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Viral nervous necrosis (VNN) an emerging disease caused by Nodaviridae in aquatic hosts: Diagnosis, control and prevention: A review

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Abstract

Betanodavirus is one of the two genera making up the family Nodaviridae and is the etiological agent of viral nervous necrosis (VNN, also known as viral encephalopathy and retinopathy or VER). The virus infects a large range of host species in more than 50 species of marine and freshwater fish worldwide from different geographical areas and the known host range continues to expand as new species of fish are used for aquaculture. The disease is characterized by vacuolating necrosis of neural cells of the brain, retina and spinal cord and causes up to 100% mortality in larval and juvenile fish, and can cause significant losses in older fish. The lack of knowledge about control and prevention of the disease makes the problem serious and impedes development of management approaches. Therefore this review focuses on current knowledge and future perspectives of viral nervous necrosis in the aquaculture industry with special focus on the type of diagnosis, control and prevention of the disease.

Keywords: Viral nervous necrosis, Betanodavirus, Diagnosis, Control and prevention

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Introduction

Viral nervous necrosis (VNN) is a disastrous fish disease and one of the main reasons for great economic losses in marine fish and the aquaculture industry. The agent of VNN is a virus belonging to the genus Betanodavirus and family Nodaviridae (Peducasse *et al*., 1999; Mu *et al*., 2013; Jia *et al*., 2015; Pascoli *et al*., 2016). Actually the first documented nodavirus infection was detected in Japanese parrotfish (*Oplegnathus fasciatus*) in Japan (Yoshikoshi and Inoue, 1990). Then the disease was reported in barramundi (*Lates calcarifer*) farmed in Australia (Glazebrook *et al*., 1990), and then a year later in turbot, *Scopthalmus maximus* (Bloch *et al*., 1991), European sea bass *Dicentrarchus labrax* (Breuil *et al*., 1991), red spotted grouper, *Epinephalus akaara* (Mori *et al*., 1992) striped jack, *Pseudocaranx dentex* (Mori *et al.*, 1992) and several other species such as the golden grey mullet, *Liza aurata* (Zorriehzahra *et al*., 2005). Currently occurrence of VNN is reported in some African countries including: Tunisia (Chérif *et al*., 2009) and Algeria (Kara *et al*., 2014). It revealed that early unknown mortality occurred in fry and juveniles of some marine fish in decade of 1980-1990 in the world. Now this virus has been recognized as a major problem and has been increasing in importance in Mediterranean and Asian marine aquaculture (Costa and Thompson, 2016; Vendramin *et al*., 2016). Betanodavirus is non-enveloped and icosahedral with a diameter of 20–30 nm, with two positive-sense RNA strands known as RNA1 and RNA2. RNA1 encodes RNA dependent RNA polymerase (RdRp), a mitochondrial enzyme, and is responsible for viral replication (Nopadon *et al*., 2009; Jia *et al*., 2015). On the other hand, RNA2 encodes the capsid protein (Wu *et al*., 2016). The existence of four genotypes characterized by high homology has been approved on the basis of the viral genome analysis, designated bar fin flounder nervous necrosis virus (BFNNV), tiger puffer NNV(TPNNV), striped jack NNV (SJNNV), and red spotted grouper NNV (RGNNV) (Nishizawa *et al*., 1997; Shetty *et al*., 2012). The main target tissues are the nerve tissues especially the central nervous system (CNS) and the eye (retina). The characteristic lesions of VNN are necrosis and vacuolation of the central nervous system and retina of the affected larvae and juvenile fishes showing abnormal swimming behavior (Liu *et al*., 2015). To date, the disease has been reported in more than 120 species belonging to 30 families from 11 different orders, mainly marine fish being susceptible to infection (Munday *et al*., 2002; Su *et al*., 2015; Costa *et al*., 2016). On the other hand, several freshwater fish species such as Chinese catfish (*P. asotus*); Australia catfish (*Tandanus tandanus*); Barramundi (*L. calcarifer*); Medaka (*Oryzias latipes*); Guppy (*Poicelia reticulata*) and Zebrafish (*Danio rerio*) showed outbreaks of the disease (Hegde *et al*., 2003; Shetty *et al*., 2012). Affected fish may reveal different clinical signs related to species, age and temperature of the environment; an acute and subacute form characterized by atypical signs and a chronic form was observed for the first time only in affected fish in the Caspian Sea up to now (Zorriehzahra *et al*., 2016). The most specific and common symptoms observed among the different species is an abnormal swimming behavior, lethargy and swim bladder hyperinflation that lead to abdominal extension. The viral etiology has been emphasized following the identification of small, non-enveloped, RNA agents definitively specified to the Nodaviridae family, genus *Betanodavirus*. Although horizontal transmission surely appears to be the most common transmission route, vertical transmission has also been already proved in some species. According to the OIE protocol, VER/VNN is officially diagnosed through the isolation of the causative agent in susceptible cell line and then identified with immunological or molecular methods such as FAT or by real time RT-PCR or nested RT-PCR (OIE, 2018). The control of the disease is intricate with difficulties in applying biosecurity procedures, emphatic hygiene and preventive actions in open environments like the ocean and in selecting brood fish free of pathogen. Furthermore, commercial vaccines are currently not available.

