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Short communication

Isolation and confirmation of viral nervous necrosis (VNN) disease in golden grey mullet (*Liza aurata*) and leaping mullet (*Liza saliens*) in the Iranian waters of the Caspian Sea

M.E.J. Zorriehzahra[a,*], M. Ghasemi[b], M. Ghiasi[c], S.Haghighi Karsidani[d], G. Bovo[e], A. Nazari[f], M. Adel[a], V. Arizza[g], K. Dhama[h]

[a] *Aquatic Animal Health & Diseases Dept., Iranian Fisheries Science Research Institute (IFSRI), Agricultural Research Education and Extension Organization (AREEO), Tehran, Islamic Republic of Iran*
[b] *Department of Fish Diseases, Inland Water Aquaculture Institute, Iranian Fisheries Science Research Institute (IFSRI), Agricultural Research Education and Extension Organization (AREEO), Anzali, Gilan, Islamic Republic of Iran*
[c] *Department of Fish Diseases, Caspian Sea Ecology Research Center, Iranian Fisheries Science Research Institute (IFSRI), Agricultural Research Education and Extension Organization (AREEO), Sari, Islamic Republic of Iran*
[d] *Department of Fisheries, Bandar-e-Anzali Branch, Islamic Azad University, Gilan, Islamic Republic of Iran*
[e] *OIE Reference Lab. for Encephalopathy and Retinopathy, Instituto Zooprofilattico Sperimentale delle Venezie,Viale dell'Università, Padova, Italy*
[f] *Department of Aquaculture, Falavarjan Branch, Islamic Azad University, Falavarjan, Islamic Republic of Iran*
[g] *Department of Stebicef, Università degli Studi di Palermo, Via Archirafi, Palermo, Italy*
[h] *Division of Pathology, ICAR—Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India*

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ABSTRACT

The present study was conducted on 428 moribund mullet fish samples to isolate and identify the causative agent of a mysterious acute mortality which recently occurred in wild mullets in Iranian waters of Caspian Sea, suspected to be due to viral nervous necrosis (VNN) disease. Disease investigation was carried out employing various diagnostic procedures such as virology, bacteriology, parasitology, haematology, histopathology, IFAT, IHC and nested RT-PCR. Brain and eye samples of affected fishes were collected in sterile conditions and then kept at −80 °C for cell culture isolation and nested RT-PCR detection of the causative agent. Other tissue samples were also collected and fixed for histopathology, IHC and EM examinations. CPE was observed in cell cultures at 6 days after inoculation. Nine samples were found positive with virological assay. Nested RT-PCR, performed on suspected tissues and CPE positive samples, showed that about 21 tissue samples and all the CPE positive samples were positive for VNN virus (VNNV). IFAT was selected as a confirmatory method for detecting the presence of *Betanodavirus* antigen, cell culture isolation results and nested RT-PCR findings. Moreover, VNNV particles with 25–30 nm in diameter were also visualized in the infected brain and retina. In pathogenicity studies, guppy fishes bathed in VNNV-infected tissue culture (10^{-4} $TCID_{50}$) showed clinical signs similar to naturally infected mullet after 15 days post infection (dpi), with mortality rates reaching up to 100% at 30 dpi. Affected organ samples as examined by cell culture isolation, IFAT, IHC and histopathology, revealed the presence of VNNV in the guppy fishes. In conclusion, it was confirmed that VNNV was the main causative agent for the disease outbreak in mullet fish in the Caspian Sea, and this is such first official report of VNN disease from Iran.

1. Introduction

The Caspian Sea as the largest lake on the planet was bordered by Russia, Kazakhstan, Turkmenistan, Azerbaijan and Iran. In its southern part, there live more than 120 fish species, of which 23 species are commercially important.

Unidentified acute mortality has been long occurring in wild golden grey mullet of the Caspian Sea since 1998. The Caspian Sea Ecology Research Centre reported some mortality in wild mullet fish (golden grey mullet) on the Feridon Kenar beach of the Caspian Sea in the summer of 2001 for the first time, but which was forgotten without any published reports. The Caspian Sea Bony

* Corresponding author.
E-mail address: zorrieh@yahoo.com (M.E.J. Zorriehzahra).

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Table 1
Sampling table for VNN detection in mullet fish in the Caspian Sea [2006–2010].

Province	Year	Sampling Season	Fish Species	No. Samples	Weight of fish (g)	Clinical signs	Histopathology	Haematology	Bacteriology	Parasitology	Virus isolation	IHC	IFAT	RT-PCR
Guilan (Southern West of Caspian Sea)	2006–2010	Autumn and Winter	*Liza aurata* *Liza saliens*	312	50–250	Imbalance in swimming, darkening in skin, abdominal distension and inflation of the swimbladder	350 (Brain and Eye) 350 (liver, kidney, intestine, stomach, gill, skin and muscle, gall bladder and gonads)	–	–	20	312	100	100	300
Mazandaran (Southern Mid of Caspian Sea)	2007	Winter	*Liza aurata*	56 + 30 (Affected and Health apparently fish)	100–250	1st Group: Abdominal distension, swimbladder inflation, lethargy, bilateral exophthalmia, bleeding at the base of the fins & eyes.	100 (Brain and Eye)	86	56	20	56	50	50	56
			Liza saliens			2nd Group: Muscles atrophy, large head and thin body, spinal cord diversion, corneal opacity and cataract.	90 (liver, kidney, intestine, stomach, gill, skin and muscle, gall bladder and gonads)							
Golestan (Southern East of Caspian Sea)	2007	Summer	*Liza saliens*	30	100–200	Hyperemia or Hemorrhage in the abdominal fins and the base	40 (Brain and Eye) 30 other tissues	30	30	10	10	20	20	30
Total				428			960	116	86	50	378	170	170	386
Total Samples	2744													

fishes Research Centre reported some moribund and mortality in golden grey mullet along the Zibakenar coastline in February 2003.

