub> Fish<sup>-1</sup>) were examined at 72 h post infection, striped jack nervous necrosis virus (SJNNV; the type species of the genus *Betanodavirus*) was frequently detected from striped jack and flounder while kelp grouper nervous necrosis virus (KGNNV) were detected from kelp grouper, striped jack, and flounder. No constant growth of the virus was noticed in the brains of threeline grunt and yellow tail. Considering that natural outbreaks of VNN (viral nervous necrosis) have been frequently reported in the former three species but not in the later two. The present result supports that the brain cell-tropism might be one of the tools to perform the host-specificity of betanodavirus.

**Key words:** Betanodavirus, marine fish, host-specificity, intravitreal injection, brain-tropism

### Introduction

Betanodavirus cause highly destructive disease, viral nervous necrosis (VNN) in hatchery-reared larvae and juveniles of a variety of marine fish species (Munday *et al.*, 2002). The virus is neuropathogenic and for this reason the disease has variously been designated as viral nervous necrosis (VNN) (Yoshikoshi and Inoue 1990), fish encephalitis (Breuil *et al.*, 1991) or viral encephalopathy and retinopathy (VER) (Munday *et al.*, 1992). Affected fish is characterized by a corkscrew or whirling swimming behavior and exhibit a range of neurological signs, which are noticed by vacuolation and cellular necrosis in the central nervous system (CNS) and retina. By genetic analysis based on the nucleotide sequence of partial (T4) region of coat protein gene, betanodavirus has been classified into 4 genotypes: SJ type, RG type, BF type and TP type (Nishizawa *et al.*, 1995). Among these genotypes, representative viruses are: SJNNV (striped jack nervous necrosis virus) for SJ type, RGNNV (redspotted grouper nervous necrosis virus) for RG type, BFNNV (barfin flounder nervous necrosis virus) for BF type and TPNNV (tiger puffer nervous necrosis virus) for TP type. Previous published reports reveal that natural hosts for SJ and TP types are very limited, while for RG and BF types, natural host range is broad (Totland *et al.*, 1999; Tanaka *et al.*, 1998). Genetic diversity of nodaviruses reflects host specificities. However, there is very limited information directly demonstrating differences on the host specificity between genotypic variants of betanodaviruses. So in the present situation to reveal the host specificity it was essential to study the growth of betanodaviruses in the experimentally infected fishes by enumerating the number of infectious virus. Differences of accumulated virus in the brain, the most devastating target organ for infection, were observed between two betanodaviruses, striped jack nervous

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necrosis virus (SJNNV), SJ-type and kelp grouper nervous necrosis virus (KGNNV), RG-type. The strategy is to develop a new method *in vivo* model to perform the host specificity in which betanodavirus (SJ- and RG-type) is introduced directly into eyes (intravitreally) where they replicate and travel to brain as betanodavirus is apparently neuroinvasive.

## Materials and Methods

**Experimental fish:** Five species of marine young fish, striped jack, *Pseudocaranx dentex* (average body weight 104 g), kelp grouper, *Epinephelus moara* (97 g); Japanese flounder, *Paralichthys olivaceus* (70 g); Threeline grunt, *Parapristipoma trilineatum* (323 g) and yellowtail, *Seriola quinqueradiata* (620 g) were used for the experiment.

**Cell line:** The E-11 cell line (total number of cell culture passages: 72), which had been cloned from the striped snakehead fry cell line (SSN-1; Frerichs *et al.*, 1996) by Iwamoto *et al.* (2000), was used to isolate and propagate betanodaviruses. The cell was cultured at 25 °C using Leibovitz L-15 medium supplemented with 5% fetal bovine serum (FBS).

**Virus:** SJNNV, SJNag93 strain (SJNNV-genotype) and KGNNV, KGOit97 strain (RGNNV-genotype), which had been isolated from diseased larval striped jack in Nagasaki prefecture in 1993 and diseased young kelp grouper in Oita prefecture in 1997, respectively, were used in this study and stored at -80 °C until being used.

**Preparation of viral solution:** Before infection experiment, the viral samples were thawed in ice and viruses were propagated in the E-11 cells at 25 °C, TCID<sub>50</sub> mL<sup>-1</sup> were identified and then adjusted to 10<sup>7</sup> TCID<sub>50</sub> mL<sup>-1</sup> using Hanks' balanced salt solution (HBSS).

