



Molecular Monitoring and Phylogenetic Analysis of *Betanodavirus* in Groupers (*Epinephelus* spp.) and Asian Sea bass (*Lates calcarifer*) of Iranian Northern Waters of the Persian Gulf

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Abstract

The emergence of diseases has caused much health and economic damage. Viral Nervous Necrosis (VNN) is considered as one of the most important threats to aquatic ecosystems. VNN can cause severe mortality and economic loss in fish farms. The high water temperatures in southern Iran and the observed incidences of fish mortality in the Persian Gulf led to the hypothesis of the possible emergence of VNN. Therefore, this study aimed to monitor two species of fish susceptible to VNN using PCR, and Nested PCR methods and comparing the sensitivity of these methods to the identification of *Betanodavirus* infection in apparently healthy and symptomatic fish. About 850 Grouper (*Epinephelus* spp.) and Asian Sea bass (*Lates calcarifer*) fish of the Persian Gulf were collected randomly and examined. Molecular methods were used to identify VNN in visibly healthy and symptomatic fish of the Persian Gulf of Iran. The results of the PCR showed no positive cases, but the Nested PCR revealed some positive results. Then, the phylogenetic analysis of the virus sequence was performed. The nucleotide sequence of Nested PCR products revealed a 98–100% homology with Red Spotted Grouper Viral Nervous Necrosis (RGNNV). This is the first report on VNN tracing and detection as well as phylogenetic analysis of the virus from the Persian Gulf of Iran. Therefore, considering the importance of emerging viral diseases and the irreparable damage they cause, continuous monitoring and epidemiological studies of VNN were recommended by authorized organizations.

Introduction

Viral Nervous Necrosis (VNN) is an emerging viral disease in a wide variety of wild and farmed fish. This important aquatic pathogen belongs to the Nodaviridae family and the genus *Betanodavirus*, which targets nervous tissue preferentially the brain and retina. *Betanodaviruses* are small, non-enveloped viruses with a genome comprised of two positive-sense ssRNA molecules. RNA1 is 3100 bp in length and encodes RNA-dependent RNA polymerase, whereas RNA2 is 1400 bp in length and encodes the capsid coat protein [1, 2]. The basis for *Betanodavirus* identification in fish is the

detection of the variable region of the coat protein gene on RNA2 [3].

The virus causes acute and latent infections [4]. Acute infection of VNN causes severe mortality in larval and juvenile fish whereas persistent infection of adults remains unknown [2]. Biological and environmental factors can induce reactivation of latent infection to an acute infection. Infected fish also can cause vertical and horizontal transmission of the virus [4].

In Iran, this disease was first identified and reported of the golden grey mullet (*Liza auratus*) in the Caspian Sea in 2005 [5]. Also, in recent years, some evidence of this disease was reported [6, 7]. Unfortunately, continuous casualties and fish losses were observed in the north of Iran [8, 9]. In 2012, for the first time in the south of Iran, the mortality of fish was observed with some typical clinical signs of *Betanodavirus* and examined using histopathological assays. Necrosis and vacuolation of the fish brain and eye tissue were observed and researchers reported the possible presence of the VNN in the region [10].

Iran is surrounded by the Persian Gulf and Oman Sea in the south [11] and fishery is the second most important

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natural resource in the region [12]. The waters of the Persian Gulf and Oman Sea are also unique because of the special environmental conditions (high temperature and salinity) and the existence of many important commercial species. Different types of fish and aquatic resources have provided nutrients, job opportunities and potential export earnings in the south of Iran [11–13]. In 2014, Iran was in the 28th place in world fishery production, the 20th place in world aquaculture production and 27th place in overall production. Iran is the biggest fish producer (947,354 tons) in the Middle East and Western Asia [11]. VNN can spread in tropical marine areas and cause disease in wild and breeding fish [14]. Groupers species (particularly *Epinephelus* spp.), one of the most important commercial fish in the Persian Gulf and the Oman Sea [15], and also Asian Sea bass (*Lates calcarifer*) are found highly susceptible to VNN [16, 17]. Since more than 60% of the marine fish production in Iran is in the south of Iran [11] and the Persian Gulf is a semi-closed water body, connected to the Oman Sea [12], there is no significant difference in the abundance of fish species in these two seas [11]. Therefore, the spread of this emerging disease between wild and farmed fish and especially its vertical and horizontal transmission as well as the significant economic damage it causes to the aquaculture industry makes it necessary to carry out VNN detection [9]. Due to the loss of wild and cultured fish in the south of Iran, molecular monitoring of VNN was first carried out in the northern waters of the Persian Gulf and the sequence of the detected genome was compared with the previous genotype available in the GenBank.