In collaboration with reference laboratory of the World Organisation for Animal Health (OIE) at the Japan and Taiwan National University first occurrence of a new viral disease, possibly the viral nervous necrosis (VNN) disease was reported in Iran for first time (Zorriehzahra et al., 2005). VNN is a serious disease causing significant economic damages to the marine aquaculture industry, having a wide geographical distribution (Maltese and Bovo, 2007; Zorriehzahra et al., 2016). It was first described in 1990 in hatchery-reared Japanese parrotfish, *Oplegnathus fasciatus* in Japan (Yoshikoshi and Inoue, 1990) and barramundi (Asian sea bass), *Lates calcarifer* in Australia (Glazebrook et al., 1990). Affected fish may show different symptoms depending on the species and age of the fish and the temperature; furthermore, acute and sub-acute forms with slightly different symptoms and mortality rates are also known. The most characteristic and common clinical sign observed among the different fish species is an abnormal swimming behaviour characterized by showing difficulties in maintaining normal static and dynamic equilibrium, speed, swimming direction and inflation control of the swimbladder (Maltese and Bovo, 2007). The viral aetiology has been confirmed through the identification of small, non-enveloped RNA agents definitively assigned to the Nodaviridae family, genus *Betanodavirus*. The existence of four genotypes, characterized by high homology, has been proposed on the basis of viral genome analysis. Although horizontal transmission undoubtedly represents the most common transmission route, vertical transmission has also been highly suspected, at least for some species. VER/VNN is commonly diagnosed after isolation of the causative agent in cell cultures, followed by identification with immunological or molecular methods (Maltese and Bovo, 2007).

The aim of this present disease investigation study was to employ diagnostic tools such as virology, bacteriology, parasitology, haematology, histopathology, IFAT and IHC, and molecular tools of nested RT-PCR, in a multidisciplinary project to investigate the possibility of a new viral disease in marine fish species such as mullet and the Acipenciridae family. Therefore, approximately 2997 tests were conducted on all available samples using the previously mentioned diagnostic tests. This multidisciplinary investigation was done according to OIE protocols as first time in Iran.

2. Materials & methods

2.1. Fish sampling

Approximately 428 affected mullet fishes, each weighting 250 ± 50 g, were collected between October 2006 and September 2010. Additionally, 136 sturgeon samples (46 brains, 43 eyes, 24 eggs and 23 milt of broodstock) were also collected. Moribund fishes were examined clinically and paraclinically. Sampling procedures are provided in Tables 1 and 2.

2.2. Histopathology

Approximately 960 samples comprising of liver, kidney, intestine, stomach, gill, skin, muscles, gall bladder, gonads, brain and eye from apparently healthy and moribund fishes in three sampling provinces were collected. Samples were fixed and processed using an automatic tissue processor (Shandon Citadel 1000, USA) according to standard methods. 5 μm sections were prepared using a rotary microtome (Jung Multicut 2045, LEICA Instruments, Germany) and these were stained using haematoxylin and eosin (H&E) and examined under a compound light microscope (Nikon 100, Tokyo, Japan).

Table 2
Sampling table for VNN detection in sturgeon fish in the Caspian Sea [2006–2008].

Province	Year	Sampling Season	Fish Species	Brain	Eye	Eggs	Sperm	Total	Weight of fish (g)	Histopathology	Virus isolation	IHC	FAT	RT-PCR
Guilan (Southern West of Caspian Sea)	2006	Winter	*Acipenser persicus*	11	11	5	5	32	1700–2200	22	11	10	10	7
	2007			10	10	–	–	20		20	10	9	8	5
	2008			13	13	–	–	26		26	13	11	10	6
Mazandaran (Southern Mid of Caspian Sea)	2006	Autumn and Winter	*Acipenser persicus*		3	9	8	20	1500–2300	3	2	2	2	5
	2007						6	6		–	–	–	–	7
	2008			–	–	–	–	–		–	–	–	–	4
Golestan (Southern East of Caspian Sea)	2006	Winter	*Huso huso*	12	6	10	4	32	1800–2500	18	9	8	7	3
	2007			–	–	–	–	–		–	–	–	–	3
	2008			–	–	–	–	–		–	–	–	–	2
Total				46	43	24	23	136		89	45	40	37	42
Total Samples				253										

2.3. Haematology

Blood samples were collected in anticoagulant (heparin, 0.2 mg/ml) from the caudal veins of the two groups, moribund fishes (n = 56) and apparently healthy fishes (n = 30) Erythrocyte count (RBC), leukocyte count (WBC), haemoglobin (Hb), packed cell volume (Hct), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and leukocyte differential count were measured (Stoskopf et al., 1993). Furthermore, total protein, albumin, total IgM, C3, C4, alanine aminotransferase (ALT), and aspartate aminotransferase (AST), were estimated on fish sera samples by using commercial kits (Pars Azmoon Company, Tehran, Iran) and a biochemical auto analyzer (Eurolyser, Belgium).

2.4. Virology

Approximately 378 suspected samples were examined by methods recommended by Nakai (OIE, 2013), collected from three sampling provinces and diagnostic tests were conducted according to the steps described below.

2.4.1. Cell culture and virus inoculation

The SSN-1 cells were cultured using Leibovitz L-15 medium (Gibco, BRL, USA), supplemented with 5% foetal bovine serum (FBS) in a 25-cm^2 tissue culture flask (Sumitomo Bakelite Co., Ltd., Japan) seeded in 24 well plates. Fish brain and eye samples were homogenised in nine volumes of Eagle's minimum essential medium (EMEM) (GIBCO), centrifuged and filtered through a 0.45 μm membrane. A 150 μl of this supernatant was inoculated to the cells. After standing at room temperature for 60 min, the plate was washed once with HBSS, supplied with EMEM (5% FBS), and incubated at 25 °C (OIE, 2013).