Clinical signs and symptoms

The clinical sings of viral nervous necrosis are linked to the neuro-invasive nature of viruses of the family Nodaviridae, causing this disease, as well as the consequences of the lesions present in the retina and brain of the infected fish, ultimately leading to abnormal swimming, coloration, sight and swim bladder control (Munday *et al*., 2002; Costa and Thompson, 2016,). In general, the clinical signs of VNN are observed in the specific behavior of affected individuals. These behavioral changes include: loss of appetite, erratic swimming patterns like whirling, spiral, looping swimming and belly up at rest, loss of equilibrium, minimized nervous coordination, uncoordinated swimming and alterations in pigmentations (Nopadon *et al*., 2009). These signs are accompanied by some general sings such as anemia, lethargy and anorexia (Munday *et al*., 2002). The infected individuals also adopt a peculiar stationary position, such as vertical position keeping caudal fin and head above the water surface. Some VNN infected fish swim straight forward so swiftly that they are unable to discontinue their speed and crash into tanks' walls and they experience traumatic and harrowing lesions on their jaws and nose (Maltese and Bovo, 2007; Binesh, 2014; Keawcharoen *et al*., 2015). Other changes that coexist are hyperinflation of the swim bladder (Mori *et al*., 1992; Hellberg *et al*., 2010; Kara *et al*., 2014; Vendramin *et al*., 2016) (Fig. 1). The presence or absence of any of these signs or deviation in pigmentation change may be due to species and water temperature (Binesh, 2014).

 

Figure 1: Clinical signs in VNN: hyperinflation of swim bladder (left) and abdominal extension (right) in infected *Lisa aurata* in the Caspian Sea (Zorriehzahra *et al*., 2014).

Virus isolation

A number of cell lines are now available for the culture of betanodaviruses (Qin *et al*., 2006). The striped snakehead cell line (SSN-1) originally developed by Frerichs *et al*., (1996) has been shown to be permissive for 17 isolates of fish nodaviruses, encompassing the RGNNV, SJNNV, TPNNV and BFNNV types (Iwamoto *et al*., 2000; Dalla Valle *et al*., 2001; Iwamoto *et al.*, 2001; Chi *et al.*, 2003). Iwamoto *et al*., (2000) reported that six cell clones were derived from the SSN-1 cell line, which is composed of a mixed cell population and persistently infected with a C-type retrovirus (SnRV). These clones were susceptible to 4 piscine nodavirus strains belonging to different genotypes SJNNV, RGNNV, TPNNV and BFNNV (striped jack, redspotted grouper, tiger puffer and bar fin flounder nervous necrosis viruses). Three clones, designated A-6, E-9, and E-11, were highly permissive to nodavirus infection and production (Iwamoto *et al*., 2000). The virus-induced cytopathic effects appeared as cytoplasmic vacuoles and intensive disintegration at 3 to 5 days post-incubation (Frerichs *et al*., 1996; Chi *et al*., 1999; Iwamoto *et al*., 2001). These observations were highly reproducible and formed the basis for a successful virus titration system. Quantitative analysis using the cloned E-11 cell line clearly revealed differences in the optimal growth temperatures among the 4 genotypic variants: 25 to 30° C for strain SGWak97 (RGNNV), 20 to 25 degrees C for strain SJNag93 (SJNNV), 20 degrees C for strain TPKag93 (TPNNV), and 15 to 20° C for strain JFIwa98 (BFNNV) (Iwamoto *et al*., 2001). Electron microscopy demonstrated SnRV retrovirus particles only in A-6 and E-9 cells, but PCR amplification for the pool gene and LTR region of the proviral DNA indicated the presence of the retrovirus in the other clones, including E-11. The cell clones obtained were more useful for qualitative and quantitative analyses of piscine nodaviruses than the SSN-1 cell line. Further susceptible cell cultures (GF-1) have been developed from the groupers *Epinephelus coiodes* (Chi *et al*., 1999; Mu *et al*., 2013) and may be used for research and diagnostic purposes provided sensitivity is regularly monitored (OIE, 2018). Lai *et al*., (2001) have developed another cell line from *E. awoara* (Lai et al., 2001), and Qin *et al*., (2006) have made other cell lines (GS) from the groupers, *E. coiodes* that support grouper nervous necrosis viruses, but the lines have not been tested for other types of fish nodaviruses (Qin *et al*., 2006).