**Investigation of brain cell susceptibility in fish:** Five species of marine young experimental fishes (25) were used for intravitreal injections (Fig. 1) with viruses at a dose of 10<sup>6</sup> TCID<sub>50</sub> fish<sup>-1</sup> (dose volume 10 µl) during March, 2003 at Kamiura station of Japan Sea-Farming Association (JASFA), and fish pathology laboratory, Higashihiroshima, Japan. Mock infected control groups were also set up in the same manner using comparable injections of HBSS instead of virus solution. Experimental fishes were artificially produced and reared at Kamiura station. Fish were reared in 500 L separate aquaria (tank) at 22±2 °C and were unfed throughout the entire experimental course. Five fish were sacrificed to collect the organ samples (brain, eye) at 0 h (just before injection), 24 h and 72 h after virus injection. These samples were stored at -80 °C until used for virus titration. Prior to experiments, RT-PCR tests using the F2-R3 primer set (Nishizawa *et al.*, 1994) were carried out for each of the fish groups to confirm that these fish groups were free from betanodaviruses.

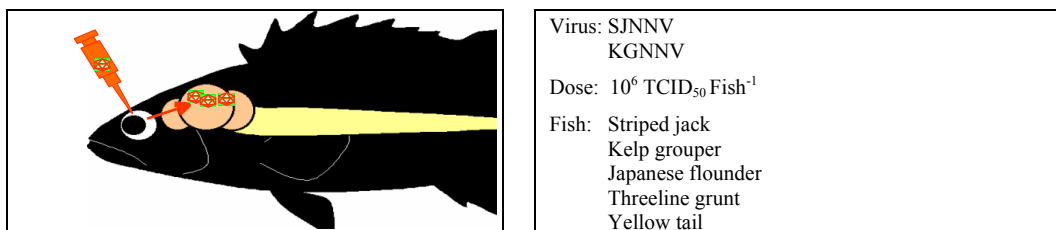


Fig. 1. Schematic diagram of intravitreal injection to investigate the brain cell tropism.

**Virus titration:** The organs of brain and eye samples were weighed and homogenized with 9 volumes of HBSS. After centrifuging at 2000 g for 10 min, the supernatant was filtered by a millipore membrane filter (0.45 µm). Serial 10-fold dilution from 10<sup>-2</sup> to 10<sup>-12</sup> of the supernatant were inoculated (50 µl) into 96-well plate containing E-11 cells at about 70% confluent and incubated at 25 °C for 10 days. The virus titer (TCID<sub>50</sub> g<sup>-1</sup>) was determined by Reed and Muench method (1938).

## Results

The results of brain cell susceptibility of betanodaviruses have been shown in the Table 1. Neither behavioral abnormalities nor mortalities were noticed in any of the fish groups for 72 h after injection and no virus was isolated from any of mock-infected control group fishes. Both SJNNV and KGNNV were detected from eye

at 24 and 72 h post-injection (PI) from the examined fishes, striped jack, kelp grouper and Japanese flounder, which are natural hosts of betanodaviruses. However, at 24 h no virus was detected from any brain samples while at 72 h PI both of the viruses were significantly detected at higher titer levels (with the exception of kelp grouper injected with SJNNV) from all the brain samples of striped jack, kelp grouper, Japanese flounder, indicating susceptibility of those fishes to betanodavirus. On the other hand, no virus was detected from the brains of threeline grunt (with only one exception when it was injected with SJNNV) and yellow tail, at 24 h and 72 h PI, suggesting that the brain of those fishes were not susceptible to betanodavirus. Among susceptible fishes, Japanese flounder was more susceptible to both SJNNV and KGNNV, though it was found more susceptible to KGNNV.

Table 1: Viral concentrations in the tissues of intravitreally injected five young marine fishes.