2.4.2. Monitoring incubation

Cell cultures were observed for CPE induction daily for 10d and then tested for virus detection by nested RT-PCR or by indirect fluorescent antibody technique (IFAT).

2.5. Indirect fluorescent antibody test (IFAT)

This confirmation of virus detection was conducted on 170 suspected samples in three classifications according to the following methods.

2.5.1. IFAT on cell line monolayers

This was performed using rabbit anti-nodavirus monoclonal antibody and fluorescein isothiocyanate–conjugated anti-rabbit Ig (Aquatic diagnostics Ltd., Scotland) according to OIE procedure (OIE, 2006).

2.5.2. IFAT on the tissue smears (imprinting method)

Brains or eyes of suspicious fishes were fixed on slides by the imprinting method, kept at room temperature, fixed with chilled acetone, packaged in aluminium foil and kept at −20 °C (OIE, 2013).

2.5.3. IFAT on fixed tissue

Tissues from suspected fishes were fixed according to the procedure described above and embedded in paraffin and processed by Leica RM2265 (Automated Rotary Microtome) in order to prepare 5 μm sections. The sections were washed with a cool phosphate buffer saline (PBS) and processed following the above described methods.

2.6. Immunohistochemistry (IHC)

Approximately 170 paraffinated blocks with 5 μm sections were prepared by ultramicrotome (Leica Ultracut T), and these were used to perform immunohistochemistry using anti-Betanodavirus rabbit serum and biotinylated goat anti-rabbit Ig (Lai et al., 2001).

2.7. Polymerase chain reaction (PCR)

PCR was performed on 386 mullet and 42 sturgeon samples using a commercial Nodavirus Detection Kit (VNN) (IQ2000© Co.). Sample tested included fish brain with the frontal brain, lips visual midbrain, cerebellum and medulla oblongata. These were divided into two parts and one part was placed in 70% alcohol for subsequent molecular testing with PCR. Additionally, one of the eyes was used to perform the above experiments. The same analysis was performed on sturgeon broodstock sperm and eggs by the International Sturgeon Research Institute (Rasht). This molecular diagnosis method consisted of five steps as follows: 1) sample preparation and RNA extraction; 2) RT-PCR; 3) Nested RT-PCR; 4) electrophoresis of the PCR products and 5) diagnosis (IQ 2000, guideline manual).

2.8. Electron microscopy

Samples under study were examined by transmission electron microscopy (TEM) (Hitachi Model HT7700, Japan) employing negative and positive staining following standard prescribed protocols for observing virus specific changes.

2.9. Bacteriology and parasitology studies

Skin, intestine, brain and kidney samples from 23 fishes were examined for bacteriology studies. Part of the brain samples were inoculated aseptically to brain heart infusion agar (BHIA) plates and kidney samples were cultured on blood agar. All the cultured plates and brain samples were incubated aerobically for 24–48 h at 20–24 °C. If no growth occurred at 24 and 48 h, the result was recorded, while if no growth occurred after 96 h, the samples were discarded. Furthermore, 50 samples consisting of gills, skin, fins, intestine and the swim bladder, were analysed for parasitological examinations

2.10. Pathogenicity test (challenge studies)

Pathogen (virus) isolated in the previous steps was used to infect healthy fish (challenge study). Guppy (*Poecilia retriculata*) and sturgeon fingerlings (*Acipenser persicus*) were selected as experimental hosts. Two basic methods were employed for the aforementioned challenges: the bath challenge (BC) and the injection challenge (IC).

Additionally, 10^{-4} $TCID_{50}$ dose of virus was selected for challenging, and at 15 days post infection (dpi), the guppies were bathed with VNN-infected tissue culture. Furthermore, the same supernatant was injected into 80 sturgeon fry weighing 5–10 g through intravitreous injection (Thiéry et al., 1997).

Paraffin blocks of samples for complimentary testing were sent to the OIE reference laboratory in Italy, to be confirmed by IHC, real-time PCR and nested PCR tests.

2.11. Statistical analysis

All the tests were performed in triplicate and analysed using SPSS software version no. 18 by one way analysis of variance (ANOVA) (SPSS Inc., Chicago, IL, USA)., followed by Duncan's multiple range tests. P-value of <0.05 was considered significant.

3. Results

3.1. Clinical signs and macroscopic observations

Fishes examined during this disease investigation, showed imbalance in swimming, skin darkening, and abdominal distension. Autopsy revealed no abnormality in Guilan samples, except for the inflation of the swim bladder (Fig. 1), while the samples from Mazandaran and Golestan showed different symptoms.

Generally, two groups of clinical signs were found in the above mentioned provinces. In the first group, abdominal distension, swimbladder inflation, lethargy, bilateral exophthalmia, bleeding at fin base, pale kidney were the main clinical symptoms (Fig. 1). Extremely severe muscle atrophy; a very large head and a very thin body characterized the second group. The gastrointestinal tract was completely empty of food and the body surface was scabious. Moribund fishes showed spinal cord diversion, corneal opacity and cataracts. The group was languishing or floating in the water (Fig. 2).

In the first sampling at Golestan, the fish showed no illness, except for certain cases with hyperemia or haemorrhage at the base of the abdominal fins. None of them showed abdominal distension. Certain consisted of some pathognomonic signs of VNN such as abdominal distention or belly up orientation in affected adult fishes were seen.

