Viral Nervous Necrosis (VNN) in White Seabass, *Atractoscion nobilis*, Cultured in Southern California, and Implications for Marine Fish Aquaculture

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Abstract-The organism responsible for causing Viral Nervous Necrosis (VNN) or Viral Encephalopathy and Retinopathy (VER) has been demonstrated to belong to the group Nodaviridae [1] [2]. Since its initial identification in 1990 by Yoshikoshi and Inoue [3], in Japanese parrotfish, its distribution and/or identification has become global.

Methods of identification developed and results from outbreaks of VNN in white seabass between 1992 and 1999 have been published in Curtis et al. [4]. The white seabass cultured at the Hubbs-SeaWorld Research Institute's hatchery in Carlsbad California (CBD), have experienced VNN, of varying severity, since 1992. Disease in 1992 was thought to be due to a picrona-like virus but the indirect fluorescent antibody technique (IFAT) performed in 1999 on samples from 1992, indicated the same nodavirus was responsible. Confirmation of the nodavirus in white seabass was done using IFAT, reverse transcription- polymerase chain reaction (RT-PCR), electron microscopy and virus isolation via cell culture. The strain of nodavirus isolated from white seabass was shown to be similar to that recovered from redspotted grouper in Japan. The most serious and extensive outbreak experienced in white seabass at CBD extended from August of 2002 to May 2003. Since 1999 a concerted effort has been made to further the understanding of VNN epidemiology in white seabass. Recently, enzyme linked immunosorbant assays (ELISA) performed on sera samples from wild fish have demonstrated VNN exposure in wild juvenile white seabass.

With the apparent lack of species specificity of VNN and its global distribution, continued devastating impacts on new marine species development are a distinct possibility. Along with cooperative measures for identification and epidemiology, there needs to be a drive towards development of preventative husbandry protocols and methods of prophylaxis.

I. Introduction

The organism responsible for Viral Nervous Necrosis (VNN) or Viral Encephalopathy (VER) is a nodavirus (Nodaviridae). Nodaviruses were classically known as insect pathogens. Since the identification of nodavirus in fish, they have been divided into two groups, the insect strains with the genera Alphanodavirus and fish strains the genera Betanodavirus [5]. The virus is small in size, ranging from 22 to 34 nm, is icosahedral in symmetry, lacks an envelope and contains two molecules of single-stranded, positive-sense RNA.

Typical clinical signs in white seabass, *Atractoscion nobilis*, vary with fish age. Larvae and fry ages 12 to 40 days post hatch (dph) typically present with hyper inflated swimbladders and appear lethargic, laying on their sides at the surface. If disease persists past 40 dph, infected fish appear disoriented and swim in a spiral pattern. White seabass generally do not show signs of infection after 45 dph when grown at 23°C, unless disease started prior to 45 dph.

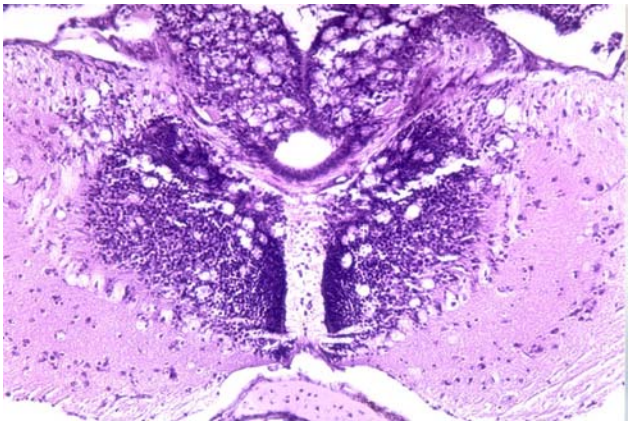

Fig. 1. Photo of brain from diseased 40 dph white seabass with mild vacuolation (100x).

The typical lesions in white seabass are associated with the brain, spinal cord and retina. Vacuolation of these tissues causes degeneration and death. Severity can vary from focal vacuoles in one site (Fig. 1) to diffuse multifocal vacuolation of all three tissues (Fig. 2).

