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Original Article

Fate of Betanodaviruses in the Experimentally Infected Natural and Non-Natural Host

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To investigate the fate of betanodavirus, the causative agent of viral nervous necrosis (VNN) in cultured fish, the tissue distribution of virus in experimentally infected fish was investigated. Two genetically different betanodaviruses, striped jack nervous necrosis virus (SJNNV; SJNNV-genotype) and kelp grouper nervous necrosis virus (KGNNV) belonging to red spotted grouper nervous necrosis virus (RGNNV)-genotype, were intramuscularly cross-injected with a dose of 10^6 $TCID_{50}$/fish/200 µl in striped jack *Pseudocaranx dentex* or kelp grouper *Epinephelus moara* and sacrificed at scheduled to titre the virus in the spinal cord, brain, eye, kidney and blood at day 0, 1, 3, 5, 14 and 21. As a result, intramuscularly inoculated virus was recovered at high titres from the brain, spinal cord, and eye of their natural host species, *i.e.*, striped jack for SJNNV and kelp grouper for KGNNV during 3-week experimental period, while the virus titres were relatively low in the organs of non-natural hosts, particularly in kelp grouper injected with SJNNV. In every case, no virus was detected in blood samples, suggesting that infection did not develop to be systemic.

Keywords: Betanodavirus, Viral nervous necrosis, Fate, Tissue distribution, *Pseudocaranx dentex*, *Epinephelus moara*, 50% tissue culture infectious dose ($TCID_{50}$)

Introduction

Betanodaviruses (genus: Betanodavirus, family: Nodviridae) cause highly destructive diseases in hatchery reared larvae and juveniles of a variety of marine fish species and often damage fish at grow-out stages[1]. Affected fishes are characterized by a corkscrew or whirling swimming behaviours and exhibit a range of neurological signs, which are noticed by vacuolation and cellular necrosis in the central nervous system (CNS) and retina.

The genome of the betanodaviruses is bi-segmented, single-stranded, positive-sense RNAs, RNA1 (3.1 kb) and RNA2 (1.4 kb)[2]. Based on the partial sequence of the coat protein gene (RNA2), betanodaviruses are divided into four genotypes[3]: striped jack nervous necrosis virus (SJNNV)-, redspotted grouper nervous necrosis virus (RGNNV)-, tiger puffer nervous necrosis virus (TPNNV)-, and berfin flounder nervous necrosis virus (BFNNV)-types. Natural hosts for SJNNV and TPNNV genotypes are limited, only larval striped jack *Pseudocaranx dentex* for SJNNV and only larval or juvenile tiger puffer *Takifugu rublipes* for TPNNV[4-5]. BFNNV-genotype also has a limited number of natural host species, flounder (*Paralichthys olivaceus* and *Verasper moseri*), turbot (*Scophthalmus maximus*) and halibut (*Hippoglossus hippoglossus*)[6-9]. In contrast, RGNNV-genotype was isolated from a variety of warm-water fish species such as seabass (*Lates calcarifer*, *Dicentrarchus labrax* and *Lateolabax japonicus*) and grouper (*Epinephelus* spp., *Chromileptes altivelis*) at any developmental stages[10-16]. Isolating viruses in nature is indicating that various fishes are infected by this virus throughout their life stages - from larvae to adult stages.

Investigation to find the susceptibility of various fishes to betanodavirus have been experimentally carried out by many researchers[6,11,16-23]. However, the growth of viruses in natural and non-natural hosts through experimental infection had not been investigated in terms of quantifying viruses in various organs of infected fishes. Bearing this point in mind, infection characteristics of kelp grouper nervous necrosis virus (KGNNV, RG-genotype) and striped jack nervous necrosis virus (SJNNV, SJ-genotype) in young marine fishes of kelp grouper *Epinephelus moara* and striped jack *Pseudocaranx dentex* had been studied and the fate of these two strains of betanodavirus in vivo had been compared.

The prime objective of the study was to know the replication kinetics or growth pattern of betanodaviruses inside natural and non-natural young host body. Till the date, various studies like this have been made on betanodavirus. However, infection tests with quantified number of viruses have never been conducted.