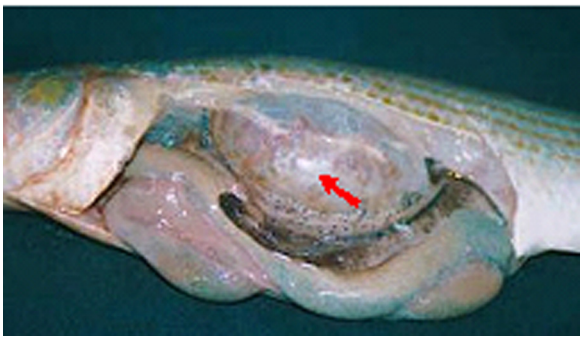

Fig. 1. Swimbladder inflation in *Liza aurata.*

Fig. 2. Naturally infected *Liza aurata* shows lethargy and emaciated body.

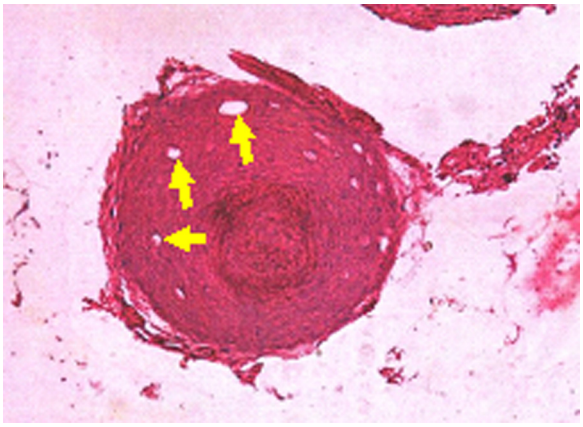

Fig. 3. Vacuolation in optic nerve of *Liza aurata* (cross section, 50×; H&E).

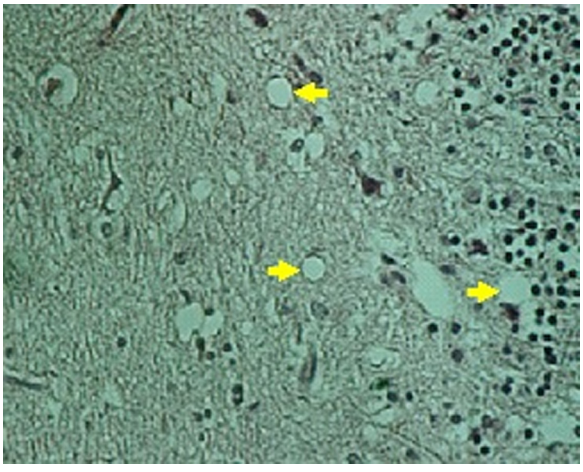

Fig. 4. Vacuolation in grey matter of brain in challenged sturgeon fry (20×; H&E).

Table 3
The comparison of blood parameters (M ± SD) between caught mullet in winter of 2003 and summer & winter of 2007 in the Caspian Sea.

Blood Parameters	Sample				
	Health Mullet fish (Caught in 2003 winter)	Diseased Mullet fish (Caught in 2003 winter)	Suspected Mullet fish (Caught in 2007 summer)	Diseased Mullet fish (Caught in 2007 winter)	Health Mullet fish (Caught in 2003 winter)
Total Count of RBC ($N \times 10^6$)	4.06 ± 0.22^{b}	2.98 ± 0.24^{a}	2.6 ± 0.1^{a}	3.3 ± 0.2^{ab}	4.3 ± 0.1^{b}
Hemoglobin (Hb)	11 ± 0.5^{c}	9.1 ± 0.1^{b}	7 ± 0.2^{a}	10.5 ± 0.6^{b}	13.5 ± 0.5^{d}
Hematocrit (%)	45.5 ± 1.3	33.1 ± 2.8	24.3 ± 0.7	34.5 ± 1.7	43.6 ± 1.5
MCH (pg)	27.9 ± 1.1^{a}	30.5 ± 0.2^{b}	26 ± 0.9^{a}	31.8 ± 0.4^{b}	35.2 ± 0.06^{c}
MCV (fl)	115.2 ± 5.2^{c}	111 ± 0.4^{c}	92.6 ± 2.9^{b}	85.1 ± 5.2^{b}	59.7 ± 6.6^{a}
MCHC	24 ± 0.5^{a}	27.2 ± 1.1^{b}	28.9 ± 0.9^{b}	29.8 ± 0.2^{b}	30.8 ± 0.8^{c}
Total Count of WBC	8033.3 ± 459^{a}	19357 ± 216^{d}	15177.4 ± 651^{c}	9837.5 ± 534^{a}	11933.3 ± 515^{b}
lymphocytes	65.7 ± 2.3^{a}	60.5 ± 2.1^{a}	83.3 ± 1.7^{b}	61.6 ± 2.5^{a}	64.5 ± 1.7^{a}
Neutrophils	28.4 ± 1.7^{b}	29.3 ± 1.7^{b}	10.9 ± 1.4^{a}	26.3 ± 1.7^{b}	28.6 ± 2.1^{b}
Monocytes	0.6 ± 0.1^{a}	0.9 ± 0.2^{a}	0.7 ± 0.1^{a}	0.8 ± 0.2^{a}	0.4 ± 0.2^{a}
Myelocytes	5.5 ± 0.9^{a}	9.3 ± 1.4^{b}	5.1 ± 1^{a}	11.3 ± 1.6^{b}	6.5 ± 0.7^{a}

Data are presented as mean ± S.D. Values in each row with different superscripts shows significant difference ($P < 0.05$).

3.2. Histopathology

Out of 960 samples, 490 samples of brain and eye revealed pathognomonic lesions such as widespread and massive vacuolation in gray matter of brain and spinal cord, optic nerve and retina (Fig. 3). Wild sturgeon's brain and retina did not show any sign of vacuolation, except in the challenged sturgeon fry pathogenicity test group (Fig. 4). Furthermore, no pathognomonic lesions were observed in other tissues such as the liver, kidney, intestine, stomach, gill, skin, muscle, gall bladder and gonads.