In SSN-1 cells, the cytopathic effect appears on the 3rd day post infection and is characterized by the appearance of intracellular vacuolar lesions unevenly distributed throughout the cell monolayer (Iwamoto *et al*., 2000). These vacuolar lesions initially are isolated and began assuming the form of vacuolized cellular aggregates after the passage of hours. Seventy-two hours post infection, their number and size increase considerably and the cellular monolayer is gradually replaced by cellular lysis until complete destruction (Maltese and Bovo, 2007; Nishi *et al*., 2016).

Histopathology and immunohistochemistry

The histological lesions of VNN include severe degeneration, pyknosis, shrinkage and basophilic cells in affected areas and vacuolation throughout the central nervous system (CNS) of the fish and all retinal layers (Peducasse *et al*., 1999; Munday *et al*., 2002; Shetty *et al*., 2012). Infected larvae have large vacuoles in the brain and retina, together with severe congestion of the blood vessels in the brain. Larger fish also showed vacuoles and congestion in nervous tissue. Vacuolated cells and vacuoles are mainly present in the bipolar and ganglionic nuclear layer of the retina in the eyes (Glazebrook *et al*., 1990; Le Breton *et al*., 1997; Grotmol *et al*., 1997; Nakai *et al*., 2009). A common finding in the CNS is gliosis (Shetty *et al*., 2012, Costa and Thompson, 2016). Vacuolated cells and larger vacuoles were mainly apparent in the telencephalon, the diencephalon and the cerebellum (Le Breton *et al*., 1997; Lai *et al*., 2001). In the nerve cells small vacuoles and strong basophilic inclusions are seen. The most prominent vacuolation is usually found in the grey matter of the optic tectum and cerebellum and there is often involvement of Purkinje cells (Starkey *et al*., 2004; Shetty *et al*., 2012). Vacuolation can also be seen in the white matter, adjacent to the ventricles. These vacuolations appear to be intracytoplasmic, but their exact position cannot always be determined (Munday and Nakai, 1997; Su *et al*., 2015). Sections can be stained by immunohistochemistry (IHC) with the avidin-biotin peroxidase complex technique using hydrogen peroxide and DAB as chromogen and substrate and show strong positive reactions in the same layers (Le Breton *et al*., 1997). The location of immunopositive cells is revealed by red or brown colour. This demonstrated that the virus enters the CNS along nerves and blood vessels during the viremic stage of infection (Le Breton et al., 1997; Johansen *et al*., 2004; Shetty *et al*., 2012).