Fish	Virus	Organs	Concentration ( $\log_{10}$ TCID <sub>50</sub> g <sup>-1</sup> ) of viruses at									
			24 h PI					72 h PI				
			Fish number					Fish number				
			1	2	3	4	5	1	2	3	4	5
Striped jack ( <i>P. dentex</i> )	SJNNV	Eye	7.5	7.5	8.2	7.5	7.6	8.5	7.5	7.4	7.6	7.5
		Brain	-	-	-	-	-	4.5	5	4.8	4.9	4.1
	KGNNV	Eye	7.4	7.5	7.5	6.5	6.4	7.9	3.7	7.2	6.6	4.7
		Brain	-	-	-	-	-	6.5	6.1	6.1	6.3	4.8
Kelp grouper ( <i>E. moara</i> )	SJNNV	Eye	4.6	4.4	4.3	4.1	4	5.5	3.4	4.6	4.8	4.1
		Brain	-	-	-	-	-	-	-	-	-	4.4
	KGNNV	Eye	4.2	4.2	7	5.6	4.2	7.3	7.7	5.2	6.5	7.9
		Brain	-	-	-	-	-	7.1	6.2	7.1	6.2	8
Japanese flounder ( <i>P. olivaceus</i> )	SJNNV	Eye	5.6	5.3	6.7	5	6.8	6.5	6.9	6.8	6.4	6.6
		Brain	-	-	-	-	-	5.8	5.7	3.8	5.2	5.1
	KGNNV	Eye	6.6	6.8	5.8	5.3	5.5	8.2	8.5	8.5	8.1	8.8
		Brain	-	-	-	-	-	7.2	7.1	5.5	6.2	7.5
Threeline grunt ( <i>P. trilineatum</i> )	SJNNV	Eye	4.5	4.9	4.5	-	4.3	5.6	6.3	6.2	3.5	5.7
		Brain	-	-	-	-	-	-	-	-	-	3.9
	KGNNV	Eye	-	3.2	5.4	4.6	-	4.3	3.4	-	-	-
		Brain	-	-	-	-	-	-	-	-	-	-
Yellow tail ( <i>S. quinquerediata</i> )	SJNNV	Eye	4.3	4.1	4.6	4.5	4.8	8.8	4.5	5.4	7.6	4.8
		Brain	-	-	-	-	-	-	-	-	-	-
	KGNNV	Eye	-	-	-	-	-	4.9	4.9	5.7	4.3	nd
		Brain	-	-	-	-	-	-	-	-	-	nd

∴ <3; nd: not done

## Discussion

Knowledge of betanodavirus diversity is important for better understanding the wide variety of pathogenesis of disease, as well as host specificity of genetically different genotypes, which is still unknown. Vacuolation and necrosis, frequently reported in the VNN infections are common in brain cell. It is important to determine the genetic diversity and the replication kinetics of SJ type and RG type of betanodavirus in vivo to assess the ability to replicate in the brain of different natural and non-natural host species. If the virus can pass from the inoculated site to brain of the investigated fish and viral replication occurs there then it can be said that the particular fish has susceptibility to that virus, i.e., susceptibility of brain cells to betanodavirus will indicate total susceptibility of viruses. Therefore, it can be hypothesized that the differences in the brain cell tropism indicate the host specificity of different genotypes of viruses.

To investigate the brain cell tropism in different host species, intra-cerebral inoculation of different viruses belonging to different genotypes would be the best method to solve this issue. But there are some technical problems in the injecting and handling fish in this case. To overcome this problem, virus was directly injected in the vitreous body of eye where the inoculated virus particles are expected to get easy access to the retinal cells and subsequently transmitted to the brain via unidentified route, probably via the optic nerve (Nguyen *et al.*, 1996; Peducasse *et al.*, 1999; Ikenaga *et al.*, 2002).

According to the Table 1, intravitreally injected viruses retained in the eye and brain in fishes of natural hosts, striped jack, kelp grouper and Japanese flounder, within 72 h indicating susceptibility to both SJNNV and KGNNV, while no virus was detected in the brain of yellowtail, and very rarely isolated from threeline grunt indicating their non-susceptibility to betanodaviruses. Failure to replicate productively in the host yellowtail and threeline grunt indicates that betanodavirus is unable to affect the replication kinetics in vivo, and as well as to produce infection in those species.

Among the susceptible fishes (out of 5 tested fishes), injected KGNNV grew well in the eye and brain of natural host kelp grouper and as well as in non-natural hosts striped jack and Japanese flounder, indicating a broad host specificity of that virus. While, on the other hand, SJNNV scarcely recovered from the brain of kelp grouper, indicating that kelp grouper's brain is less susceptible to SJNNV.

In case of Japanese flounder, both SJNNV and KGNNV were recovered from brain within 72 h with the maximum titer of  $10^{5.8}$  and  $10^{7.5}$  TCID<sub>50</sub> g<sup>-1</sup> respectively, indicating a broad susceptibility of this species against different genotypic variants of batanodavirus. This result is consistent with the finding of Nishizawa *et al.* (1997). Furthermore, it was observed that Japanese flounder was more permissive to KGNNV as virus could be detected with the titer ranging from  $10^{5.5-7.5}$  TCID<sub>50</sub> g<sup>-1</sup>, which was comparatively higher than that of SJNNV (Table 1). Our model is novel in that sense virus was introduced directly in the eye and thus virus is delivered into the unknown route, which subsequently enters in the brain and cause infection.

