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Research Article

Detection of betanodavirus in wild golden grey mullet (*Chelon aurata*) in southern parts of the Caspian Sea using Real-time RT-PCR and immunohistochemistry

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Abstract

Viral Nervous Necrosis (VNN), caused by betabodavirus, is considered as one of the most important threats for mariculture and aquaculture globally. The disease was previously reported in wild golden grey mullet (*Chelon aurata*) from the Caspian Sea in 2004. To update these findings and to examine the phylogenetic relationship of the betanodavirus involved in disease outbreaks in Iran with other betanodavirus genotypes, forty golden grey mullets were screened for the presence of betanodavirus using real-time RT-PCR targeting the T4 region of RNA2 coat protein gene. The results showed that all 40 fish has positive test for the virus, which was also confirmed by immunohistochemistry. The phylogenetic relationship of betanodavirus involved in disease outbreaks in Iran from 2016 to 2017 was compared with betanodavirus genotypes sequenced from elsewhere, and the 300 bp product from the real-time RT-PCR sequenced was found to have a 98–100% homology with red-spotted grouper nervous necrosis virus (RGNVV), confirming that the betanodavirus detected in wild golden grey mullet in the southern parts of the Caspian Sea adjacent to Iran determined to be the RGNNV genotype.

Keywords: Betanodavirus, Viral Nervous Necrosis, Phylogenetic analysis, Real-time RT-PCR, Immunohistochemistry, Golden grey mullet.

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Introduction

The Caspian Sea, located between Europe and Asia, is composed of brackish water and is the world's largest inland lake with approximate salinity of 12 ppt (Harlioglu and Farhadi, 2017). Iran is located on the southern coast of the Caspian Sea and has the deepest area. Fish living in the Caspian Sea, such as killka (*Clupeonella Cultriventris*), Kutum (*Rutilus kutum*), mullets and sturgeons, migrate to this region to feed and reproduce (Harlioglu and Farhadi, 2017). Golden grey mullet (*Chelon aurata*) is particularly important to capture fisheries in this region, but since 2002 these catches have been steadily decreased (Fazli *et al*., 2008, 2020); from 20,950 tons in 2002 to less than 8,800 tons in 2017-2018 (Zorriehzahra *et al*., 2014; Fazli *et al*., 2020). Several different reasons have been appointed for this decline, such as pollution, illegal fishing and diseases (Fazli *et al*., 2012, 2020). Betanodavius is believed to be one of the main agents involved in golden grey mullet mortality (Soltani *et al*., 2010), being first reported in golden grey mullet in Iran in 2004 (Zorriehzahra *et al*., 2020).

Viral Necrosis Virus (VNN), the disease caused by betanodavirus, is considered as one of the most important threats for mariculture and aquaculture worldwide (OIE, 2019). Infection with this virus can lead to 100% mortality in larval and juvenile fish, and can also cause significant losses in older, market-size marine and freshwater species. The disease affects both farmed and wild fish and has been reported in more than 120 species (Costa and Thompson, 2016) mainly from marine sites, although outbreaks have been recorded at freshwater sites (Chi *et al*., 2003; Bovo *et al*., 2011). Fish infected with betanodavirus exhibits a range of neurological signs, including abnormal swimming behaviour and vacuolation in their central nervous system and retina (Yamashita *et al*., 2005). Betanodavirus, belonging to the family Nodaviridae (https://talk.ictvonline.org/taxonomy/p/taxonomyhistory?taxnode_id=201903931), is a non-enveloped, icosahedral virus with a diameter of 25-35 nm and a genome comprised of two positive-sense non-polyadenylated ssRNA (Nishizawa *et al*., 1997). RNA1 is 3,100 bp in length and encodes protein A of 100 kDa, which is the RNA-dependent RNA polymerase; while RNA2 is 1,400 bp in length and encodes the capsid protein of about 42 kDa (Nagai and Nishizawa, 1999; Lin *et al*., 2001). Moreover, there is a third RNA segment, RNA3, synthesized sub-genomically from RNA1and encodes protein B2 (Iwamoto *et al*., 2005). Four species have been identified according to the partial nucleotide sequence of the T4 region (381 bases) of the coat protein gene, and include Tiger Puffer Nervous Necrosis Virus (TPNNV), Striped Jack Nervous Necrosis Virus (SJNNV), Barfin Flounder Nervous Necrosis Virus (BFNNV) and Red spotted Grouper Nervous Necrosis Virus (RGNNV), (Nishizawa *et al*., 1995, 1997). Betanodavirus has been reported in wild fish indistinct sea around the world, e.g. Hainin Island (China) (Ma *et al*., 2015),

South China Sea (Liu *et al*., 2015), along with Korean peninsula (Gomez *et al*., 2004, 2008a), Japan (Sakamoto *et al*., 2008), Norway (Nylun *et al*., 2008), southern Italy (Vendramin *et al*., 2013), Sicilia (Ciulli *et al*., 2006), central Mediterranean (Giacopello *et al*., 2013) or south Atlantic Iberian peninsula (Moreno *et al*., 2014).