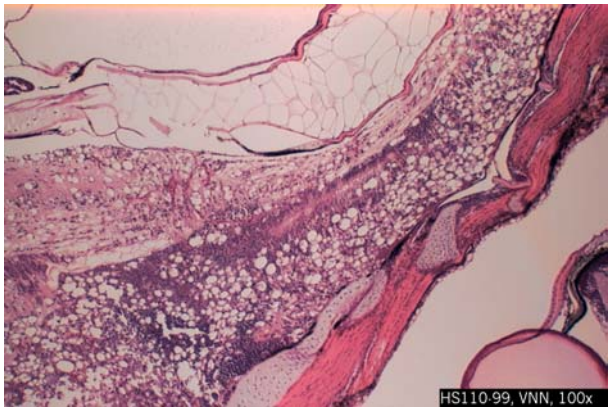

Fig. 2. Photo of brain and spinal cord from diseased 40 dph white seabass with severe vacuolation

Since the first description and identification of VNN [3] [6], it's distribution has streached globally. By 1997 it had been reported worldwide in 19 teleost species from 10 families [7]. In 2002, Munday et al. [8] reported in a review on Betanodavirus, that the virus has been identified in fish on all continents of the world, except Africa. There are now 16 families and 32 species of fish known to be affected (Fig. 3). The lack of species specificity may account for the broad global distribution of Betanodavirus.

The combination of global distribution and lack of species specificity makes the VNN virus a dangerous pathogen that could seriously hinder the growth and diversification of the aquaculture industry. VNN has already had a dramatic impact on the development of commercial flatfish and cod culture along the east coast of North America (King, Pers. comm.). Hatcheries have had to depopulate in order to eliminate the organism from their culture facilities. Japan has also felt the impact with 12 species of fish under commercial development. Japanese species affected include flatfish, jacks and groupers [8]. In Europe, cultured European seabass, *Dicentrarchus labrax*, have also suffered from VNN epizootics [9] [10] [11]. As the number of species, and the intensity of culture increases, so does the list of species affected.

II. Background and History of VNN in Cultured White Seabass

Intensive rearing of white seabass larvae is undertaken by the Hubbs-SeaWorld Research Institute at two locations (HSWRI). One location is on Mission Bay (MB) in San Diego, California; the second on the Agua Hedionda lagoon (AH) in Carlsbad, California.

The HSWRI hatchery in CBD is a full-scale marine larval rearing facility on the coast of California and is where the majority of the fish culture is undertaken. The facility houses approximately 200 broodstock in four independently temperature and photoperiod controlled recirculating systems. Each system contains one 45500 l tank housing approximately 50 fish. Twelve incubators, each holding 1600 l of water are operated on an independent temperature controlled recirculating system. Two large temperature controlled recirculating systems are present for early rearing. Six 9500 l tanks are used to rear fish from 12 to 45 dph and a second group of eight 9500 l, and eight 1000 l tanks for growout from 45 to 90 dph. Seawater is supplied to the hatchery at approximately 3785 l/min from the Agua Hedionda lagoon after passing through sand filters.

The white seabass produced by the HSWRI are part of the Ocean Resource Enhancement and Hatchery Program (OREHP) administered by the California Department of Fish and Game (CDF&G). Because the fish are being released into the ocean intentionally, strict health certification requirements have been established by the CDF&G. The identification of any virus of unknown origin in cultured fish disqualifies them from being released and most often results in euthanasia. The CDF&G policy is such that if the virus can be identified as naturally occurring (i.e. found in the wild population), fish surviving a disease outbreak would be eligible for release.

The first diagnosed incidence of VNN in white seabass was in July 1992 at the MB laboratory, with acute mortality in 40 dph fish. Infected fish swam in a spiraling motion when excited yet otherwise were listless and drifted in the water column. Histologic samples were characterized by severe vacuolation of the brain and spinal cord. Transmission electron microscopy (TEM) revealed aggregates of small picrona-like virus particles in the spinal cord and brain.