3.3. Haematology

Infected fishes during winter exhibited a significant decrease ($p < 0.05$) in RBC, Hb, PCV and MCHC (g/dl) as compared to healthy fishes. In contrast, MCV (fl) was significantly higher in infected fishes while MCH (pg) did not differ significantly between the two groups. Infected fishes showed a significant decrease ($p < 0.05$) in total IgM, protein and albumin compared to healthy fishes. Although C3 and C4 were lowered, yet were not significant. In contrast, AST and ALT enzymes were significantly increased ($p < 0.05$) in the infected fishes compared to the healthy fishes (Tables 3 and 4).

3.4. Virology

The brain and eye tissue extracts of the infected samples exhibited first CPE at 6 dpi in the SSN-1 cell line observed as round vacuoles in the cytoplasm in a limited area that gradually spread across the cell layer. The CPE rate increased in the next three passages (Fig. 5). The virus titration was calculated as 10^{-4} $TCID_{50}$ in each ml. Brain and eye extract of the wild sturgeon showed no CPE (Fig. 6).

3.5. Indirect fluorescent antibody test

IFAT test conducted on 170 tissue smears, bright fluorescent spots were observed in 21 samples of the brain and retina from moribund fishes (Fig. 7). Moreover, at 7 dpi viral antigens were observed in the SSN-1 cell line, inoculated with infected brain and eyes extracts. The golden grey mullet did not show the presence of viral antigens (Fig. 8).

3.6. Immunohistochemistry

Out of 170 samples of golden grey mullet, only 21 were found positive for IHC, with typical red-brown pigments on tissue sections (Fig. 9). The test was negative in all sturgeon samples tested.

3.7. Polymerase chain reaction (PCR)

Nucleic acid was extracted from the tissue samples of brain and eyes of fishes showing clinical signs or moribund and from the SSN-1 cell monolayers inoculated with tissue extracts from moribund fishes. Results indicated that *Betanodavirus* RNA was present in the brain, eye and also in the supernatant of the SSN-1 cell line infected with fish tissue extracts that revealed CPE (Fig. 6). Presence of 289 bp and 479 bp bands indicated medium VNN infection (P+) while bands at 289 bp and 665 bp indicated light VNN infection (P+). Negative cases revealed a single band at 665 bp N(−). PCR results from 56 mullet samples, processed at the Ecology Research Centre in Mazandaran and 42 wild sturgeon processed at the Sturgeon International Research Institute, revealed only a single band at 665 bp, (N(−)). The nested RT-PCR products using deproteinized nucleic acids extracted from eye and brain and CPE-positive cells revealed a band of 289 bp (Fig. 10). PCR

Table 4
The comparison of serum parameters (M ± SD) between caught mullet in summer & winter season of the 2007 in the Caspian Sea.

Serum Parameters	Samples		
	Suspected mullet fish (caught in 2007 summer)	Diseased mullet fish (caught in 2007 winter)	Health mullet fish (caught in 2007 winter)
Serum total protein (g/dL)	2.4 ± 0.1^{a}	3.4 ± 0.2^{b}	4.5 ± 0.2^{c}
Albumin (mg/L)	0.8 ± 0.1^{a}	2 ± 0.1^{b}	2.8 ± 0.2^{c}
Serum total IgM	54 ± 4.7^{a}	90.8 ± 5.4^{b}	112.4 ± 7.6^{c}
C3 (mg/L)	27.1 ± 2.3^{a}	22.6 ± 1^{a}	24.2 ± 1.4^{a}
C4 (mg/L)	5.6 ± 1^{a}	14.8 ± 1.7^{b}	17.5 ± 3.1^{b}
AST(U/L)	NA*	193 ± 20.3^{b}	69.5 ± 8.1^{a}
ALT(U/L)	NA	16.1 ± 2^{b}	3.7 ± 0.3^{a}

Data are presented as mean ± S.D. Values in each row with different superscripts shows significant difference ($P < 0.05$).
* Not available.

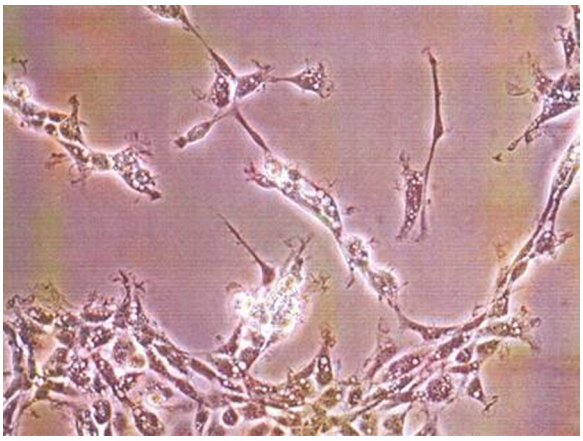

Fig. 5. Infected SSN-1 inoculated with retina filtrate showing vacuolation at 3–4 dpi.

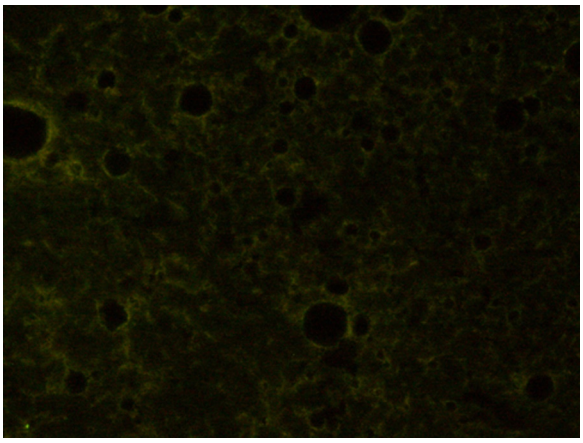

Fig. 8. IFAT negative results in wild sturgeon (IFAT, ×100).