Several environmental factors can influence virus survival in the marine environments include heavy metals, sewage, sunlight, and the presence of microorganisms (Salo *et al*., 1976; Berry and Noton, 1976; Fujioka *et al*., 1980; Gomez *et al*., 2008a). Unfortunately, the Caspian Sea has been subjected to severe pollution events in recent years including heavy metals, oil and urban wastes (Harlioglu and Farhadi, 2017). It has been shown that virus can be easily adsorbed by sediment and survives for long time in the presence of sediment compared to seawater alone (La Belle and Gerba,1979). Mullets are bottom feeders, eating detritus and small invertebrates; thus large amounts of soil can be found in their intestine (Coad, 2017). Feeding on betanodavirus-contaminated organisms such as crustaceans, shellfish or aquatic plants may also play an important role in transmitting the virus to wild fish since betanodavirus has been detected in wild marine invertebrates (Gomez *et al*., 2008b). The Iranian government has the initiative to develop aquaculture cage culture in the Caspian Sea, intending to produce 50000 MT of marine fish through within the next decade, particularly high valued species such as sturgeon (Harlioglu and Farhadi, 2017). A greater understanding of the significance of betanodavirus in wild fish in the southern Caspian Sea region will help the biosecurity needed to reduce the risk of wild fish transmitting the virus to farmed fish as part of Iran's new aquaculture initiative.

Sensitive and specific detection techniques have been developed to identify betanodavirus in environmental samples and in fish tissues, helping to prevent and control NNV. These include polymerase chain reaction (PCR)-based method targeting the T4 variable region of the RNA2 coat protein, for example, reverse transcription (RT)-PCR, nested RT-PCR (Nishizawa *et al*., 1994, 1996; Thiéry *et al*., 1999; Grotmol *et al*., 2000; Dalla Valle *et al*., 2000), real-time PCR (Dalla Valle *et al*., 2005; Grove *et al*., 2006; Hick and Whittington, 2010; Panzarin *et al*., 2010; Kuo *et al*., 2011), reverse-transcription loop-mediated isothermal amplification (RT-LAMP) (Suebsing *et al*., 2013) and microfluidic chip system (Kuo *et al*., 2012).

In the present study, real-time RT-PCR and immunohistochemistry (IHC) were used to investigate the occurrence of betanodavirus infections in wild golden grey mullet populations collected from the Caspian Sea, and also phylogenetic analysis of the nucleotide sequence obtained for the partial RNA2 coat protein to characterize the relationship between the nodavirus identified in this study with other betanodavirus genotypes.

Materials and methods

Sample collection

A total of forty golden grey mullets, suspected of being infected with NNV based on clinical signs, were collected from the coastal areas of the Caspian Sea in Iran from October 2016 to February 2017. The mean weight of collected fish was 86±4 g. Their brains were aseptically collected as the target tissue for betanodavirus analysis. These were placed in sterile tubes, and stored at -80˚C for further process. Additional samples from the same fish were fixed in Bouin's solution for immunohistochemistry (IHC) analysis.

RNA extraction

The tissues were homogenized in 1 ml of RNX-Plus reagent (Sinaclon, Iran) and incubated for 5 min at 22˚C. Two hundred microliters of chloroform (Merck, Germany) were added to the suspensions, which were incubated for 5 min at 4˚C, and then centrifuged at 11,000×g for 15 min at 4˚C. The RNA in the aqueous upper phase was precipitated by adding an equal volume of isopropanol (Merck, Germany). The mixture was incubated for 15 min on ice and then centrifuged at 11,000×g for a further 15 min at 4˚C. The pellet obtained was washed with 1 ml of 75% ethanol and centrifuged at 4000×g for 8 min at 4˚C. The RNA pellet was air dried and dissolved in 50 ml of DEPC-treated water. The quality of RNA was examined by observing on an ethidium bromide-stained 1% agarose gel (w/v) using UV illumination.