Materials and Methods

Study Site

In this study, the marine and farmed fish of the Persian Gulf located in the south of Iran were studied (Fig. 1).

Sample Collection

About eight hundred and fifty fish were collected from the rocky shores of the Persian Gulf in the south of Iran, from December 2016 to July 2018. The water temperature was measured at the time of sampling and was reported ranging from 22 to 35 °C in different sampling seasons. The samples were divided into several groups; the caught wild Grouper and farmed Grouper weighing 150–200 g, imported Asian Sea bass fingerlings and mariculture juvenile Asian Sea bass weighing 150–200 g. Only a very small number of juvenile fish had clinical signs like abdominal distension, hyperemia and exophthalmia (Fig. 2). The samples were collected and transferred on ice, to the Laboratory of Microbiology of



Fig. 1 The sampling sites of the Persian Gulf in the present study

Jahrom Azad University. Then, the retina and brain of both apparently healthy groups as well as symptomatic fish were separated as target tissues and kept at – 80 °C until use.

RNA Extraction

Different fish species were tested individually. Sea bass fingerlings were mixed with some similar and also small sized Groupers were mixed separately. Total RNA was extracted directly from the brain and eyes of fish separately using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Then the quality of the extracted RNA was checked using a BioPhotometer® (Eppendorf, Germany). Finally, samples were examined with OD 1.6–1.8.

cDNA Synthesis

cDNA synthesis was performed using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's instructions, and cDNA samples were stored in a freezer at – 20 °C for 3–7 days [18].

PCR

The PCR was done using primers F (5'-CGTGTCAGTCATGTGTCGCT-3') and R (5'-CGAGTCAACACGGGTGAA GA-3') to amplify the coat protein gene of RNA2 [19]. PCR was carried out in a final volume of 30 µl using a product from Sinaclon Co. The reaction mixture contained 1 × PCR Buffer (AMS), 0.2 mM of dNTPs, 1.5 mM MgCl₂, 0.5 µM of each forward and reverse primer, 0.5 U of Taq polymerase (SmartTaq DNA Polymerase), Template cDNA, and autoclaved distilled water. To ensure the accuracy of the PCR, a negative control (no template) and positive control were used at each step of the reaction. As a positive control,

Fig. 2 Clinical signs of fish. Left: abdominal distension in wild Grouper; right: hyperemia and exophthalmia of eye in Sea bass



the RNA or cDNA extracted from the larva of the Orange-Spotted Grouper (*E. coioides*) was used and was obtained through Dr. C. Nallathambi from the Department of Biotechnology, Rajalakshmi College of Engineering in India. The cycling conditions were as follows: 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and finally 72 °C for 5 min [20].

Nested PCR

For the Nested PCR, the first step is similar to the first round of PCR and its product was used as the Nested PCR template. In the Nested PCR, the amplification was performed using a pair of internal primers, F'2 (5'-GTTCCCTGTACA ACGATTCC-3') and R'3 (5'-GGATTTGACGGGGCTGCTCA-3') (about 300/294) corresponding to the *Betanodavirus* coat protein gene [21]. Nested PCR was carried out in a final volume of 30 µl of a product of Sinaclon Co. The reaction mixture contained, 1 × PCR Buffer (AMS), 0.2 mM of dNTPs, 1.5 mM MgCl₂, 0.5 µM of each forward and reverse primer, 0.5 U of Taq DNA polymerase (SmartTaq DNA Polymerase), and the first round of PCR products. Meanwhile, a positive control, RNA extracted from the larva of Orange-Spotted Grouper (*E. coioides*), and negative control (water instead of the sample) was used. The cycling conditions in the second stage were as follows: 94 °C for 5 min followed by 25 cycles of 94 °C for 40 s, 50 °C for 40 s, 72 °C for 40 s and finally 72 °C for 10 min. After completing the steps, the product was added to the 2% electrophoresis gel, and bands were observed by staining with ethidium bromide [21].