When VNN was discovered in several groups of seabass in 1999 at AH, a more concerted effort was initiated to determine and document prevalence, epidemiology and histopathology. TEM examination of 1999 samples revealed virus particles similar to those from the 1992 case. In follow up studies, the indirect fluorescent antibody technique (IFAT) was applied to 36 fish sampled in 1999. Of these, 11 were positive for virus. The virus was subsequently identified as a nodavirus (Nodaviridae) [4].

Severe infections curtailed production at the AH hatchery from mid-2002 to mid-2003 causing mortality in fish 13 to 45 days of age. Once fish reached approximately 45 dph, they no longer appear susceptible to mortality caused by the VNN virus. At approximately 45 dph, white seabass undergo a metamorphosis, after which they more closely resemble the adult form. It is interesting that this period of metamorphosis coincides with the timing of reduced sensitivity to VNNV, although a direct cause and effect relationship has not been established. Along with the

above-mentioned diagnostic tools, an enzyme linked immunosorbant assays (ELISA) was developed to help evaluate the distribution of exposure in both hatchery reared and wild fish. The VNN ELISA therefore became an important tool for the success of the hatchery by indicating exposure in wild seabass.

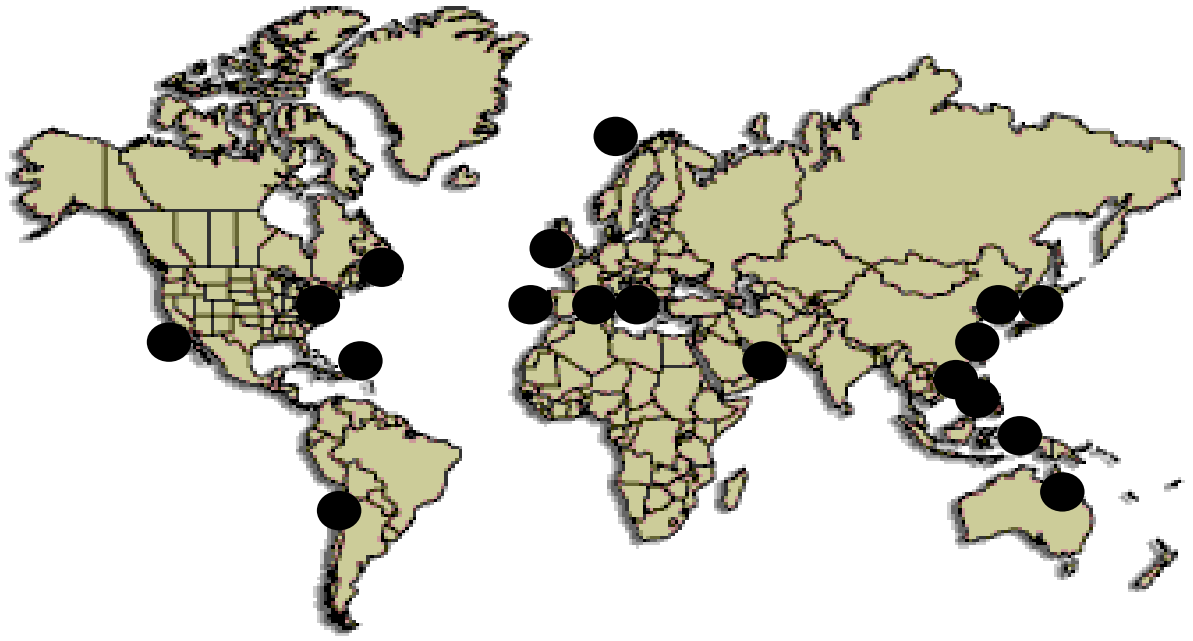

Fig. 3. Global Distribution of VNN outbreaks

VNN has been a reoccurring problem at many hatcheries globally, and thus the development of husbandry techniques and protocols to avoid or control the organism from affecting sensitive life cycle stages is imperative.

III. Discussion

A. California White Seabass Production

Betanodavirus has had a significant impact on hatchery production of white seabass. The need to determine appropriate husbandry techniques to avoid or manage around the organism has driven the effort to understand its pathology, epidemiology and control. Over the last four years, a great deal of advancement has been made towards this end, and production has improved.