Real-time RT-PCR

Reverse transcription was performed using a ready-to-use reverse transcription kit (AccuPower® CycleScript RT PreMix (dN6), Bioneer, Daejeon, Republic of Korea) according to the manufacturer's protocol, whereby 0.1~1.0 µg of extracted total RNA was used as the template, and the reaction volume made up to 20 µl with DEPC-treated distilled water. The lyophilized transparent pellet was dissolved by vortexing and the cDNA synthesis reaction was performed with 12 cycles of 20˚C for 45 s and 45˚C for 4 min and a final step of 95˚C for 5 min.

The real-time RT-PCR was performed on a CFX Connect™ thermocycler (Bio-Rad, Hercules, California, USA) using a 96-well plate. The real-time RT-PCR amplification was performed using primers F'2 (5'-GTT CCC TGT ACA ACG ATT CC-3') and R'3 (5'-GGA TTT GAC GGG GCT GCT CA-3') (Thiéry et al.1999). Reactions contained 6.25 µl of 2x Quantinova SYBR Green PCR Kit (Qiagen, Zist Baran, Tehran, Iran), 0.15 µl each of forward and reverse primers, 4.95 µl RNAse free water and 1 µl cDNA, making a final volume of 12.5 µl. Thermocycling conditions selected were: 1 cycle of 95°C for 10 min, 45 cycles of 95°C for 15 s and 60°C for 1 min, followed by a melt cycle between 55 and 95°C with 0.5°C increments. Therefore, melting curves analysis was performed by CFX Manager (Version: 1.1.308.1111) are automatically converted to melt peaks. To ensure the

quality and reproducibility of data, positive and negative controls were added to each plate run. RNA from NNV infected orange-spotted grouper (*Epinephelus coioides*) larvae were used as positive control (Kindly provided by Dr. Nallathambi Chakravarthy, Biotechnology Department, Rajalakshmi Engineering College, Chennai, India) and nuclease-free water was used as a negative control. Five µL of real-time RT-PCR products were electrophoresed on a 1.5 % agarose gel in 1× TBE buffer for 1 h at 85 V. The gel was stained with ethidium bromide and photographed in a gel documentation system (Bio-Rad).

Nucleotide sequence analysis

The band containing the real-time RT-PCR amplified product was visualized on a 1.5 % agarose gel. The band was recovered and purified using an AccuPrep Gel Purification kit (Bioneer, Korea) as described in the manufacturer's instructions. The purified real-time RT-PCR products were sequenced by Macrogen Genomics Division, South Korea.

Phylogenetic analysis

Sequencing data were assembled and edited using Chromas software V2.6.4, and the analysis of sequence similarities was determined using the Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov/ blast/) of NCBI. The multiple sequence alignment was performed with Clustal W v1.2.4 (European Bioinformatics Institute; www.ebi.ac.uk/Tools/clustalw). A phylogenetic tree of the betanodavirus sequences was constructed using the maximum likelihood method and Jukes Cantor model with 1000 bootstrap replicates by MEGA6 software (Tamura *et al*., 2013). GenBank accession numbers of known betanodavirus sequences used in this analysis are listed in Table 1.

Immunohistochemistry (IHC)

The brains of PCR positive golden grey mullet were screened with IHC to visualise the location of virus in the brain of infected fish. Briefly, 5 µm tissue sections of Bouin-fixed brain tissue of sampled fish were placed on glass microscope slides (Superfrost plus slides, Thermo Fisher Scientific, Loughborough, UK) and the tissue sections were dewaxed and dehydrated through an alcohol series (x2 xylene for 5 min, 100 % ethanol for 5 min, 70 % ethanol for 3 min, ultrapure H_2O for 5 min)

Table 1: Betanodavirus isolates and accession numbers of sequences used in the present study.

Strain	GenBank accession no.	Source of strain	Country	Reference
CS-GM	MK214434	*Chelon aurata*, golden grey mullet	Iran	This study
GNNV	AF499774	*Poicelia reticulate*, guppy	Singapore	Hegde *et al.*, 2003
B00GD	AY140793	*Lates calcarifer*, barramundi	Taiwan	Chi *et al*., 2003
BVN16	JF412273	*Lates calcarifer*, barramundi	India	Binesh, 2013
TGNNV0109	HQ859940	*Mycteroperca tigris*, tiger grouper	Malaysia	Ransangan and Manin, 2012
GNNV-Cage1	KX608916	*Epinephelus coincides*, orange spotted grouper	India	Sekar *et al*., 2016
SBN147	KP455642	*Lateolabrax japonicus*, sea perch	China	Jia *et al*., 2015
SG14	AF175516	*Dicentrarchus labrax*, european seabass	Singapore	Skliris *et al*., 2001
GGNNV20131122	KM588181	*Epinephelus lanceolatus*, giant grouper	Taiwan	Chen *et al*., 2015
RG-TO91	D38636	*Epinephelus akaara*, red-spotted grouper	Japan	Nishizawa *et al*., 1995
BF93Hok	D38635	*Verasper moseri*, barfin flounder	Japan	Nishizawa *et al.*, 1995
Tp93Kag	D38637	*Takifugu rubripes*, tiger puffer	Japan	Nishizawa *et al.*, 1995
05SaiJJM-12	LC180356	*Pseudocaranx dentex*, striped jack	Japan	Nishioka *et al*., 2016

