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PORTAL ENTRY AND PROGRESSION OF BETANODAVIRUS CAUSING VIRAL NERVOUS NECROSIS IN SEVENBAND GROUPER *EPINEPHELUS SEPTEMFASCIATUS*

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ABSTRACT

A study was conducted to identify the entry site for the betanodavirus, the causative agent of viral nervous necrosis (VNN) in 375 fish (150 spawn, 150 fry and 75 fingerling) during the period from March to August 2003. Highly susceptible fish, sevenband grouper *Epinephelus septemfasciatus* to sevenband grouper nervous necrosis virus (SGNNV) was used as experimental fish. Fish were placed into the seawater containing 10^4 $TCID_{50}$ mL^{-1} of the viral isolate for 1 h. Samples for virus isolation were randomly collected at an interval of 3 h, 12 h, 24 h and daily, and continued until 5 to14 days depending on the mortality. The inoculated virus could first recover from the mouth including nostril at 3 h of exposure and then from fin at 12 h, and within 1 d it was detected from all the organs examined at titers ranging from 10^4 to 10^7 $TCID_{50}$ g^{-1}. The highest titer was found at day 5 in the target organs of central nervous system (CNS), i.e., the brain, bone including spinal cord, and eye. The results suggest that nasal route the initial route for the portal entry of betanodavirus into fish.

Key words : Betanodavirus, viral nervous necrosis, virus titration, portal entry, nasal route, sevenband grouper.

INTRODUCTION

Viral nervous necrosis (VNN) or viral encephalophathy and retinopathy (VER) is one of the major constraints on culture of a number of marine fish species (Anon., 2003) that is caused by betanodavirus, a bipartite, single stranded, positive sense RNA virus that belongs to the family *Nodaviridae*. VNN is a highly destructive disease in hatchery reared larvae and juveniles, or grow-out stages in some species such as grouper and sea bass (Munday *et al.*, 2002). The major clinical entities of VNN are characterized by cellular necrosis and vacuolation in the central nervous system (brain and spinal cord) and retina and common behavioral changes such as lack of appetite, erratic, spiral, or belly-up swimming and dark coloration of body. The disease was first reported in Japanese parrotfish *Oplegnathus fasciatus* in Japan (Yoshikoshi and Inoue, 1990) and in barramundi *Lates calcarifer* in Australia (Glazebrook *et al.*, 1990). Thereafter, with the development of marine finfish aquaculture, the disease has spread worldwide in Asia, Europe and USA (reviewed by Munday *et al.*, 2002). Betanodaviruses have been detected or isolated as the causal agent of VNN in a variety of diseased marine fishes including 32 marine fish species or more, belonging to 15 families.

General clinical signs, appearances, physical and biological properties, and histopathology of infection causing death have been well detailed in many studies (Mori *et al.*, 1992; Nguyen *et al.*, 1996; Totland *et al.*, 1999; Grotmol *et al.*, 1999). However, detailed information on penetration, multiplication and dissemination of this virus in fish are scare. Moreover, the complete infection cycle of VNN in fish has not been determined. Many questions still remain concerning the route of viral entry. Since nodavirus predominantly infects neuronal and retinal cells, the first critical step in the infection process is entry of the virus into the cell. Viral invasion from external environment to host intracellular environment is an essential step in the pathogenesis of the disease. There have been some reports on the entry of some pathogenic viruses in fish. Gills have been suggested as the site of viral entry for fish rhabdovirus infections, such as spring viraemia of carp (Ahne, 1978), hemorrhagic septicemia of trout (Neukirch, 1984), infectious hematopoietic necrosis (Yamamoto and Clermont, 1990). In case of Betanodaviruses, virological tests were carried out by various authors using several infection and diagnosis methods (Nguyen *et al.*, 1996; Grotmol *et al.*, 1999), and skin, gill and intestinal epithelia were importantly indicated as initial multiplication sites of the viral infection. However, to the best of our knowledge there is no available information on quantified infectivity of virus or on determining virus yield from individual organ to identify the entry point for nodavirus. As a first step to determine the potential route (s) of infection used by the pathogen to gain access to fish host, the present study was carried out in order to obtain information concerning entry, multiplication and spread of virus. For this purpose, sevenband grouper of different age groups, viz., spawn, fry and fingerling had been sequentially used in this study. The objective of this study was to develop a better understanding about the route of the viral entry and progression of the virus through fish tissues.