Nucleotide Sequence Analysis

In the present study, the Nested PCR product was sent to South Korea (Macrogen Genomic Division) for analyzing the nucleotide sequence and the relevant information was modified using the Chromas software. To determine the genome of *Betanodavirus*, the sequence of coat protein gene of the present study was compared with known sequences of the previous studies within the GenBank, Basic Local Alignment Search Tool (BLAST, <https://www.ncbi.nlm.nih.gov/blast>)

of NCBI was employed. The phylogenetic tree of the *Betanodavirus* sequences was designed using Test Maximum Likelihood Tree [13], 1 thousand fast bootstrap replicates were performed to assess the robustness of individual nodes of the phylogeny [22] and MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets [23].

The nucleotide accession numbers used in this study were KX608916- KX608915- AF175516- JX194164- D38636- EF558369- EF591367- HM017076- EU380202- FJ617262- MF668946- EU236147- EU236149- D38637- LC180356- LC180347.

Results

In the present study, all of the samples, apparently healthy and symptomatic, were used for the molecular detection of *Betanodavirus* using PCR and Nested PCR methods. Of all of the symptomatic and apparently healthy Groupers, 64 were positive and among all of the Asian Sea bass, 210 samples (30%) were positive. The total number of samples tested and the number of positive samples of each species by Nested PCR are specified separately in Table 1.

The primers designed by Nishizawa (1994) were used to test the samples using the PCR method. As expected, a band of about 429 bp was observed for the positive control sample but no bands were observed in test samples (Fig. 3). On the other hand, a specific band of 294 bp was observed, as we expected in the test and positive control samples when the Nested PCR method was used using the primer designed by Thiery (1999) (Figs. 4, 5). Except for the positive control, all samples that were positive in this method were negative in the PCR method.

Nucleotide Sequence Analysis

In the test samples, several isolated coat protein sequences were analyzed using the Blast program of NCBI. Two partial sequences were from wild and cultured Grouper (*E. coioides*) and two sequences were from the imported and

Table 1 The number and species of fish used and the percentage of positive samples

Fish species	Wild/Farmed	No. clinical signs	No. Test	No. positive	%Positive
Duskytail grouper (<i>Epinephelus bleekeri</i>)	Wild	0	20	8	5.33
Orange-spotted grouper (<i>Epinephelus coioides</i>)	Wild/farmed	5	70	32	21.33
Epaullet grouper (<i>Epinephelus stoliczkae</i>)	Wild	0	35	14	9.33
Thornycheek grouper (<i>Epinephelus diacanthus</i>)	Wild	0	25	10	6.66
Asian Sea bass (<i>Lates calcarifer</i>)	Farmed	23	700	210	30
Total		28	850	274	32.23

Fig. 3 Detection of *Betanodavirus* by PCR. Lane 1: ladder (Jena Biosciencia, 50 bp to 1 kb linear scale); Lane 2–11: samples; Lane 12: *Betanodavirus* positive control; Lane 13: negative control