Transmission of Betanodavirus is believed to occur both vertically and horizontally. Evidence for vertical transmission is strong in striped jack [12]; lesser so with European seabass [2], Japanese and barred flounder [13] and Atlantic halibut [14]. When vertically transmitted, VNN can be catastrophic. The culture of striped jack was seriously hindered as most groups of 1 to 4 dph larvae became infected [1] [12]. Once a Betanodavirus broodstock screening program was put in place, disease free groups of larvae were produced [15]. Though the possibility exists that all susceptible species may be vulnerable to vertical transmission, Munday et al. [8] speculated that the virus may only be present in the ovary at specific times, and stressful events such as repeat spawning may exacerbate infection and transmission. If vertical transmission does occur, surface egg sterilization may help prevent infection. Grotmol and Totland [16] were able to inactivate the virus using ozonated seawater baths on Atlantic halibut eggs, reducing larval mortality.

Vertical transmission in white seabass, though not demonstrated, is a possibility as with any species that is prone to infection. As wild fish are the source of broodstock, and new fish enter the system periodically possibly acting as a vector. The antibody based ELISA was developed to determine if wild fish have been exposed to the virus. A preliminary examination of blood sera from 20 wild fish indicated 2 were positive. A rapid diagnostic test, like this ELISA, can be used for eliminating sera positive newly collected animals from the broodstock pool and to test broodstock currently in captivity.

Observations obtained from VNN outbreaks in white seabass suggest a horizontal mode of transmission [4]. Of larval sibling groups, set up in two different geographical locations, only fish at one location became infected with the VNN virus. If the virus was being passed vertically, it would be reasonable to expect that both groups would succumb. The white seabass outbreaks of 2002-2003, also appear to support horizontal transmission of Betanodavirus. As the virus is very hardy, it is difficult to eliminate from an infected system. Inadequate sterilization of systems in 2002 appeared to allow the reinfection of new larval batches entering the system. Once adequate sterilization was achieved, no further outbreaks were observed.

As the white seabass cultured by HSWRI are released to the ocean, and Betanodavirus is transmitted horizontally, it was important that VNN exposure was detected in wild fish so there was no possibility of introducing the virus into a naive wild stock. The potential of horizontal transmission inhibited white seabass production due to DFG policy mentioned above. The ELISA therefore became a very important tool for the culture and release program.

B. Global Significance

The discovery of Betanodavirus around the globe opens questions as to how it became so well distributed. Isolates are generally detected once a species is under development for commercial production. It is therefore difficult to determine how long the virus may have been present in wild fish populations. Munday et al. [8] suggests that the spread of VNN within a species is due to commerce. Infected fish are transported to markets, thus distributing the organism.

In Curtis et al. [4], speculation on distribution through other species was discussed. The genotype identified in white seabass was the RGNNV strain, which has also been identified in several fish species in Japan, European seabass from the Mediterranean, Malabar reefcod from Thailand and ombrina from Italy [17] [18] [19]. With a broad number of species affected and lack of species specificity, the possibility of highly migratory fish distributing the organism from one geographical location to another exists. Once local fish are exposed, they may then become carriers and distribute the virus. The presence of virus without active disease has been identified by: Nishizawa et al. [17] in red seabream and pacific cod; in brown meager by Dalla et al. [20]; and possibly in salmon by Grotmal et al. [14]. These fish therefore have the potential to act as carriers.

One of the most critical factors dictating the degree of mortality in a diseased group is the fish's age at the onset of infection. Munday et al. [8], compiled data illustrating that in most affected species, it is the larvae and early juveniles that are usually impacted. Disease has however been identified in older animals including cultured Atlantic halibut [21] and European seabass [10]. The virus has also been recovered from 1 adult white seabass. The onset of disease in white seabass has been observed between 12 to 40 dph. If white seabass contract the disease when they are 12 to 20 dph, mortality is usually 100% over a 1 to 2 week period. Once they pass 20 dph, an inverse relationship is observed between mortality and age. Once the fish complete a metamorphosis at approximately 40 dph, they no longer appear susceptible to the VNN virus.