MATERIALS AND METHODS

Fish

Sevenband grouper *E. septemfasciatus* of different age groups viz., 150 spawn (2 days old), 150 fry (22 days old), and 75 fingerling (59 days old) of the same population were obtained from Kamiura station of Japan Sea-Farming Association, Oita Prefecture was used as experimental fish during the period from March to August 2003. The fish were maintained as the specific pathogen free (SPF) examining by polymerase chain reaction (PCR).

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Virus

Sevenband grouper nervous necrosis virus (SGNNV) was isolated in 1997 from diseased sevenband grouper cultured in commercial fish farm in Wakayama, Japan was used in this study. The isolate was passed thrice in E-11 cell having L-15 medium, and was used in all challenged cases.

Culture of virus

The E-11 cell line, which had been cloned from striped snakehead (SSN-1) cell line (Iwamoto *et al.*, 2000), was used to isolate and propagate the virus. The cell line was cultured in 25 cm^2 tissue culture plates having Leibovitz' L-15 medium supplemented with 5% fetal bovine serum (FBS) at 25°C. For experimental purpose viral suspension was made in Hank's balance salt solution (HBSS) with the supernatant after centrifuging the culture at 3000 x g for 10 min.

Infection challenge

Three experimental set up were used for three different age groups of sevenband grouper in the experimental infection as experiment I for spawn, experiment II for fry and experiment III for fingerling. In experiment I and II, infection with betanodavirus (SGNNV) was carried out by placing about 150 spawn and fry randomly into the 2 L jar (s) of static aerated seawater containing 10^4 $TCID_{50}$ mL^{-1} of virus. The fish seeds were unfed and ambient temperature was maintained at 25°C. In experiment-III, 200 µl of same viral solution was added into 2 L of seawater containing 75 fingerlings (size 2.5 – 3.5 cm) for 1 h under aeration and then gently placed in 100 L tanks. Water temperature was maintained at 25°C and juveniles were fed dry pellets lightly on daily basis. Each of the experiments was repeated twice and continued until 5 to 14 days depending on the mortality.

In each experiment five groups were maintained among which three for virus exposure and the other two for control (mock infected). Of the three virus-exposed groups two were monitored daily for mortality, and the other one was used to determine the extent of virus by titration methods. Out of two control groups, one was used for monitoring mortality and the other for virus titration.

Sampling

Samples were collected at an interval of 3 h, 12 h, 24 h and daily until death (5 to 14 days). Depending on fish size, seeds were either assayed whole or individual organs separately after dissection. In every sampling time, 10 fish seed (whole) were randomly collected for experiment I and experiment II while for experiment III, a pooled samples (3) of organs viz., skin, fins, gills, eye, brain, mouth including nostril, bone with kidney and spinal cord, muscle, and visceral organs were removed and examined twice for virus isolation.

Virus titration

The samples, whole body of fish seed or individual organs were weighed and homogenized using tissue homogenizer in eppendroff tubes and diluted using HBSS (9 : 1) under sterile condition. The tissue suspension was centrifuged (2000 x *g*) for 10 min and the supernatant was filtered using 0.25 µm millipore membrane filter, and inoculated in E-11 cell line in 10 fold serial dilution from 10^{-2} to 10^{-12}. Virus titration was performed by end point dilution in 96-well microtiter plates using 4 well per dilution, and virus infectivity titer was calculated as per method described by Reed and Muench (1938) and was expressed as tissue culture infective dose ($TCID_{50}$ g^{-1} tissue).

RESULTS AND DISCUSSION

In the course of the experimental infection, nodavirus was recovered from all challenged groups of fish (Table 1 and Table 2) while no clinical signs appeared nor virus was detected in mock-infected control groups in all trials. In experiment I, virus could isolate at relatively lower titers ($10^{5.5}$ $TCID_{50}$ g^{-1}) from pooled samples of 3 h, 12 h and 1 d after virus exposure (Table 1). After two days exposure, when the first death of fish was recorded, titer levels rapidly increased and reached to 10^{11} $TCID_{50}$ g^{-1} at 4 to 6 days post exposure. The cumulative mortalities of challenged groups after immersion challenge with virus and of mock challenged groups are presented in Fig. 1. In experiment I, the mortality initiated at day 2 in both virus, challenged and control groups, and all fish seed died within 6 days in virus-challenged groups, and within 7 days in control group (Fig. 1a).