Molecular diagnostic techniques

Many molecular methods have been used in diagnosis of NNV. These methods were developed for the rapid, convenient and sensitive diagnosis of the NNV pathogen in the fish and include: conventional or nested polymerase chain reaction (PCR) (Muroga, 1994; Dalla Valle *et al*., 2000; Grotmol *et al*., 2000; Mu *et al*., 2013), real-time PCR (Starkey *et al*., 2004; Dalla Valle *et al*., 2005; Panzarin *et al*., 2010; Hodneland *et al*., 2011; Baud *et al*., 2015; Mekata *et al*., 2015) and nucleic-acid sequence amplification (NASBA) (Starkey *et al*., 2004). For the first time during the nineties Nishizawa et al. established NNV RT-PCR detection method (Nishizawa *et al*., 1995). With the development of this technology, the nucleic acid extraction technique improved and now allows an easy, fast, and high-quality RNA preparation, and the availability of more NNV genome sequences facilitating the primer design and optimization (Mu *et al*., 2013). More recently, RT-PCR assays with or without nested PCR have been developed as a powerful diagnostic tool alone or in combination with cell culture (Iwamoto *et al*., 2001; Dalla Valle *et al*., 2005). The most used target is generally a portion of the coat protein gene (RNA2) of betanodavirus, a powerful and sensitive target for identification of the infection (Nishizawa *et al*., 1997; Grotmol *et al*., 2000; Barke *et al*., 2002; Azad *et al*., 2005). These PCR protocols have greatly improved test sensitivity, allowing better control of VNN infection through identification and stamping out of infected spawners (Dalla Valle *et al*., 2005). For example, Striped jack (*P. dentex*) broodstocks were screened for NNV to prevent vertical transmission of this pathogen to the larval offspring (Muroga, 1994). Some authors showed that nested RT–PCR is 10–100 times more sensitive than the previously reported RT–PCR methods (Thiery *et al*., 1999; Dalla Valle *et al*., 2000).

Moreover, since conventional PCR is a non-quantitative technique, the actual copy number of the viral template in samples cannot be determined (Starkey *et al*., 2004; Dalla Valle *et al*., 2005). So, Dalla Valle and his colleagues described the setting up of two real-time, SYBR Green I-based, PCR diagnostic assays targeting both RNA1 and RNA2 of betanodavirus for its quantitative detection in biological samples (Dalla Valle *et al*., 2005). The sensitivity of this technique was compared with that of conventional RT-PCR assays previously developed for betanodavirus (Dalla Valle *et al*., 2000; Grotmol *et al*., 2000; Mu *et al*., 2013) and with the results of routine virus isolation test (Delsert *et al*., 1997; Iwamoto *et al*., 2001), to check for a correlation between measured viral RNA load and virus isolation response. Also, other quantitative real time methods have been developed (Dalla Valle *et al*., 2005; Kuo *et al*., 2011; Lopez Jimena *et al*., 2012; Souto *et al*., 2015). They have been used as powerful tools to study transmission and development of this viral infection in juveniles (Hodneland *et al*., 2011). NASBA is another useful method which consists of an isothermal method for nucleic acid amplification that is particularly suited to RNA targets (Deiman *et al*., 2002). The method amplifies a target-specific product through oligonucleotide primers and the co-ordinated activity of 3 enzymes: reverse transcriptase, RNase H, and T7 RNA polymerase (Deiman *et al*., 2002; Starkey *et al*., 2004). This method has been developed for the detection of betanodavirus and the sensitivity of this procedure was compared to a conventional single-tube RT–PCR assay showing comparable results (Starkey *et al*., 2004).

Compare of methods and diagnostic applications

The real-time PCR assays were more sensitive than the one step RT-PCR for betanodavirus (Dalla Valle *et al*., 2005; Hodneland *et al*., 2011; Panzarin *et al*., 2010; Baud *et al*., 2015, Mekata *et al*., 2015). This enhanced sensitivity can be exploited to reveal sub-clinical VNN infections in carrier fish and to screen out infected spawners to reduce or prevent the vertical transmission of the virus (Costa and Thompson, 2016). It is important to point out that the real-time PCR can only detect the presence of the viral genome, but is not able to estimate its infectious potential (Dalla Valle *et al*., 2005; Mekata *et al*., 2015). Hence, PCR techniques will never replace the virus cultivation test and both approaches should be used according to their specific benefits.

Phylogenic analysis of NNV

The genome of NNV viruses consists of two single-stranded, positive-sense RNA molecules (RNA1 and RNA2) of about 3.0 and 1.4 kb in length, respectively, without poly(A) extension at the 3’ end (Delsert *et al*., 1997; Jia *et al*., 2015) and sometimes possesses an additional segment designated RNA3 (Shetty *et al*., 2012). RNA1 encodes a non-structural protein, RNA-dependent RNA polymeras (RdRP) and RNA2 encodes a capsid proteins (CP) of about 37-42 kDa (Jia *et al*, 2015). Using molecular phylogenetic analyses based on partial sequences of the CP gene, the betanodaviruses have been classified into four main clades: striped jack nervous necrosis virus (SJNNV), tiger puffer NNV (TPNNV), bar fin flounder NNV (BFNNV) and red spotted grouper NNV (RGNNV) (Nishizawa *et al*., 1997; Aspehavg, 1999; Dalla Valle *et al*., 2001; Gomez *et al*., 2004; Jia *et al*., 2015).