C. Control of Disease

In the absence of a treatment for the disease, avoiding exposure is the most effective method of minimizing the chance of outbreaks. Methods employed have included changes in the husbandry of juveniles, water treatment, screening of broodstock and egg disinfection as discussed above.

The VNN virus is a very hardy organism and can survive outside the host for extended periods. It could survive in both seawater and freshwater for greater than a month (Nakai, unpublished data). Frerichs et al. [22] were able to recover viable virus from seawater stored at 15°C for more than a year. In trying to sterilize systems, water or equipment, the tolerance of VNN virus to various compounds must be considered. Arimoto et al. [23] found VNNV to be resistant to ether and chloroform and to formalin between the concentrations of 50 to 1600 µg/ml. Alcohol concentrations of 50% to 60% were required for complete deactivation. Freirichs et al. [22] also found the virus resistant to pH levels between 2 and 9. Some of the other standard compounds proving more effective were sodium hypochlorite, iodine and benzalkonium chloride at 50 ppm for 10 minutes at 20°C [23].

In attempts to sanitize the contaminated systems and equipment after outbreaks between July 2002 and May 2003, sodium hypochlorite (bleach) was added to system water. After sterilization, disease returned to subsequent spawn groups leading to questions of transmission. Precautions were taken to avoid aerosols by covering degassing systems and reducing the level of aeration. Footbaths of 500 ppm iodine, cordoned off systems, restricted access and other biosecurity measures were put in place. Infections continued to occur and the effectiveness of the bleach treatments in seawater was questioned. Because of the interactions of bleach with seawater, sterilizing levels of bleach were never attained. By using fresh water, hypochlorite levels were brought up to 100 ppm and circulated for two hours. Once systems were sterilized with bleach in freshwater, fish completed the growth cycle to 60 days with no signs of viral infection.

Seawater is supplied to the hatchery directly from the Agua Hedionda Lagoon in Carlsbad California. The lagoon contains a great deal of marine life including a large number of fish species, some of which may be vectors for VNN virus. Sterilization measures were put in place to eliminate the possibility of virus entering the facility through the influent water. Ultraviolet (UV) sterilizers were installed to treat all of the makeup water entering the larval and juvenile recirculating systems. Arimoto et al. [23] demonstrated that at least 100,000 uwsec cm^{-2} were required to eliminate the virus. UV sterilizing units installed were all sized to provide at least this level of protection.

Ozone is also an effective method of sterilization. Arimoto et al. [23] demonstrated that residual ozone of 0.1 mg/l with a 2.5 minute exposure would deactivate the VNN virus. All of the water that is supplied to recirculating systems at the Carlsbad hatchery is now ozonated at a minimum of 0.5 g/l for 4 minutes. The ozone will not only protect cultured fish from microorganisms but also many toxic organic compounds entering the water supply.

Among the identified strains of VNN, there appear to be different optimal temperatures for growth in culture [24] [25]. When Totland et al. [26] tried to cross infect striped jack and Atlantic halibut with strains recovered from each species, they were unable to induce disease. The lack of infection could have been due to host specificity and/or environmental conditions such as temperature. Genetic variations within and between genotypes may have arisen from adaptation to specific hosts and environments, thus warm water adapted organisms may be much less pathogenic at colder water temperatures. Tanaka et al. [27] determined that red spotted grouper were much more susceptible to the seven band grouper genotype (SGNNV) as temperatures increased.

Temperature may therefore be a good management tool for controlling outbreaks of disease. By understanding the temperature relationship of the infecting genotype, temperature manipulation may reduce mortality. Preliminary temperature manipulations at HSWRI appeared to help reduce mortality somewhat during an outbreak. When water temperature was dropped from 23°C to 18°C mortality tended to decline whereas tanks kept at 23°C tended to climb or remain high.

Since the initial test of dropping temperature, the same temperature manipulation was applied to another group yielding similar results. The viral strain infecting white seabass may be acclimated to higher temperatures, and the temperature decrease could reduce virus replication, allowing the fish to better cope with the infection. If more outbreaks are experienced, this procedure will continue to be used to further evaluate its effectiveness. Controlled studies are needed to substantiate these observations empirically.