Materials and Methods

Cell line

The E-11 cell line[24], which had been cloned from striped snakehead fry cell line[25] (SSN-1), was used to isolate and propagate betanodaviruses. The cell line was cultured at 25°C using Leibovitz L-15 medium supplemented with 5% fatal 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 *Epinephelus moara* in Oita prefecture, Japan in 1997 respectively, were used in this study. These viruses were propagated in the E-11 cells at 25°C, adjusted to 10^7 $TCID_{50}$ per ml using Hanks' balanced salt solution (HBSS) and stored at –80°C until being used.

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Experimental fish

Two species of marine fish, striped jack (average body weight 84 g), kelp grouper (112 g), were used for intramuscular injections with viruses (SJNNV and KGNNV). These fishes were artificially produced and reared at Kamiura station of Japan Sea-Farming Association (JASFA). Fish were reared in 500-l aquaria (tank) at 22 ± 2°C throughout entire experimental course. Prior to experiments, RT-PCR tests using the F2-R3 primer set[26] were carried out for each fish group to confirm that these fish groups were free from betanodaviruses.

Experimental infection

Groups of 35 fishes (striped jack and kelp grouper) were intramuscularly cross injected with viruses at a dose of 10^6 $TCID_{50}$/fish/200 µl. Fishes were sacrificed to collect organ samples (brain, spinal cord, eye, kidney, blood) from each five fish at day 0 (just before injection) 1, 3, 5, 7, 14 and 21 after virus injection. Mock-infected control groups received HBSS in the same manner. These samples were stored at –80°C until used for virus titration.

Virus titration

The organ and blood samples were homogenized and mixed with 9 volumes of HBSS. After centrifuging at 2,000x *g* for 10 min, the supernatant was 10-fold diluted with HBSS and filtered through a Millipore membrane filter (0.45 µm). Virus infective titres were determined using the E-11 cell line. Serial 10-fold dilution from 10^{-2} to 10^{-12} of the supernatant were inoculated into 96-well plate containing the cells at about 70% confluent and incubated at 25°C for 10 days. The virus titre ($TCID_{50}$/g) was determined by Reed and Muench Method[27].

Results

No external and internal pathological signs were noticed in fish groups injected with any of the either viruses or Hanks' balanced salt solution (HBSS). Only one fish of kelp grouper injected with kelp grouper nervous necrosis virus (KGNNV), died 7 days after injection. The virus titres in the dead fish were $10^{11.0}$ $TCID_{50}$/g for the brain sample.

In the infection experiment against natural hosts, *i.e*., kelp grouper injected with KGNNV and striped jack injected with striped jack nervous necrosis virus (SJNNV), both viruses were detected by cytopathic effects (CPE) on E-11 cells from spinal cord, brain, eye and kidney but never from blood samples (Table 1). In case of kelp grouper, the growth of KGNNV occurred in the spinal cord with infective titres ranging from $10^{7.1\text{-}9.1}$ $TCID_{50}$/g at day 3 and simultaneously in the brain with $10^{3.7\text{-}6.6}$ $TCID_{50}$/g, and then in the eye with $10^{4.2\text{-}9.4}$ $TCID_{50}$/g at day 7. The maximum titers were obtained at day 5 in spinal cord ($10^{9.5}$ $TCID_{50}$/g), at day 14 in the brain ($10^{9.8}$ $TCID_{50}$/g), and at day 21 in the eye (10^{10} $TCID_{50}$/g).

Table 1. *Tissue distribution of betanodaviruses in natural host fish after intramascular injection of viral genotype KGNNV in kelp grouper (Epinephelus moara) and genotype SJNNV in striped jack (Pseudocaranx dentex)*