Table 1. Virus titers (Log $TCID_{50}$ g^{-1}) in the whole body of sevenband grouper after exposure to betanodavirus SGNNV

Experiment*	Sampling time (h = hour & d = day)							
	3 h	12 h	1 d	2 d	3 d	4 d	5 d	6 d
I	5.5	5.6	5.6	7.5	9.5	11.6	11.5	11.4
II	< 3[a]	< 3	6.6	7.4	9.8	11.8	10.7	10.7

[a]Detection limit, *Experiment I for fish spawn and experiment II for fish fry.

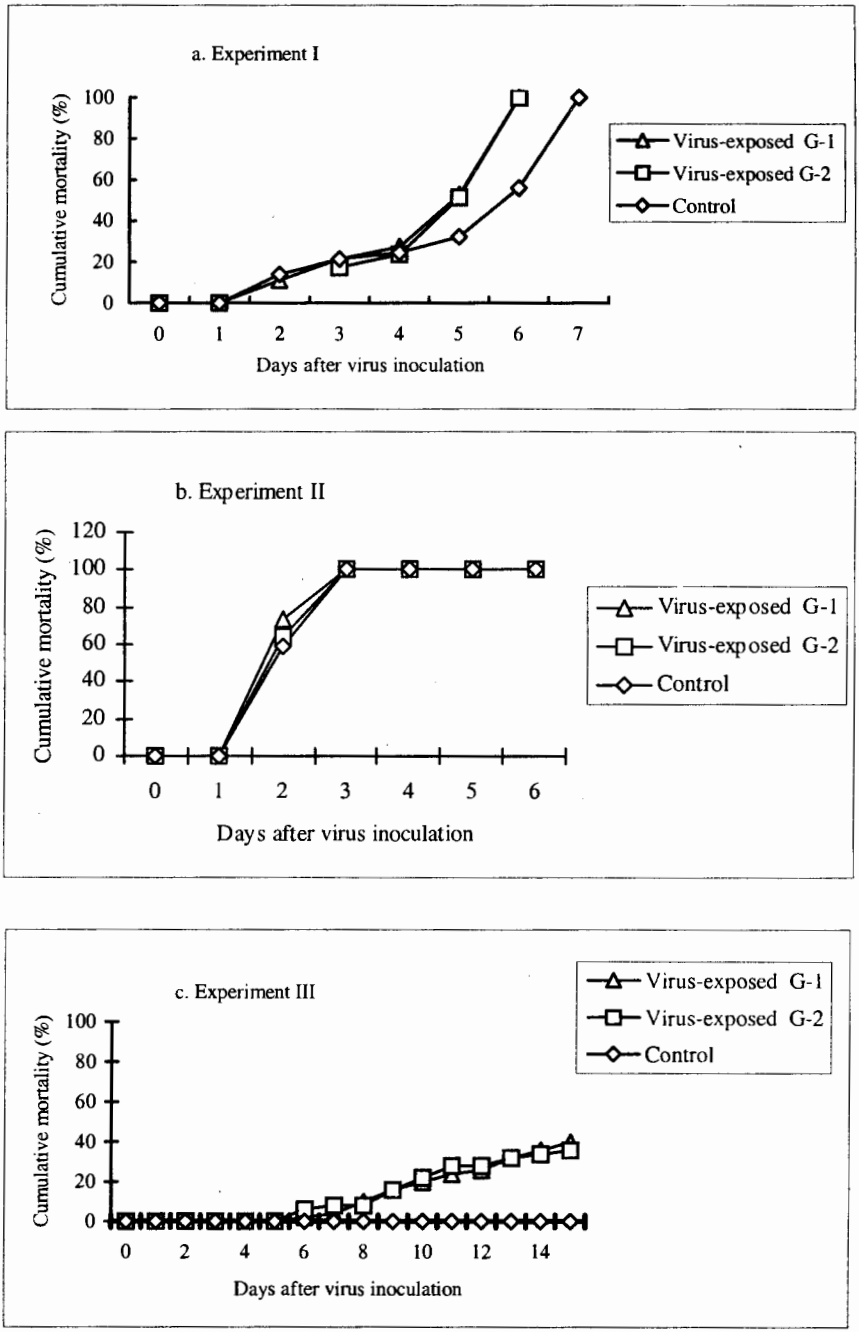

Fig. 1. Cumulative mortalities of sevenband grouper after exposure to betanodavirus SGNNV