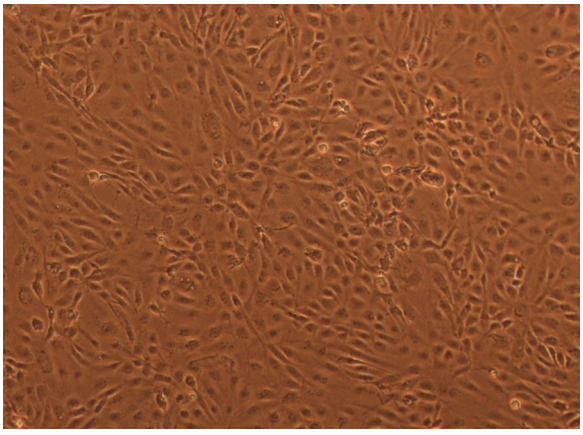

Fig. 6. SSN-1 cell line inoculated with retina and brain filtrate that not showing CPE.

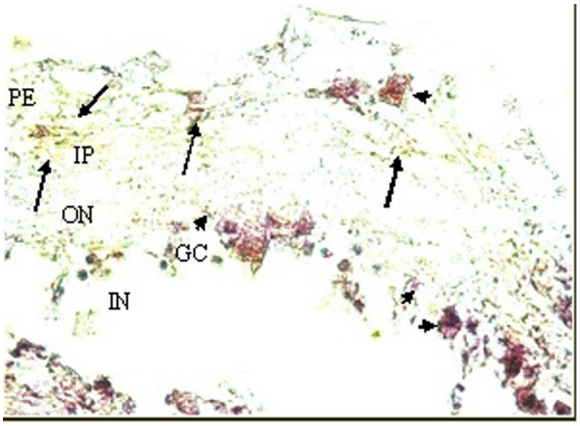

Fig. 9. Positive reactions in IHC against VNN in retina of infected *Liza aurata*. Immunolabeling (large arrows) in the PE: pigment epithelium, ON: outer nuclear layer, IN: inner nuclear layer, IP: inner plexiform layer, GC: ganglion cell layer. Basophilic cells (small arrows). Mag, 100×.

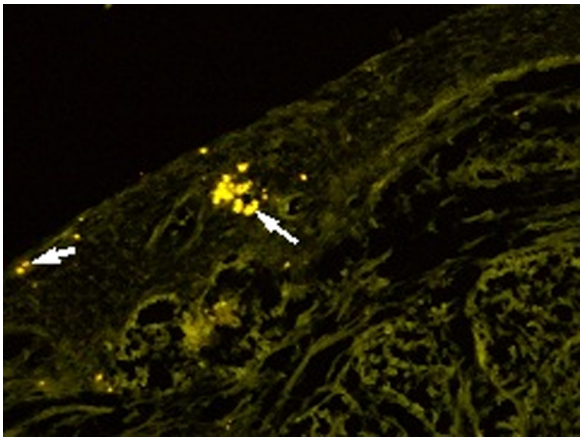

Fig. 7. IFAT showing fluorescent nodavirus particles (arrows) in the retina of affected *Liza aurata* (IFAT, ×100).

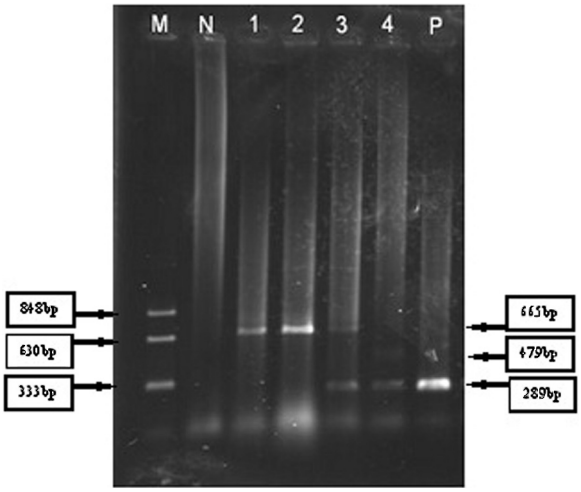

Fig. 10. Agarose gel electrophoresis of the Nested-RT-PCR products samples using the IQ2000™ VNN detection kit; Lane M = molecular weight marker, N = negative control (no template), P = positive control, 1 and 2 = non infected SSN-1. Cells show 665 bp, 3 = Infected SSN-1 Cells show 289 bp and 665 bp, 4 = infected SSN-1 cells show 289 bp and 479 bp products.

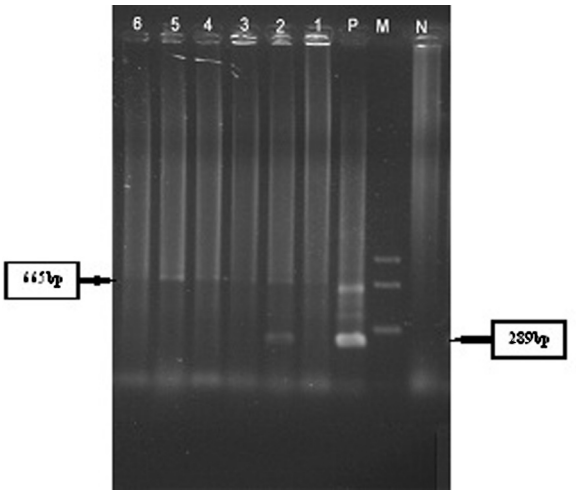

Fig. 11. Agarose gel electrophoresis of a nested RT-PCR product showing 289 bp for the amplicon of the test sample. Lane M = molecular weight marker; N = negative control (no template); P = positive control; Lanes 1, 3 and 5 = brain samples; Lanes 2, 4 and 6 = eye samples. The template cDNA was prepared from naturally infected fish.

products of few eye and brain samples presented two bands of 289 and 479 bp, which indicated medium VNN infection (P+) (Fig. 11).