The IHC procedure was performed at room temperature (21±1°C) and the reagents were acquired from Sigma-Aldrich (Gillingham, Dorset, UK). The tissues were blocked first with 3 % H_2O_2 (v/v) in methanol (100%) for 20 min, washed 3 times with PBS (0.02 M phosphate, 0.15 M NaCl, pH 7.2) and then incubated with 10 % (w/v) normal goat serum diluted in PBS for 30 min. The goat serum was tapped off and rabbit polyclonal anti-RGNNV betanodavirus antibody (Aquaculture Research Group, Moredun Research Institute) was added to the tissues (1/200 dilution in PBS), and the slides were incubated for 30 min. The tissues were then washed 3 times by rinsing the slides with PBS containing 0.1 % Tween (PBST) and incubated with an anti-rabbit IgG-HRP conjugate (Pierce, Thermo Fisher Scientific, UK) (1:200 dilution in PBS) for 30 min. The tissues were once again washed in PBST and incubated with Vector VIP peroxidase substrate kit (Vector Laboratories Ltd, UK) for 10 min. The tissues were counterstained with Vector Methyl Green QS (Vector Laboratories Ltd, UK) for 3 min at 60°C, washed for 1 min in water and mounted with VectaMount Permanent Mounting Medium (Vector Laboratories Ltd). Pre-immune rabbit serum (diluted 1/200 in PBS) was used as a negative control. Positive staining appeared purple.

Results

Clinical signs and gross pathology

The golden grey mullet sampled for this study were showed pathognomonic signs of NNV infection, such as abdominal swelling and bilateral exophthalmia in most fish (Fig. 1A), hyperaemia in the mouth region (Fig. 1B) and the abdomen ventricular (Fig. 1C), also, the intraperitoneal cavity presented melanosis on its walls, the gallbladder was distended and full of bile, the intestine was swollen with white patches over the surface (Fig. 1D), while the swim bladder was distended with hyperaemia evident on its surface (Fig. 1E).

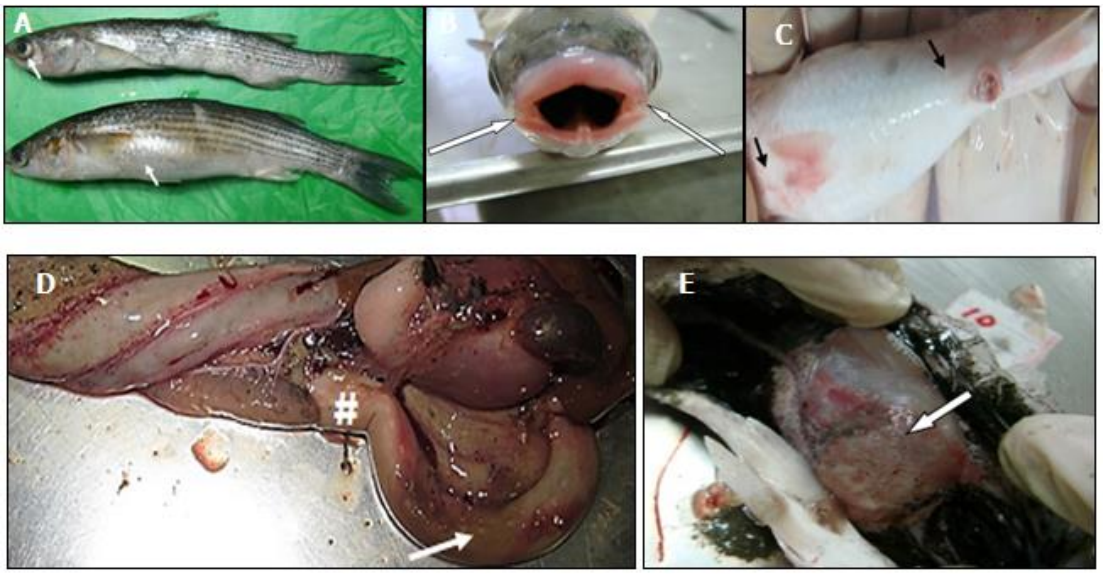

Figure 1: Gross signs observed in wild Golden grey mullet. Externally: A) swollen abdomen and with exophthalmia (small arrow) and (large arrow); B) swollen and hyperaemia (red) maxillary and mandibular lips (arrows); C) hyperaemia on the ventral abdomen. Internally: D) swollen intestine (#), white patches (thin arrow); E) distension and hyperaemia on swim bladder (B & E with permission of Nazari, 2011)