In experiment II, the virus could not isolated from fish at 3 h and 12 h post exposure. The virus could first isolate at 1 day after exposure with the titer of $10^{6.6}$ $TCID_{50}\,g^{-1}$ and subsequently increasing titers reaching to 10^{11} $TCID_{50}\,g^{-1}$ at day 4 to 6 post exposure (Table 1). A marked mortality (about 70%) was found at 2 days post exposure in both challenged and control groups (Fig. 1b).

Table 2. Virus titers (Log $TCID_{50}\,g^{-1}$) in the organs of sevenband grouper after exposure to betanodavirus SGNNV in experiment III

Organs	Trial No.	Sampling after virus exposure							Dead fish
		3 h	12 h	1 d	2 d	3 d	4 d	5 d	
Nostril	1	4.6	4.8	4.3	5.2	5.4	5.2	5.5	4.7
	2	4.3	4.7	4.5	4.9	5.6	4.3	5.1	4.6
Fin	1	< 3[a]	4.5	5.4	6.2	5.7	6.3	6.8	7.0
	2	< 3	4.4	5.1	6.8	6.9	6.7	6.1	7.1
Gills	1	< 3	< 3	5.3	5.6	6.4	6.7	6.7	7.6
	2	< 3	< 3	4.2	5.6	6.5	5.8	7.5	7.0
Skin	1	< 3	< 3	5.5	6.8	7.9	7.3	7.4	7.6
	2	< 3	< 3	5.2	6.4	7.5	6.9	7.1	7.3
Muscle	1	< 3	< 3	6.7	7.5	7.6	7.5	6.9	7.1
	2	< 3	< 3	7.3	7.1	6.9	7.4	7.3	6.8
Bone	1	< 3	< 3	7.5	6.4	7.0	7.8	8.3	8.8
	2	< 3	< 3	6.9	7.1	7.5	8.4	8.1	8.4
Eye	1	< 3	< 3	6.3	7.2	7.6	8.8	10.1	11.0
	2	< 3	< 3	7.2	7.5	8.3	8.7	9.2	10.7
Brain	1	< 3	< 3	5.4	6.6	7.8	8.2	7.8	9.8
	2	< 3	< 3	5.6	6.3	7.6	8.1	8.3	9.7

[a] Detection limit

In experiment III, the clinical signs of lethargy, an up side down swimming behavior were generally observed at day 4. Virus was first detected in mouth with nostril at 3 h of exposure (10^4 $TCID_{50}\,g^{-1}$) and then in fin at 12 h of exposure with titers ranging from 10^{4-5} $TCID_{50}\,g^{-1}$, and the virus was found to be widely distributed within 1 d in gills, skin, eye, brain, bone and even visceral organs (Table 2). Virus detection in various tissues of the exposed fish indicated that virus infection and replication was wide spread in the body of the infected fish. No mortality was recorded in the control group of experiment III (Fig. 1c), while in challenged groups, it was about 40% during the experimental period (15 days). The virus titers in brain, bone with kidney and spinal cord, and eye of the sampled dead fish were 10^9, 10^8, and 10^{11} $TCID_{50}\,g^{-1}$, respectively (Table 2). The onset of death of fish occurred earlier in spawn (experiment I) and fry (experiment II) than those of fingerling (experiment III). Typical signs of viral infection were not observed in the control group of spawn and fry. Even virus could not trace from these fishes although high mortality occurred in these groups. The cause of mortality was difficult to determine; starvation and handling problem may have contributed to the mortality.