VNN appears to be one of the next big hurdles to be conquered in the development of new marine aquaculture species. As new species emerge the list of affected species grows. New methods of control and prevention are needed to facilitate this development. The impact on several species in Japan and on European seabass in Europe has been so great that several university laboratories have evolved, devoting tremendous resources towards researching this virus. On the east coast of North America, Atlantic halibut and cod farms have been severely hampered by the disease. Concern over the impact of VNN on halibut and cod, has elicited the creation of a "VNN working group" to help address some of the above issues. The VNN working group subsequently organized the first ever (in North America) "Nodavirus Workshop" in November of 2002. Sponsored by the Memorial University of Newfoundland, the workshop brought academia, government and industry together to help understand and control the disease.

Cooperation between academia, government and commercial producers will be imperative for the development of management strategies to lessen the biologic and economic impact of VNN on aquaculture expansion. With the ever increasing impact on the industry, corporate interest in developing diagnostic tools, treatments and vaccines may grow through encouragement from the industry.

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Referneces

[1] K. Mori , T. Nakai, K. Muroga, M. Arimoto, K. Mushiake and I. Furusawa, Properties of a new virus belonging to Nodaviridae found in larval striped jack, *Pseudocaranx dentex,* with nervous necrosis. Virology 187, 368-371, 1992.

[2] M. Comps, F.J. Pepin and J.R. Bonami, Purification and characterisation of two fish encephalitis viruses (FEV) infecting *Lates calcarifer* and *Dicentrarchus labrax*. Aquaculture 123, 1-10, 1994.

[3] K. Yoshikoshi and K. Inoue, Viral nervous necrosis in hatchery-reared larvae and juveniles of Japanese parrotfish, *Oplegnathus fasciatus* (Temminck & Schlegel). J Fish Dis 13, 69-77, 1990

[4] P.A. Curtis, M. Drawbridge, T. Iwamoto, T. Nakai, R.P. Hedrick and A.P. Gendron, Nodavirus infection of juvenile white seabass, *Atractoscion nobilis*, cultured in southern California: first record of viral nervous necrosis (VNN) in North America. J Fish Dis 24, 263-271, 2001.

[5] M.H.V. Van Regenmortel, C.M. Fauquet, D.H.L Bishop, E.B. Carstens, M.K. Estes, S.M. Lemon, J. Maniloff, M.A. Mayo, D.J. McGeoch, C.R. Pringle and R.B. Wickner (eds.). *Virus taxonomy*, *Classification and Nomenclature of Viruses*, 7th edn. Academic Press, San Diego, CA, 2000.

[6] J.S. Glazebrook, M.P. Heasman and S.W. deBeer, Picorna-like viral particles associated with mass mortalities in larval barramundi, *Lates calcarifer* (Bloch). J Fish Dis 13, 245-249, 1990.

[7] B.L. Munday and T. Nakai, Special topic review: Nodaviruses as pathogens in larval and juvenile marine

finfish. World J Microbiol Biotechnol 13, 375-381, 1997.

[8] B.L. Munday, J. Kwang and N. Moody, Betanodavirus infections of teleost fish: a review. J Fish Dis 25, 127-142, 2002

[9] G. Breuil, J.R. Bonami, J.F. Pepin and Y. Pichot, Viral infection (picorna-like virus) associated with mass mortalities in hatchery-reared sea-bass, *Dicentrarchus labrax,* larvae and juveniles. Aquaculture 97, 109-116, 1991

[10] A. Le Breton, L. Grisez, J. Sweetman and F. Ollevier, Viral nervous necrosis (VNN) associated with mass mortalities in cage-reared sea bass, *Dicentrarchus labrax* (L.). J Fish Dis 20, 145-151, 1997.

[11] G.P. Skliris, R.H. Krondiris, D.C. Sideris, A.P.Shinn, W.S. Starkey and R.H. Richards, Phylogenetic and antigenic characterization of new fish nodavirus isolates from Europe and Asia. Virus Res 75, 59-67, 2001.