Real-time RT-PCR amplification

Real-time RT-PCR detection was based on the binding of the fluorescent SYBR Green dye to the amplicon and emission of specific fluorescence. All forty samples of golden grey mullet collected from the coastal areas of the Caspian Sea were positive for betanodavirus target genome detection. The melting curve of the PCR product showed an obvious temperature at which the double-stranded amplicon was denatured (Tm - mean melting temperature - 80±0.5°C), and there was no obvious increase in fluorescence signal from primer-dimers or other non-specific products (Fig. 2A). The size of the real-time RT-PCR amplicon was 300 bp (Fig. 2B).

Nucleotide sequence analysis

Partial nucleotide sequences obtained for the T4 region of the betanodavirus coat protein gene from golden grey mullet were analyzed using the BLAST programme of NCBI.

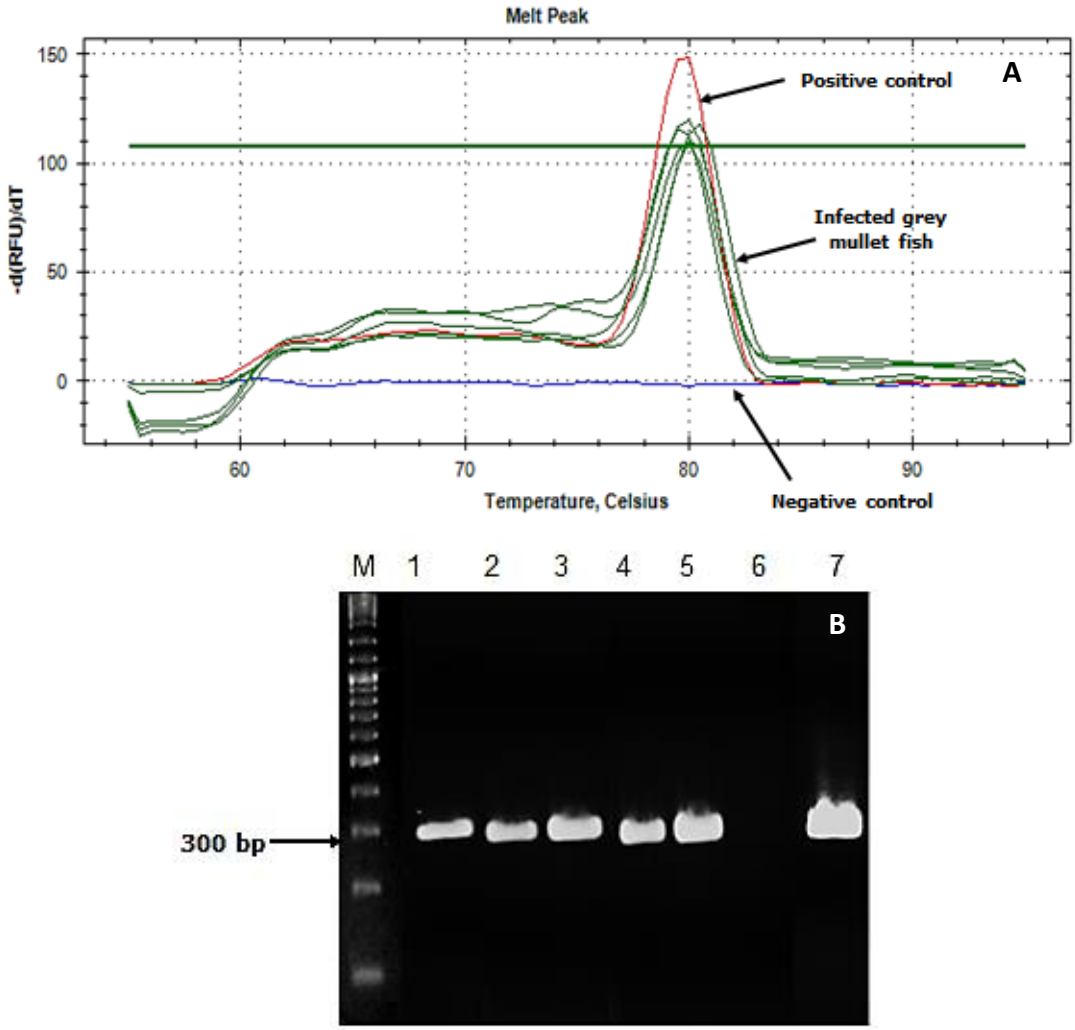

Figure 2: Analysis of the specific amplification of betanodavirus RNA2 T4 region in five samples of golden grey mullet, positive and negative controls. A) melting curve obtained for the samples using SYBR Green as fluorescence marker and specific primers F2-R3; B) agarose gel electrophoresis of the real-time PCR products: M- 100 bp DNA ladder; lanes 1 to 5 - brain sampled from infected golden grey mullet; lane 6 - negative control and lane 7 - Betanodavirus positive control.

The genomic sequence alignments of betanodavirus showed that the RNA2 of golden grey mullet has a homology of more than 95% with the RGNNV. Of the RGNNV genotype isolates used for this comparison, BVN16 and TGNNV0109 were highly related to the betanodavirus detected in the present study, with 100% homology. In contrast, the virus sequence from grey mullet shared less similarity with the genomes of the other 3 betanodavirus genotypes, with<80% homology obtained for BFNNV (77.3 %), SJNNV (71.0 %) and TPNNV (67.2%) (Table 2). To compare the relationship between the golden grey mullet betanodavirus isolated in this study (GenBank accession no. MK214434) with the other betanodavirus genotypes, a phylogenetic tree was constructed based on nucleotide sequences of the T4 region of the NNV capsid protein gene from 12 isolates available from GenBank. The result of

the phylogenetic tree analysis revealed that the current nodavirus from golden grey mullet belonged to the RGNNV genotype (Fig. 3).

Table 2: Percent nucleotide sequence similarities between the T4 region of the betanodavirus coat protein gene of golden grey mullet (accession number: MK214434) and other betanodavirus isolated from distinct fish species.

		Percent identity														
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**		
Divergence	**1**		98.2	99.0	100	99.1	98.2	99.3	99.0	100	98.2	77.3	71.0	67.2	**1**	CS-GM*
	2	1.8		98.4	97.5	97.0	97.9	99.0	98.1	97.5	98.7	82.8	68.5	79.3	**2**	GNNV
	3	1.0	1.6		98.3	97.8	98.3	90.5	98.8	98.3	99.0	83.6	68.2	80.1	**3**	B00GD
	4	0.0	3.5	1.7		99.4	98.1	98.2	98.6	100	98.3	82.8	68.5	79.1	**4**	BVN16
	5	0.9	3.1	2.2	0.6		97.0	97.5	97.8	99.4	97.2	76.5	69.9	69.1	**5**	GNNV-Cage1
	6	1.8	3.0	1.7	1.9	3.1		98.5	99.0	98.1	98.3	82.9	67.2	79.5	**6**	GGNNV20131122
	7	0.7	1.0	0.8	1.8	2.5	1.5		99.0	98.2	99.1	83.4	68.2	79.0	**7**	SG14
	8	1.0	2.8	1.2	1.4	2.2	1.0	1.0		98.6	98.8	83.3	68.2	79.4	**8**	SBN147
	9	0.0	3.5	1.7	0.0	0.6	1.9	1.8	1.4		98.3	82.2	68.5	79.1	**9**	TGNNV0109
	10	1.8	1.3	1.0	1.7	2.8	1.7	0.9	1.2	1.7		83.4	68.2	79.8	**10**	RG-TO91
	11	22.7	19.8	19.5	20.1	29.5	19.8	19.9	19.4	20.1	19.3		68.7	79.8	**11**	BF93Hok
	12	31.3	39.9	40.4	39.9	40.8	41.3	40.4	40.4	39.9	40.4	43.4		73.6	**12**	05SaiJJM-12
	13	32.8	24.8	24.7	25.1	44.2	24.4	26.2	24.6	25.1	24.9	25.3	32.7		**13**	Tp93Kag
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**		

CS-CM*: Caspian Sea– golden grey mullet.