Enzyme-linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assay is the rapid and sensitive test in order to detect specific nodavirus antibodies as well as antigens from serum samples (Fenner *et al*., 2006; Costa and Thompson, 2016; Jaramillo *et al*., 2016). This method was used to identify sero-positive virus of fish of different ages especially from vectors and broodstock in order to control vertical transmission of the disease (Arimoto *et al*., 1993; Costa and Thompson, 2016). The efficacy of this assay was confirmed by Watanabe *et al*. (2000) with the identification of nodavirus antibodies from bar fin flounder broodstock and Arimoto *et al*. (1996) from striped jack (Arimoto *et al*., 1996). Also, Fenner *et al*. (2006) could detect 10^3–10^4 TCID50 units of betanodavirus by antigen capture

ELISA from infected tissues of juvenile barramundi (*L. calcarifer,* Bloch) (Fenner *et al*., 2006). In this assay 17% and 18% sera of wild and farmed European sea bass broodstock were positive for nodavirus antibodies, respectively (Breuil *et al*., 1991). In a similar study, 9% sera of commercial barramundi were positive for antibodies (Huang *et al*., 2001) by ELISA method.

Immunofluorescence antibody test (IFT)

Immunofluorescence antibody test (IFT) that uses fluorescent-labeled antibodies to detect specific antigens from target tissues including brain, spinal cord and retina is a rapid, economical, powerful and important technique for the screening of Nodaviridae (Bigarré *et al*., 2009; Costa and Thompson, 2016). In this assay by preparing histopathological sections from CNS or other tissues such as eye, swim bladder, spleen, kidney and liver staining with specialized immunofluorescence technique the localized virus in target tissues is indicated (Sanz and Coll, 1992). The binding of antibodies to target tissues, cells or organisms can be visualized if those antibodies are directly coupled to a fluorochrome or indirectly bound by a fluorescent reagent (Grist *et al*., 1981; Furusawa *et al*., 2006). Fluorochromes emit visible light (of an ‘emission’ wavelength) when exposed to light of a different (‘excitation’) wavelength, usually in the ultraviolet range. The indirect fluorescent antibody test showed at least 20% of golden grey mullet (*L. aurata*) fish infected with VNN disease has a positive reaction to betanodavirus antigens in the optic nerve, outer molecular and granular layers of the brain and inner and outer nuclear layers of retina (Zorriehzahra *et al*., 2014).

Electron microscopy

Virus particles observed with electron microscopy are icosahedral, non-enveloped with a commonly reported diameter of about 20-34 nm. Some author showed that the virus has an electron-dense core of 13-21 nm surrounded by a clear layer of about 5 nm (Glazebrook *et al*., 1990; Grotmol *et al*., 1997; Chen *et al*., 2015; Xie *et al*., 2016b). The virions can be free in the cytoplasm of infected cell or membrane-bound by endoplasmic reticulum and may be present as paracrystalline arrays (Glazebrook et al., 1990). Cells containing virions have most often been recognized as neurons, astrocytes, oligodendrocytes and microglia (Grotmol *et al*., 1997; Munday *et al*., 2002; Xie *et al*., 2016a). Virus particles in infected Atlantic halibut have been seen in endothelial cells, pillar cells and lymphocytes attached to the endocardium, cardiac myocytes and epicardial cells by electron microscopy (Grotmol *et al*., 1997).