3.8. Electron microscopy

Supernatant from the infected SSN-1 cells showed icosahedral non-enveloped particles of 30 nm in diameter that resembled the morphology of the betanodaviruses responsible for VNN (Fig. 12).

Intracytoplasmic viral-like particles within vacuolated walls in very thin sections of approximately 30–40 nm in retina were seen (Fig. 13).

3.9. Bacteriology and parasitology survey

The 27 specimens with clinical signs that were analysed to determine the presence of bacteria and/or parasites yielded negative results.

3.10. Pathogenicity test

In Table 5, the pathogenicity results are summarized. Cumulative mortality curves of the challenged trial on *Poecilia retriculata* is

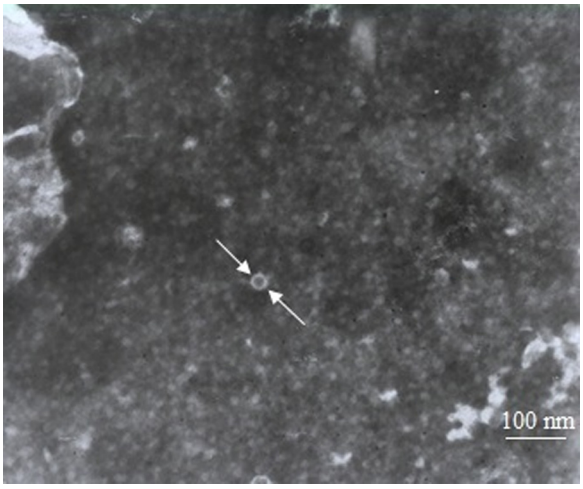

Fig. 12. Icosahedra-shaped particle was similar to VNN virus in specimen from Gilan province. PTA staining. Bar = 100 nm, Mag, 48,000×.

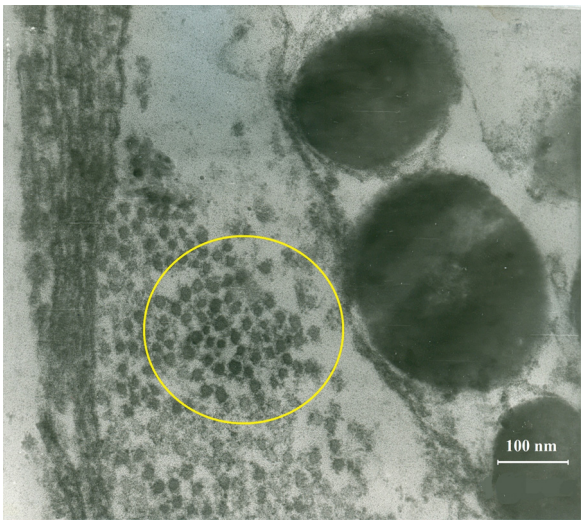

Fig. 13. TEM micrograph of *Liza aurata* eye. In the circle are showed intracytoplasmic viral-like particles in outer nuclear layer. Uranil acetate staining. Mag, 54000×.

shown in Fig. 14. First moribund fishes were from IC that appeared between 15 and 16 dpi, whereas from BC, moribund fishes appeared from 17 dpi. Infected fishes exhibited abnormal swimming behaviour such as rotation on a long axis, swimming up and down, and reached the surface with a curved body and the belly turned up at rest. Fishes showed abnormal body posture, enlarged abdomen, bilateral exophthalmia, Haemorrhagic petechiae on the skin were also observed. No clinical signs were observed in the control group.

The cumulative mortality, in the BC and IC, rose to 100% during the 30 dpi (Fig. 14) with significant value in BC and IC rising from day 17 and 15 onward, respectively. The time from infection to 50% cumulative mortality (LT_{50}) for BC and IC was 24 and 20 dpi, respectively. No mortality was observed in the control after 30 dpi.

The first clinical signs were observed in the fishes at 15 d after the BC (Figs. 15 and 16). Pathological study clearly revealed some severe and obvious vacuolation in the granular layer of the brain and retina. Also CPEs were observed in the infected SSN-1 cells inoculated with the brain and retina filtrate of the guppy (Fig. 17), and IFAT and IHC gave positive results.

Electron microscopy showed the virus components in the retinal cells at 20 dpi and the infected cells showed intracytoplasmic vacuoles (Figs. 18 and 19).

3.10.1. Sturgeon fry

Sturgeon fry showed abdominal distension and imbalanced swimming after 7 dpi, but mortality and death began on 21 dpi (Fig. 20). Histological sections of brain and eye showed severe vacuolation that continued in the cerebellum, medulla oblongata and spinal cord in some cases (Fig. 21). Results were negative except for histopathological studies.

4. Discussion

The present study was carried to investigate the cause of severe mortality in wild mullet, golden grey mullet, (*Liza aurata*) and the leaping mullet (*Liza saliens*) in the Iranian waters of the Caspian Sea.

Wild moribund mullets with observable clinical signs were collected for disease investigation. Collected fishes exhibited abnormal swimming, abdominal swollen, belly up at rest as well as lethargy, bilateral exophthalmia and haemorrhages in the skin.

Table 5
Summary of pathogenicity findings in *Poecilia retriculata* and *Acipenser persicus.*

Speices	Kind of challenge	Time of challenge (hours)	Virus titration	Clinical signs	Observation of first moribund fish (dpi)	Rate of mortality (%)
Poecilia retriculata	BC	2	10^{-4} $TCID_{50}$	Moderate	17	100 (during 30 dpi)
		4	10^{-4} $TCID_{50}$	Severe	15–16	100 (during 30 dpi)
		Control	Negative	Negative	Negative	Negative
Acipenser persicus	IC		10^{-4} $TCID_{50}$	More severe	7	100 (during 30 dpi)

BC = Bath challenge; IC = Injection challenge.