[12] M. Arimoto, K. Mori, T. Nakai, K. Muroga, and I. Furusawa, Pathogenicity of the causative agent of viral nervous necrosis disease in striped jack, *Pseudocaranx dentex* (Bloch & Schneider). J Fish Dis 16, 461-469, 1993.

[13] M. Yoshimizu, K. Suzuki, T. Nishizawa, J.R. Winton and Y. Ezura, Antibody screening for the identification of nervous necrosis carriers in flounder broodstock. In: *Proceedings NRIA International Workshop on New Approaches to Viral Diseases of Aquatic Animals*. Kyoto, pp. 124-130, 1997.

[14] S. Grotmol, G.K. Totland, K. Troud and B.K. Hjeltnes, Vacuolating encephalopathy and retinopathy associated with a nodavirus-like agent: a probable cause of mass mortality of cultured larval and juvenile Atlantic halibut, *Hippoglossus hippoglossus*, Dis Aquat Org 29, 85-97, 1997.

[15] K. Mushiake, T. Nishizawa, T. Nakai, I. Furusawa and K. Muroga, Control of VNN in striped jack: Selection of spawners based on the detection of SJNNV gene by polymerase chain reaction (PCR). Fish Pathol 29, 177-182, 1994

[16] S. Grotmol and G.K. Totland, Surface disinfection of Atlantic halibut, *Hippoglossus hippoglossus*, eggs with ozonated sea-water inactivates nodavirus and increases survaival of the larvae. Dis Aquat Org 39, 89-96, 2000

[17] T. Nishizawa, M. Furuhashi, T. Nagai, T. Nakai and K. Muroga, Genomic classification of fish nodaviruses by molecular phylogenetic analysis of the coat protein gene. Appl. Environ Microbiol 63, 1633-1636, 1997.

[18] D.C. Sideris, Cloning, expression and purification of the coat protein of encephalitis virus (D1EV) infecting *Dicentrarchus labrax*. Biochem. Mol. Biol. Int. 42, 409-417, 1997.

[19] R. Thiery, C. Arnauld and C. Delsert, Two isolates of sea bass, *Dicentrarchus labrax* L., nervous necrosis virus with distinct genomes. J Fish Dis 22, 201-207, 1999.

[20] L. Dalla Valle, L. Zanella, P. Patarnello, L. Paolucci, P. Belvedere and L. Colombo, Development of a sensitive diagnostic assay for fish nervous necrosis virus based on RT-PCR plus nested PCR. J Fish Dis 23, 321-327, 2000.

[21] V. Aspehaug, M. Devold and A. Nylund, The phylogenetic relationship of nervous necrosis virus from halibut, *Hippoglossus hippoglossus*. Bull Eur Ass Fish Pathol 19, 196-202, 1999.

[22] G.N. Frerichs, A. Tweedie, W.G. Starkey and R.H. Richards, Temperature, pH and elecrolyte sensitivity, and heat, UV and disinfection inactivation of sea bass (*Dicentrarchus labrax*) neuropathy virus. Aquaculture 185. 13-24, 2000.

[23] M. Arimoto, J. Sato, K. Maruyama, G. Mimura and I. Furusawa, Effect of chemical and physical treatments on the inactivation of striped jack nervous necrosis virus (SJNNV). Aquauculture 143, 15-22, 1996

[24] T. Iwamoto, K. Mori, M. Arimoto and T. Nakai, High permissivity of the fish cell line SSN-1 for piscine nodaviruses. Dis Aquat Org 39, 37-47, 1999.

[25] T. Iwamoto, T. Nakai, K. Mori, M. Arimoto and I. Furusawa, Cloning of the fish cell line SSN-1 for piscine nodaviruses. Dis Aquat Org 43, 81-89, 2000.

[26] G.K. Totland, S. Grotmol, Y. Morita, T. Nishioka and T. Nakai, 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. Dis Aquat Org 38, 169-175, 1999.

[27] S. Tanaka, H. Aoki and T. Nakai, Pathogenicity of the nodavirus detected from diseased sevenband grouper *Epinephelus septemfasciatus*. Fish Pathol 33, 31-36, 1998.