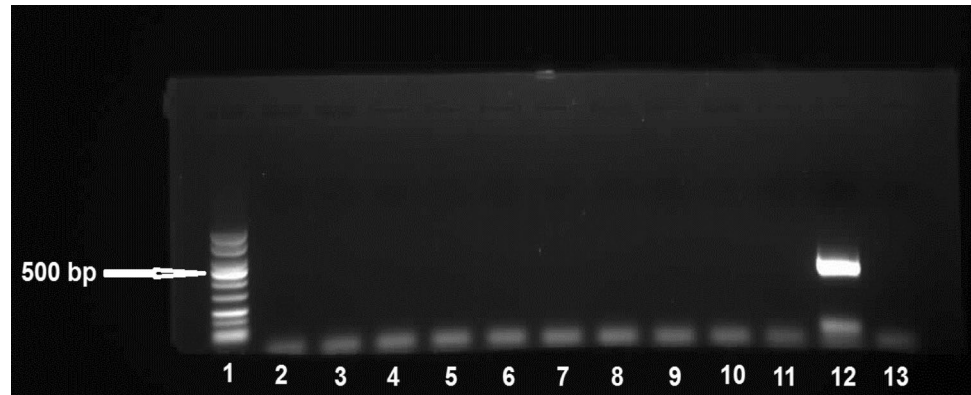


Fig. 4 Detection of *Betanodavirus* by Nested PCR. Lane 1: ladder (Jena Biosciencia, 50 bp to 1 kb linear scale); Lane 2–11: samples; Lane 12: *Betanodavirus* positive control; Lane 13: negative control



cultured *Lates calcarifer* in the cage. The result of sequence alignment showed that coat protein genes from Orange-Spotted Grouper and Asian Sea bass have the closest similarity to the Red Spotted Grouper Viral Nervous Necrosis (RGNNV). Also, the genomic sequence alignments of wild and farmed fish were similar to the coat protein gene of *Betanodavirus* in the Orange-Spotted Grouper and Asian Sea bass.

Phylogenetic Tree Analysis

In this study, to determine the relationship between *Betanodavirus* detected in wild and farmed fish with previous studies, a phylogenetic tree was designed based on the nucleotide sequences of the coat protein gene from *Betanodavirus*.

The results showed that the *Betanodavirus* detected in the fish belonged to the Red Spotted Grouper Nervous Necrosis Virus (RGNNV) genotype. The identified sequences in this study were closer to those of the Grouper fish in India (KX608915, KX608916) and relatively farther to sequences from countries such as France, Malaysia, China, Singapore, Australia, and others (Fig. 6).

Discussion

Since the discovery of VNN, there have been many reports of the *Betanodavirus* detection in marine and farmed fish in Asian, European and Australian waters. The two major