Fish oragn (Specimen No.)		Infectivity of betanodaviruses ($TCID_{50}$/g or ml)											
		Genotype KGNNV in kelp grouper						Genotype SJNNV in striped jack					
		Day 1	3	5	7	14	21	Day 1	3	5	7	14	21
Spinal cord	1	-	8.6	9.3	8.8	8.5	6.8	-	6.5	7.9	6.5	5.8	-
	2	-	7.1	9.5	7.1	8.5	6.8	-	-	7.0	6.5	6.0	-
	3	-	8.8	9.1	8.5	9.0	6.8	-	6.3	6.3	6.3	5.5	-
	4	-	9.1	9.3	8.5	8.8	6.8	-	7.3	7.9	-	-	-
	5	-	5.8	8.8	8.8	8.3	6.8	-	-	6.0	6.3	-	6.3
Brain	1	-	4.5	6.4	8.4	9.0	7.5	-	-	5.3	4.2	5.9	6.7
	2	-	3.7	6.6	7.1	8.5	-7.0	-	-	4.5	4.7	6.2	-
	3	-	6.6	5.0	7.1	9.8	6.4	-	-	4.9	4.6	4.7	-
	4	-	4.3	5.7	7.4	8.8	-	-	4.9	5.5	6.9	5.5	4.7
	5	-	4.5	-	5.7	9.2	6.0	-	-	4.9	4.9	5.0	4.1
Eye	1	-	-	-	6.1	8.8	10.0	-	-	3.4	-	-	4.9
	2	-	-	-	4.8	7.9	7.6	-	-	4.0	-	5.1	4.9
	3	-	-	-	4.2	8.6	7.1	-	4.1	-	3.0	-	4.2
	4	-	-	-	-	-	6.2	-	-	3.7	4.2	5.1	3.9
	5	-	-	-	-	9.4	6.2	-	-	-	3.3	-	-
Kidney	1	-	-	-	-	-	-	-	6.6	6.7	7.2	5.8	5.1
	2	-	-	-	-	-	-	-	6.1	8.2	6.2	7.3	6.9
	3	-	-	5.6	-	-		-	5.9	7.5	6.6	6.5	7.1
	4	-	-	-	4.1	-	-	-	6.4	7.5	7.3	5.7	6.1
	5	-	-	-	6.1	-	-	-	-	7.0	5.9	5.3	5.1
Blood	1	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-	-	-	-

KGNNV = Kelp grouper nervous necrosis virus; SJNNV = Striped jack nervous necrosis virus; $TCID_{50}$ = 50% tissue culture infectious dose; (-) = <4.8 for spinal cord, <2.8 for others; ND = Not done.

The virus was detected in some of the kidneys at day 1 to day 7. On the other hand, SJNNV injected in striped jack appeared first at day 3 in the spinal cord and brain, and the titre reached maximum at day 5 in the spinal cord ($10^{7.9}$ $TCID_{50}$/g) and at day 7 in the brain ($10^{6.9}$ $TCID_{50}$/g), while in the eye the virus appeared at lower incidence during day 3 to day 21. It was noticed that the virus was detected in the kidney at high frequency and titers from day 3 to day 21.

Compared with the multiplication of virus in these natural hosts, inoculated virus isolated from non-natural hosts showed relatively lower frequencies and lower titre levels (Table 2). KGNNV inoculated in striped jack first appeared at day 3 in spinal cord and brain, and subsequently at day 14 in the eye. Besides, the virus was also isolated from the kidney at day 3 to day 7. SJNNV injected in kelp grouper was isolated only from spinal cord and brain of few fishes throughout the experimental period.

Discussion

In the present study, by enumerating the number of infective virus in the injected fishes, a comparison was made about infectivity of two genetically different betanodaviruses, striped jack nervous necrosis virus (SJNNV) and kelp grouper nervous necrosis virus (KGNNV). The present intramuscular injection of betanodavirus into kelp grouper and striped jack revealed a significant difference between KGNNV and SJNNV in terms of multiplication in the target organs (CNS and eye). In their natural host species, kelp grouper for KGNNV and striped jack for SJNNV, the first multiplication of the virus occurred in the spinal cord, simultaneously in the brain and subsequently in the eye. This virus transmission mode is consistent with those obtained in larval striped jack infected with SJNNV[20] and in larval Atlantic halibut with a BFNNV-genotype betanodavirus isolated from diseased Atlantic halibut[28]. Virus infective titers were particularly high in kelp grouper injected with KGNNV, reaching 10^9 $TCID_{50}$/g at the maximum in brain, but this titre levels were far (100 times) lower compared with that of a dead fish (10^{11} $TCID_{50}$/g). Although the mortality of kelp grouper due to natural infection occurs at juvenile or older fish[4], unidentified stress condition to fish may be required to enhance further multiplication of virus in the target organs. Natural infection of striped jack was limited to larval stage, within 20 days after hatching[29]. However, the present quantitative analysis of SJNNV in young striped jack indicates intrinsic susceptibility of this fish species to SJNNV. Furthermore, lower multiplication (10^7 $TCID_{50}$/g at the maximum) of SJNNV in striped

Table 2. *Tissue distribution of betanodaviruses in non-natural host fish after intramascular injection of viral genotype SJNNV in kelp grouper (Epinephelus moara) and genotype KGNNV in striped jack (Pseudocaranx dentex)*