Comparative studies of the susceptibilities of commercially valuable fish species to pathogenic nodaviruses are limited, and therefore, this study involving Japanese flounder, kelp grouper, striped jack, yellow tail, and threeline grunt is expected to provide useful data on the management of aquatic ecosystem.

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### References

- Breuil, G.; Bonami, J.R.; Pepin, J.F. and Pichot, Y. 1991. Viral infection (picorna-like virus) associated with mass mortalities with hatchery reared sea-bass (*Dicentrarchus labrax*) larvae and juveniles. *Aquaculture*, 97: 109-116.
- Frerichs, G.N.; Rodger, H.D. and Peric, Z. 1996. Cell culture isolation of piscine neuropathy nodavirus from juvenile sea bass, *Dicentrarchus labrax*. *Journal of General Virology*, 77: 2067-2071.
- Ikenaga, T.; Tatecho, Y.; Nakai, T. and Uemastu, K. 2002. Betanodavirus as nobel transneural tracer for fish. *Neuroscience Letter*, 331: 55-59.
- Iwamoto, T.; Nakai, T.; Mori, K.; Arimoto, M. and Furusawa, I. 2000. Cloning of fish cell line SSN-1 for piscine nodaviruses. *Diseases of Aquatic Organisms*, 43, 81-89.
- Munday, B.L.; Kwang, J. and Moody, N. 2002. Betanodavirus infections of teleost fish: a review. *Journal of Fish Diseases*, 25: 127-142.
- Munday, B.L.; Langdon, J.S.; Hyatte, A. and Humphrey, J.D. 1992. Mass mortality associated with a viral-induced vacuolating encephalopathy and retinopathy of larval and juvenile barramundi, *Lates calcarifer* Bloch. *Aquaculture*, 103: 197-211.
- Nguyen, H.D.; Nakai, T. and Muroga, K. 1996. Progression of striped jack nervous necrosis virus (SJNNV) infection in naturally and experimentally infected striped jack *Pseudocaranx dentex* larvae. *Diseases of Aquatic Organisms*, 24: 99-105.
- Nishizawa, T.; Furuhashi, M.; Nagai, T.; Nakai, T. and Muroga, K. 1997. Genomic classification of fish nodaviruses by molecular phylogenetic analysis of the coat protein gene. *Applied and Environmental Microbiology*, 63: 1633-1636.
- Nishizawa, T.; Mori, K.; Furuhashi, M.; Nakai, T.; Furusawa, I. and Muroga, K. 1995. Comparison of the coat protein genes of five fish nodaviruses, the causative agents of viral nervous necrosis in marine fish. *Journal of General Virology*, 76: 1563-1569.
- Nishizawa, T.; Mori, K.; Nakai, T.; Furusawa, I. and Muroga, K. 1994. Polymerase chain reaction (PCR) amplification of RNA of striped jack nervous necrosis virus (SJNNV). *Diseases of Aquatic Organisms*, 18:103-107.
- Peducasse, S.; Castric, J.; Thiery, R.; Jeffroy, J.; Van, A.L. and Baudin, F.L. 1999. Comparative study of viral encephalopathy and retinopathy in juvenile sea bass *Dicentrarchus labrax* infected in different ways. *Diseases of Aquatic Organisms*, 36: 11-20.
- Reed, L.J. and Muench, H. 1938. A simple method of estimating fifty percent endpoints. *American Journal of Hygiene*, 27: 493-497.
- Tanaka, S.; Aoki, H. and Nakai, T. 1998. Pathogenicity of the nodavirus detected from diseased sevenband grouper *Epinephelus septemfasciatus*. *Fish Pathology*, 33: 31-36.
- Totland, G.K.; Grotmol, S.; Morita, Y.; Nishioka, T. and Nakai, T. 1999. Pathogenicity of nodavirus strains from striped jack *Pseudocaranx dentex* and Atlantic halibut *Hippoglossus hippoglossus*, studied by waterborne challenge of yolk-sac larvae of both teleost species. *Diseases of Aquatic Organisms*, 38: 169-175.
- Yoshikoshi, K. and Inoue, K. 1990. Viral nervous necrosis in hatchery-reared larvae and juveniles of Japanese parrotfish, *Oplegnathus fasciatus* (Temminck and Schlegel). *Journal of Fish Diseases*, 13: 69-77.