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

Zorriehzahra M.J.^{1*}; Adel M.¹; Dadar M.²; Ullah S.³; Ghasemi M.⁴

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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

¹⁻Aquatic Animal Health and Diseases Department, Iranian Fisheries Science Research Institute (IFSRI), Agricultural Research Education and Extension Organization (AREEO), Tehran, I.R. Iran.

²⁻Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran.

³⁻Fisheries and Aquaculture Lab, Department of Animal Sciences, Quaid-i-Azam University, Islamabad, Pakistan.

⁴⁻ Inland Water Aquaculture Research Center, Iranian Fisheries Science Research Institute, Agricultural Research, Education and Extension Organization Bandar Anzali, Iran.

^{*}Corresponding author's Email: zorrieh@yahoo.com

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 first documented nodavirus the 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 Asian marine and aquaculture (Costa and Thompson, 2016; Vendramin etal., 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 NNV (RGNNV) spotted grouper (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); (Poicelia reticulata) Guppy 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 Caspian Sea up to (Zorriehzahra et al., 2016). The most specific common and symptoms observed among the different species is 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 the to Nodaviridae family, genus Betanodavirus. Although horizontal transmission surely appears to be the transmission most common route, vertical transmission has also been already proved in some species. According the **OIE** protocol, to VER/VNN officially diagnosed is through the isolation of the causative agent in susceptible cell line and then identified with immunological 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 neuroinvasive 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 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 discontinue their speed and crash into 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).



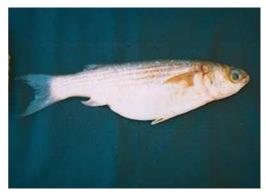


Figure 1: Clinical signs in VNN: hyperinflation of swim bladder (left) and abdominal extension (right) in infected *Lisa aurata* in the Caspian Sea (Zorriehzahra *et al.*, 2014).

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 genotypes different 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 virusinduced cytopathic effects appeared as cytoplasmic vacuoles and intensive disintegration at 3 to 5 days postincubation (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 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 ofintracellular 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 retina, together with 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 mainly apparent in were telencephalon, the diencephalon and the cerebellum (Le Breton et al., 1997; Lai et al., 2001). In the nerve cells small vacuoles and basophilic strong 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 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 improved test greatly 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 realtime, 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 compared procedure was to conventional single-tube RT-PCR showing comparable results assay (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 realtime 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 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 Thompson, 2016; Jaramillo et 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 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 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 technique immunofluorescence 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 ('excitation') wavelength, different usually in the ultraviolet range. The fluorescent antibody indirect 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, nonenveloped with a commonly reported diameter of about 20-34 nm. author showed that the virus has an electron-dense core of 13-21 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 microglia (Grotmol *et al.*, 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 viruscarrying 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 stages primary and some effective researchers reported 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 virus neutralizing production of 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 farmers (Kai et al., 2010). Unfortunately, no commercial vaccines are available at present. Also, feeding of immunostimulant components could 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 general and practices. hvgiene such the 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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