Control and prevention

VNN could be very resistant in aquatic bodies and water environments and it seems very difficult to eradicate when introduced to marine or aquaculture farms. Therefore, to recognize pathways of virus transmission is very critical for control strategies. For this reason, broodstock and larval fish could be considered as viral repertoires and responsible asymptomatic carriers for horizontal transmission (Costa and Thompson, 2016). Exclusion of virus-carrying animals from the production would be the best means of control in the vertical transmission route. Thus, the elimination or segregation of infected spawners is the best way to prevent the introduction of the virus into the hatchery (Munday *et al*., 2002; Costa and Thompson, 2016; Nakai *et al*., 2009). Muroga *et al*. (1994) reported a successful control of VNN by removal of infected striped jack (*P. dentex*) broodstock detected by PCR (Muroga, 1994). In a similar study by Breuil *et al*. (1991) the disease was controlled by exclusion of serum positive European sea bass (*D. labrax*) broodstock (Breuil *et al*., 1991). Also the use of disinfection of fertilized eggs by ozone has been recommended to control vertical transmission of betanodavirus in Atlantic halibut (*Hippoglossus hippoglossus*) (Grotmol *et al*., 2000). Arimoto *et al*. (1993) reported that 0.2 µg ml^{-1} ozone disinfects fertilized eggs in striped jack and also, 4 µg ml^{-1} was reported for halibut by Grotmol *et al.* (1997). These results indicate that VNN transmitted from the maternal sexual fluid was via the surface of the eggs (Kai *et al*., 2010).

Horizontal transmission of VNN infection may be: via contaminated influent and rearing water, utensils, vehicles and human activity (Nakai *et al*., 2009). Some effective disinfectants can inactivate the virus and prevent spread of disease such as: ozone, acid peroxygen, sodium hypochlorite and benzalkonium chloride (Arimoto *et al*., 1993; Frerichs *et al*., 1996; Shetty *et al*., 2012).

A vaccination method is essential to prevent the disease especially during the primary stages and some researchers reported effective procedures in controlling the disease (Nakai *et al*., 2009; Xie *et al*., 2016a; Vimal *et al*., 2014). Recombinant viral coat protein expressed in *Escherichia coli* injected to fish (Sommerset *et al*., 2005) or injection of virus like particles expressed in a baculovirus expression system were carried out by some researchers (Lin *et al*., 2001; Thiery *et al*., 2006). Injection of the recombinant protein in adult striped jack caused production of virus neutralizing antibodies (Munday *et al*., 2002), thus vaccination seems to be a practical and appropriate way for the control of viral nervous necrosis. Vaccinating broodfish could reduce vertical transmission of VNN and will be more acceptable by the farmers (Kai *et al*., 2010). Unfortunately, no commercial vaccines are available at present. Also, feeding of immunostimulant components could be a beneficial way to increase immunity levels in larval fish against VNN infection (Costa and Thompson, 2016).

Strict hygiene can help to control viral nervous necrosis within hatcheries (Munday *et al*., 2002; Bigarré *et al*., 2009; Shetty *et al*., 2012). No recycling of water and chemical sterilization of seawater during each hatching cycle was successful to reduce VNN disease in a barramundi hatchery (Azad *et al*., 2005). Furthermore, applying biosecurity measures and general hygiene practices, such the UV treatment, sanitary barriers, regular monitoring and disinfection of tanks and biological filters, disinfection of utensils and decreasing stress factors and density of larvae and juveniles are strongly recommended (OIE, 2018).

VNN first occurred in 13 fish species in 4 families about 23 years ago, in 1993, but this transmissible viral disease is now recorded in more than 50 species in 10 families (Munday *et al*., 2002; Costa and Thompson, 2016). Also, unlike other viral diseases such as Infectious hematopoietic necrosis (IHN), or Viral hemorrhagic septicemia (VHS) that specially affects coldwater fish, VNN virus can infect many different kinds of fish such as coldwater fish (BFNNV genotype), warm water fish (RGNNV genotype) and other fish such as ornamental fish (Nakai *et al*., 2009; Costa and Thompson, 2016) and freshwater fish such as sturgeon fish (Xylouri *et al*., 2007), tilapia (Bigarrè *et al*., 2009) and others (Pascoli *et al*., 2016).

With regard to new intensive mariculture systems being used in the Caspian Sea, Persian Gulf and Oman Sea, the risk of viral and bacterial infection could be high. This is true if we refer to recent mortality in some species that were recorded by researchers in that area (Zorriehzahra *et al*., 2016). It could be summarized that some worldwide emerging infectious diseases such as VNN could be the most important threat for the development of mariculture in the Persian Gulf and Oman Sea in the near future (Zorriehzahra *et al*., 2016).

Furthermore, the production of multivalent or recombinant vaccines against VNN virus, the increase of application of some immunostimulant drugs, the eco-epidemiological investigation on the global spreading of VNN in the new regions and new susceptible hosts should be considered in future studies.

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