Nodaviruses have diversity in the genotypic difference, pathogenesis and transmission. Researches have been performed to demonstrate the progression, tissue distribution, and transmission (Nguyen *et al.*, 1996; Grotmol *et al.*, 1999). However, normal mode of viral entry has not been conclusively demonstrated. The sequential immersion experiment in this study indicated that virus can enter into its host through a variety of tissues. The virus was first detected at very low levels (10^4 $TCID_{50}\,g^{-1}$) at 3 h from mouth region including nostrils. The initial virus isolation within 3 h suggests that primary replication of the virus takes place in this site. Samples of fish spawn at 12 h also showed the presence of low concentration of virus in the fins (10^{4-5} $TCID_{50}\,g^{-1}$), but not in any of the other organs. At 24 h, virus was detected at low levels in many of the organs assayed. With this evidence, it was understood that the response of invading virus was found to be rapid. Virus concentrations for most of the organs continued to increase and respective titers remained high until completion of the experiment. Among these organs examined the mouth with nostril was found to be highly efficient for viral attachment and considered to be the initial route of portal entry.

Report from a previous work indicated that nodavirus is efficient in attaching and invading through the skin surface (Grotmol *et al.*, 1999). In this study, failure to detect virus in substantial level in skin within 12 h suggests that skin is not main route of entry for betanodavirus. Our results support previous suggestion by Nguyen *et al.* (1996) that skin is not an important site of initial replication. The ability of virus to penetrate skin is not remarkable, because in the absence of any injury or without the assistance of other mechanical damage, invasion via skin is much more difficult to accomplish. Reason being, structurally skin presents a far more formidable barrier to penetrate than does the mouth or gills. Our result showed that virus could also detect from gills but not before 1 d of exposure. This result supports the

findings of Nguyen *et al.* (1996), Grotmol *et al.* (1999) and Chi *et al.* (2001) where it had been expressed that gills was not the main entry site of the virus although they could detect the virus from the gills.

Grotmol *et al.* (1999) also found that susceptibility of Atlantic halibut (*Hippoglossus hippoglossus*) larvae to Atlantic halibut nodavirus (AHNV) was low during first 2 weeks while our studies have shown that sevenband grouper fingerling was highly susceptible to SGNNV at 6 days or onward post exposure. After immersion challenge, SGNNV infected fish exhibited virus-induced mortality, which approached 100% within 6 days of exposure in case of spawn. High levels (10^{11} $TCID_{50}$ g^{-1}) of virus were recovered from affected fishes. Whether this change was due to merely an increase in age of the fish or to development in the immune system is yet to be known.

Grotmol *et al.* (1999) found that immunolabelled virus was simultaneously observed in the stratified epithelium of the anterior intestine and in the CNS indicating that virus must be present somewhere before coming these sites. Our immersion experiment strongly suggests that virus can enter into its host body through tissues of mouth with nostril. The nasopharynx is also reported to be an important portal of entry for rabbit haemorrhagic disease virus (RHDV) (Kimura *et al.*, 2001). The finding of this study is the first report indicating that betanodavirus (SGNNV) can enter into sevenband grouper via nasal route. So, intra-nasal inoculation of the virus is an interesting subject to determine the importance of olfactory epithelium as a portal of entry and an effective route to access to the CNS. After initial replication in the neurons located in the site of entry, used SGNNV is likely to migrate intra-axonally in an anterograde and retrograde direction (Ikenaga *et al.*, 2002) towards the CNS. The virus might initially replicate in the neuroreceptor cells of the olfactory epithelium. Subsequently, it gains access to cells of the main olfactory bulb. Following invasion of this site, further spread is then possible into target organs through polysynaptic neuronal connection.

A variety of mechanisms can be considered possible for explaining the above phenomenon. What brings about this entry is unknown (whether viral attachment factors or cellular receptors) but the powerful effects observed in our experiment may have a dramatic impact in search of entry route of betanodavirus. Information gained from expected future studies (using various species to different genotypes following intra-oral or intra-nasal inoculation) along with our results might have impact upon identifying entry of virus and cause of infection in CNS. Finally, researches to find suitable drugs that can prevent the entry of viruses to its host body and / or stimulant to increase local immunity at mouth or nasopharyngial cavity seem to be very interesting to explore.

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