Fish oragn (Specimen No.)		Infectivity of betanodaviruses ($TCID_{50}$/g or ml)											
		Genotype KGNNV in kelp grouper						Genotype SJNNV in striped jack					
		Day 1	3	5	7	14	21	Day 1	3	5	7	14	21
Spinal cord	1	-	-	-	-	-	-	-	3	8.3	-	-	-
	2	-	-	-	-	-	-	-	-	6.3	6.5	6.5	-
	3	-	8.3	-	7.5	-	-	-	7.1	8.0	-	6.0	-
	4	-	-	-	-	-	-	-	-	6.8	8.0	-	ND
	5	-	-	-	7.3	-	-	-	-	-	7.3	-	ND
Brain	1	-	-	-	-	-	-	-	4.9	5.5	-	-	-
	2	-	-	-	-	-	-	-	4.9	-	-	7.8	-
	3	-	-	-	-	-	-	-	4.2	-	-	6.0	-
	4	-	-	-	-	-	-	-	5.6	-	4.6	5.1	ND
	5	-	4.6	4.7	-	-	-	-	4.1	-	-	7.0	ND
Eye	1	-	-	-	-	-	-	-	-	-	-	3.4	4.9
	2	-	-	-	-	-	-	-	-	-	-	3.4	3.5
	3	-	-	-	-	-		-	-	-	-	4.0	7.6
	4	-	-	-	-	-	-	-	-	-	-	4.7	ND
	5	-	-	-	-	-	-	-	-	-	-	5.0	ND
Kidney	1	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	5.5	6.1	5.1	-	-
	3	-	-	-	-	-		-	4.8	6.0	5.5	-	-
	4	-	-	-	-	-	-	-	-	5.2	5.2	-	ND
	5	-	-	-	-	-	-	-	-	5.8	5.9	-	ND
Blood	1	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-	-	ND
	5	-	-	-	-	-	-	-	-	-	-	-	ND

KGNNV = Kelp grouper nervous necrosis virus; SJNNV = Striped jack nervous necrosis virus; $TCID_{50}$ = 50% tissue culture infectious dose; (-) = <4.8 for spinal cord, <2.8 for others; ND = Not done.

jack might be due to the stronger age-resistance characteristics of this species to SJNNV.

In the infection experiments to non-natural host species, *i.e*., striped jack for KGNNV and kelp grouper for SJNNV, a narrow host range of SJNNV (SJNNV-genotype) and a broad host-range of KGNNV (RGNNV-genotype) were clearly demonstrated by the infective titers in the CNS.

The failure to isolate the virus from any blood samples strongly suggest that virus, injected into the muscle could not spread via blood vascular system. This result is consistent with the result of Ikenaga *et al*.[30] as in his study labelled neurons was not observed in blood. However, the fact that the virus could be frequently isolated from the kidneys at relatively high titre levels, particularly in striped jack injected with SJNNV, suggests that viral entry and replication can occur there. Nevertheless, in general, neither characteristic histopathological lesions nor virus growth were noticed in organs other than central nervous tissues and retina, and only a few previous studies report the abnormality in the haemopoeitic tissues, the gills and intestine of affected fish[16,19-20] also showed virus multiplication in the epidermal cells of affected larval striped jack, but this was noticed only at the terminal stage of the infection. Grotmol *et al*.[28] reported that immuno-labelled lesions were also observed in the cranial region of the intestine at earlier stage of infection as well as in the CNS and later in the infection in liver, olfactory epithelium, yolk-sac epithelium, gills and pectoral fins of experimentally infected Atlantic halibut yolk-sac larvae. As melanomacrophages are well known to be involved in defense mechanism of fish[31], the appearance of infective particles into the kidneys in the present study probably indicates a process of host's clearance mechanism[32], though the possibility of the multiplication of virus in renal cells can not be ruled out.

The present study indicated that virus inoculated intramuscularly retained in the CNS or eye without apparent clinical signs in fish. Our data together with other published reports[33] lead to the conclusion that most infections in juvenile or older fish are presumably latent and overt infection, and will not occur until vigorous multiplication of the virus is triggered by unknown factors reducing host defense mechanisms. This unapparent or persistent infection in fish may suggests adaptation of betanodavirus to fish to get a diversity, particularly in RGNNV-genotype which have a wide range of natural host and genetic diversity in the coat protein gene[3].

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