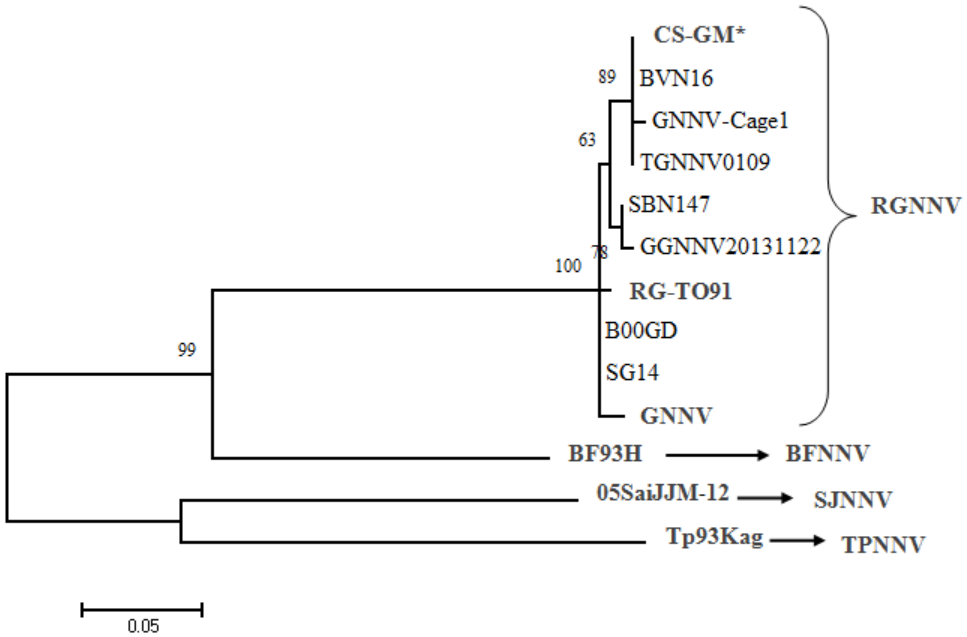

Figure 3: Phylogenetic tree of betanodavirus isolates inferred with maximum likelihood method (MEGA 6.0). The numbers on branch nodes represent bootstrap values (with 1000 replications.). *CS-CM: Caspian Sea– golden grey mullet.

Immunohistochemistry

When histological tissue sections from real-time PCR-positive fish were analyzed by IHC, the presence of porous sponge-like lesions in the brain, characteristic of NNV infection were evident (Fig. 4A). Viral antigens in the cytoplasm of degenerated cells and the spongious lesions of the brain were visualized by the presence of purple staining. Immunoprecipitation was detected in different regions of the brain

(telencephalon, mesencephalon, medulla oblongata). In 32 cases, there was a strong immunostaining present in the optic lobe (Fig. 4A) and medulla oblongata (Fig. 4B). No immuno-positive reaction was observed in the negative control (Fig. 4C).

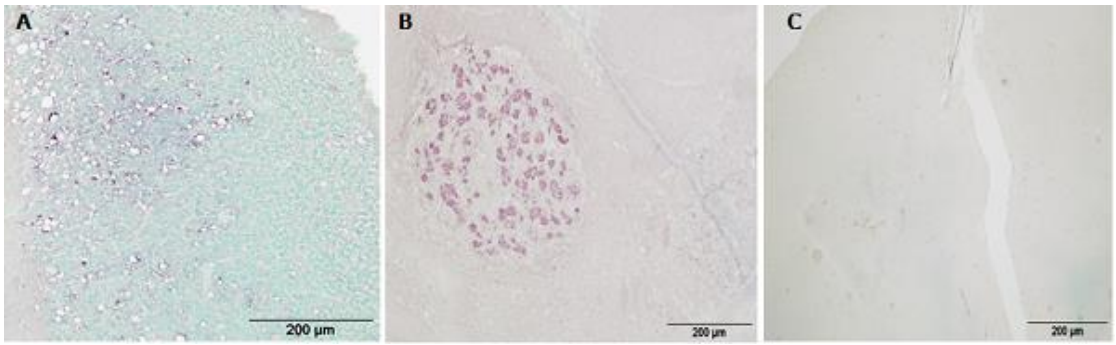

Figure 4: Nervous Necrosis Virus (NNV) immunohistochemistry in the brain of infected golden grey mullet (*Chelon aurata*) from the southern waters of the Caspian Sea screened with rabbit anti-betanodavirus polyclonal serum (raised against a RGNNV strain), indicated by the purple staining in the (A) optic lob and (B) medulla oblongata; (C) negative control with no staining.

Discussion

The use of rapid and reliable methods to detect betanodaviruses in fish carrying the virus before stocking fish farms would be a significant benefit in the management of NNV outbreaks (Hodneland *et al*., 2011). Molecular methods, particularly real-time RT-PCR, have the advantage of being highly sensitive and rapid to perform and are particularly useful for screening such as environmental samples that will contain low levels of the virus (Gomez *et al*., 2008a; Hodneland *et al*., 2011; Rajan *et al*., 2016). Even though isolation of the virus by cell culture is the recommended gold standard method to screen for nodavirus by the Office International des Epizooties (OIE, 2019), wild fish carriers tested positive by PCR that was not reported to be positive for the virus by cell culture (Gomez *et al*., 2008a; Sakamoto *et al*., 2008).