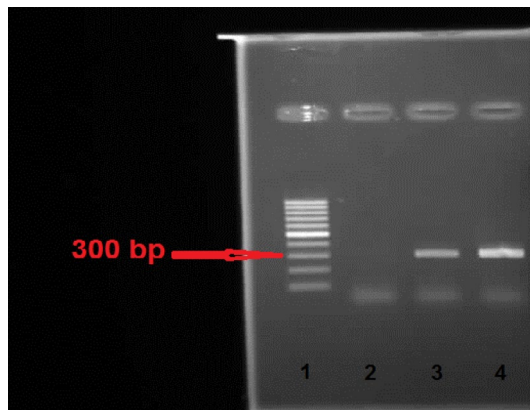


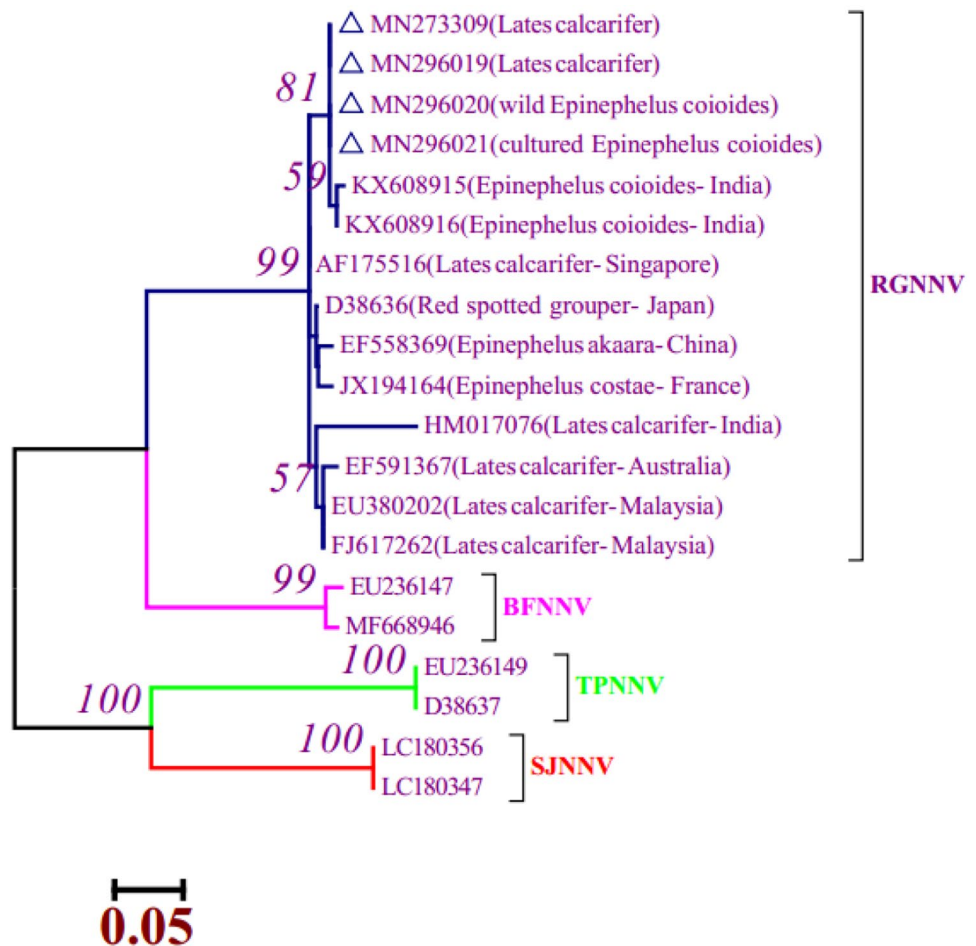
Fig. 5 Detection of *Betanodavirus* by Nested PCR: negative and positive samples together. Lane 1: ladder (Jena Biosciencia, 50 bp to 1 kb linear scale); Lane 2: negative sample; Lane 3–4: positive samples

species affected by this virus are Grouper and sea bass. Many studies have shown asymptomatic cases and in most cases, the RGNNV genotype was its cause. On the other hand, the prevalence of this genotype in Asian countries is increasing with temperature rise and available data indicate

a high prevalence among marine and farmed fish and endangered fish [24]. The Persian Gulf is a habitat for a variety of Grouper fish [15], and numerous studies have shown the reports of susceptibility and mortality of this fish to *Betanodavirus* in Asian countries such as *E. fuscoguttatus*, *E. akaara* in Japan, *E. septemfasciatus* in Japan and Korea, *E. coioides* in Philippines, *E. awoara* in Taiwan, *E. tauvina* in Malaysia, Philippines and Singapore, *E. moara* in Japan [25], *E. lanceolatus* and *E. malabaricus* in Taiwan [26], *E. marginatus* in Taiwan [27] and in European countries such as *E. marginatus*, *E. costae* in Italy [28], *E. aeneus* in the Mediterranean Sea [29], and *E. costae* in southern Europe [30].

Water is the most common way of transmitting *Betanodavirus* and the horizontal and vertical transmission paths of it have also been confirmed. Molecular methods can be referred to as appropriate methods for *Betanodavirus* identification [17]. PCR is suitable for detecting high doses of a virus and Nested PCR is more selective for detecting low doses of a virus. It is used in epidemiological studies due to its speed, high sensitivity and specificity [21, 31, 32] and it was revealed that Nested PCR is 100 times more sensitive than PCR [21]. The results of the present study only detected

Fig. 6 Phylogenetic tree of detected sequences for coat protein gene, bootstrap test:1000 replicates (Δ : the sequences of this study)



Betanodavirus in the studied fish by Nested PCR. Although some fish showed clinical signs like abdominal distension, hyperemia and exophthalmia, the PCR method was unable to detect the disease in them. This was maybe due to the presence of inhibitory compounds in the PCR tube, the sequence differences between the coat protein genes and reduced sensitivity of the PCR method. Similarly, PCR failed in the screening of *Pseudocaranx dentex* [33] and non-detection of VNN in chronic infections or low virus titers (10^3 TCID₅₀/ml) [4].

Interestingly, the most frequent *Betanodavirus* genotype in many warm water fish species associated with the RGNNV [24]. Similarly, the analysis of the genomes of *Betanodaviruses* from fish species in the close countries of the Persian Gulf and Oman Sea, such as India, indicated that viruses belonged to RGNNV [20]. Recently, the VNN prevalence caused by the RGNNV/SJNNV and SJNNV/RGNNV reassortant strains has been confirmed only in the Mediterranean region [34]. However, there is no evidence that the new reassortant strains have been spread in the Persian Gulf basin.

This is the first molecular detection of *Betanodavirus* infection in Grouper and Asian sea bass from warm southern coastal regions of Iran and also the first phylogenetic analysis of the Persian Gulf isolates of *Betanodavirus* using partial sequences of RNA2 segment. According to existing studies, RNA2 has been further screened and RNA1 is used for detection of virus reassortment, verification of genotypes identified by RNA2 segment [1, 35], and as a marker of virus origin in the phylogenetic analysis [36], therefore, we established a Nested PCR based on the detection of the RNA2 genomic sequence of the VNN, according to OIE protocol and finally the sequencing was done.

The results of sequencing in the present study detected the common genotype (RGNNV) in warm water. The temperature dependence of this genotype has been described previously [37]. The outbreak of the virus in marine and spawning fish areas such as China and Taiwan has made this issue clearer [38]. Also, in the experimental challenge of sevenband Grouper and Asian Sea bass fish at different temperatures, the relationship between the disease and the water temperature was proven [39, 40]. Due to the warm temperatures in southern Iran during most of the year, the presence of this viral genotype in fish mortality in the area should be monitored closely. In recent years, there have been numerous reports of VNN from the Persian Gulf in Iran and neighboring countries. For example, Grouper mortality due to VNN was reported in offshore equipment of Kuwait (2008) and confirmed in 2011 in the Kuwait Marine Fish Aquarium Fisheries [41], the mortality of cage cultured Asian Sea bass in the west coast of India was confirmed by sequencing [20], also the

VNN detection from seabream (Sobaity) in the waters of the region of Bahrain and its confirmation through Nested PCR and Real-Time [18] and in Iran following the catastrophic accident and severe mortality of *Liza klunzingeri* in the Persian Gulf and Oman Sea (2014), histopathological studies were performed and vacuolation of the fish brain and eyes tissue was observed but cell culture assay as a gold test and the molecular tests were not performed.

Recently, the VNN prevalence caused by the RGNNV/SJNNV and SJNNV/RGNNV reassortant strains has been confirmed only in the Mediterranean region [34]. However, there is no evidence that the new reassortant strains have been spread in the Persian Gulf basin.

Because abundant international trade, farmed fish is considered as a risk of spreading the *Betanodavirus* from one region to another, especially since *Betanodavirus* hosts can maintain infection and transfer it to later generations and other fish, and can be responsible for the transmission of disease in other fish [17].

In the current study, the high similarity in sequences of the wild and cultured fish shows the potential transmission of *Betanodavirus* infection among them in the region. Especially that broodstock of cultured Grouper was from the Persian Gulf and Sea bass are also imported from other countries. As a result, the presence of the *Betanodavirus* in the Persian Gulf fish and their genetic similarity indicates the transmission of VNN between wild and farmed fish. It was found that there is a possibility of contamination of adjacent countries and import of infected fish, so monitoring and surveillance of symptomatic fish and those without clinical signs can be effective in preventing the spread of VNN and economic damage. Using the sequencing of the T4 region of RNA2, we found that RGNNV is the predominant genotype in the Persian Gulf of Iran. However, due to the emergence of reassortant strains in some countries, further studies should be established to identify these new species.

The future epidemiological studies also are recommended to identify the VNN in other fish species, the possibility of virus reassortment by RNA1 detection, and conserve the aquatic resources of the area.

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Author Contributions MJZ designed and directed the study and prepared the manuscript, MZ prepared the samples and carried out the experiments and the analyses with the use of software, and FK and MK planned the experiments.

Data Availability All data generated or analyzed during this study are included in this published article and its supplementary information files.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

Research Involving Animal Rights All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

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