In the present study, the wild golden grey mullet sampled from the Iranian waters of the Caspian Sea were screened for the presence of betanodavirus infections by real-time RT-PCR all 40 brain samples showed positive for the virus. The real-time RT-PCR amplification of the T4 region of the betanodavirus coat protein gene gave a 300 bp amplicon with both the golden grey mullet samples and the positive control (orange-spotted grouper). To ensure no false-positive results occurred, the Tm of the target's melting curve was assessed (Joseph, 2010). Melting curve analysis allows discrimination between the primer and product peak in the presence of SYBR Green, since non-specific dyes, such as SYBR Green, do not distinguish between the intended amplicon and other products, such as primer-dimers. A single melting curve with a narrow peak indicated that the

amplification obtained was specific and lacked non-specific amplification.

Although NNV was first reported in wild golden grey mullet in the Caspian Sea in Iran in 2004 by cell culture isolation and nested RT-PCR (Zorriehzahra *et al*., 2020), the genotype of the virus isolated from wild golden grey mullet had not been determined. When the phylogenetic analysis was performed on the T4 region of the betanodavirus coat protein gene and a phylogenetic tree constructed, the partial sequences of the RNA2 coat protein gene indicated that the betanodavirus from golden grey mullet had more than 95% similarity with other RGNNV strains. According to Nishizawa *et al*. (1997), RGNNV has the greatest world distribution of the different betanodavirus genotypes, while SJNNV has only been found only in few species (Nishizawa *et al*., 1997; Skliris *et al*., 2001; Thiéry *et al*., 2004; Cutrín *et al*., 2007).

Although IHC is not generally as sensitive as molecular methods, IHC provides a more sensitive level of detection than the conventional histological staining without the requirement for molecular biology facilities. It is often considered as a confirmatory test when the presence of a suspected agent has not been confirmed through routine histology (Duraiyan *et al*., 2012). Betanodavirus particles have previously been observed in the cytoplasm of degenerated cells and in the spongious lesions of nerve cell in the brain and eye using IHC (Mladineo, 2003; Maltese and Bovo, 2007). In the present study, IHC was used to confirm the etiology of the lesions present in the brain of infected fish, corroborating the results of the PCR, with the characteristic vacuolation and necrosis of the central nervous system associated with IHC specific staining. These results are similar to staining obtained in other fish species infected with betanodavirus and in general, the anterior brain was more severely infected than the posterior brain and the spinal cord (Munday *et al*., 2002; Nopadon *et al*., 2009; Andriyanto *et al*., 2018). Le Breton *et al*. (1997) reported that the optic tectum was rarely affected in the diseased adult European sea bass (*Dicentrarchus labrax*). Lesions have also been described in the spinal ganglia in Japanese parrotfish (Yoshikoshi *et al*., 1990).

In conclusion, this is the first report analyzing the partial RNA2 sequence of betanodavirus isolate obtained from wild golden grey mullet in the southern parts of the Caspian Sea in Iran and elucidated that the RNA2 belongs to genotype RGNNV. To understand the evolutionary process of mullet betanodavirus in detail, it is necessary to use complete genomic sequencing of the isolate for more in-depth analysis and to establish if betanodavirus reassortants are present in the Caspian Sea. The results also showed that the real-time RT-PCR method is an efficient means to monitor the presence of viral genome in wild fish.

NNV is an emerging disease in Iran (both southern and northern Iran). The

presence of betanodavirus in wild fish is a warning for Iran's aquaculture and its sustainability. A more comprehensive investigation into the NNV has been started and these studies should focus as well on the impact of the environmental factors and the presence of biological organisms in the marine environment, such as invertebrates (Gomez *et al*., 2008b) and could be a source of infection to susceptible wild and farmed fish. The role of wild golden grey mullet and the implication of betanodavirus presence and possible transmission to other susceptible fish species and to the new farmed fish industry should be elucidated and understood. Such studies will permit the establishment of management strategies, reduce disease risk and allow sustainable fisheries and aquaculture industries in the Caspian Sea. A key factor for such studies and the development of management tools are the abilities to detect the presence of betanodavirus. During this study, the molecular capability has been set up, and the Iranian Fisheries Sciences Research Institute has now the ability to detect betanodavirus that will help developed research in this virus and assess its prevalence in wild populations, as well as helping the Iranian aquaculture to expand in a sustainable form.

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