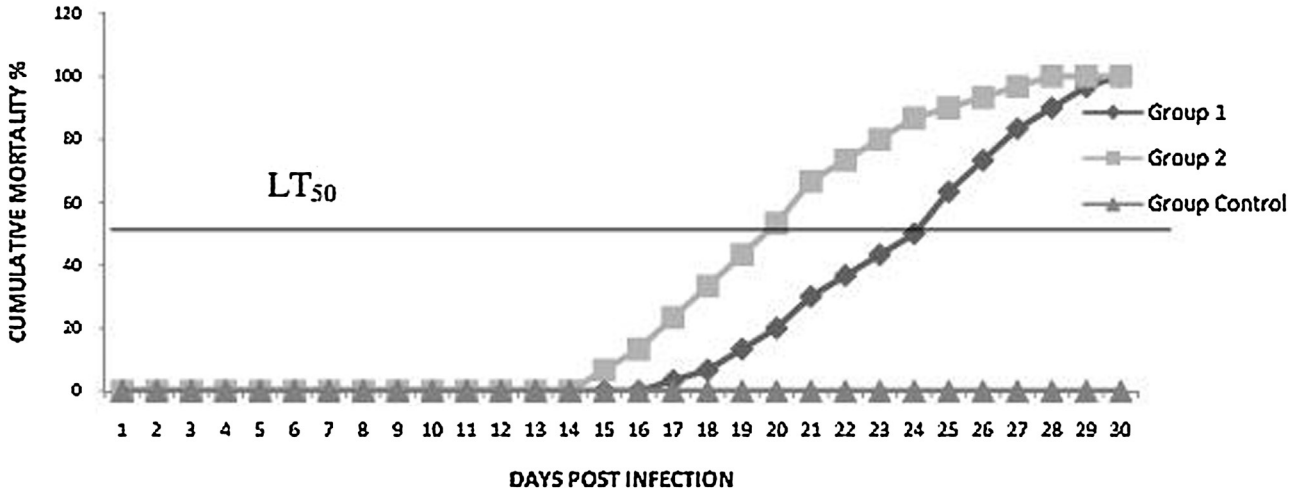

Fig. 14. Cumulative mortality curves of experimental groups of challenged trial on *Poecilia retriculata.*

L. aurata and *L. saliens* displayed abnormal swimming behaviour which were characteristic of the VNN disease as reported by several workers (Yoshikoshi and Inoue, 1990; Glazebrook et al., 1990; Grotmol et al., 1995; Boonyaratpalin et al., 1996).

The marked clinical signs in mullets were swim bladder distention. Swim bladder hyperinflation also had been reported in barramundi, European sea bass and grey mullet infected with VNNV (Zorriehzahra et al., 2005).

Gross clinical signs, i.e., lethargy and poor body condition, gallbladder distention, exophthalmia, swollen intestine, white spots on the surface of the intestine and hyperemia in the abdomen and lips were other clinical signs observed in the naturally infected grey mullets, as reported earlier (Munday and Nakai, 1997). Moreover, observations of exophthalmia, abdominal distention and haemorrhage in the skin indicated the presence of VNN viral infection in fishes (Roberts, 2001).

The same clinical signs could result from bacterial infections such as *Streptococcus* sp. (Gram positive), *Eubacterium tarantellus* and *Vibrio* sp. (Gram negative) of bacteria, and also from Pollutants such as plant toxins, heavy metals, and hydrocarbon from oil (OIE, 2006).

Furthermore, no parasitic infection was diagnosed in the sampled fish (Zorriehzahra et al., 2010). So, bacterial or parasitical agents could not infer to be the causative agents for the high mortality observed in the infected wild mullets in the Caspian Sea. The sections from the target tissues (n = 490) from the 21 wild mullet exhibited severe necrotic lesions and widespread

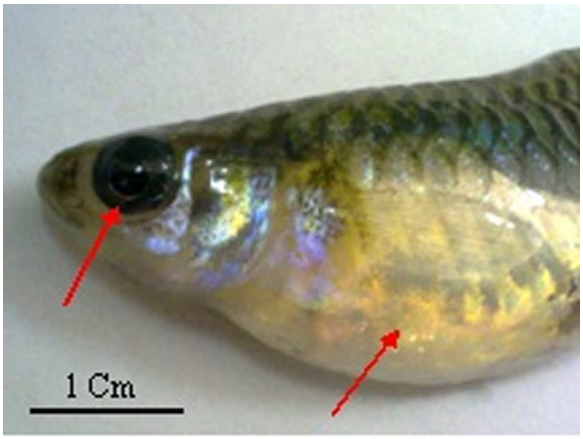

Fig. 15. Exophthalmia (white arrow) and swollen abdomen (red arrows) in affected *Poecilia retriculata.* (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

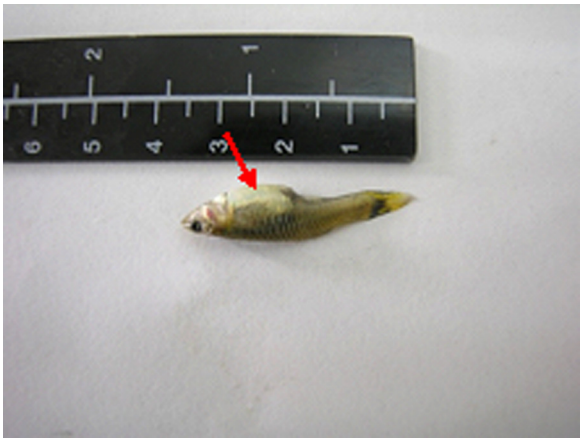

Fig. 16. Swollen abdomen in affected *Poecilia retriculata* (Arrow key).

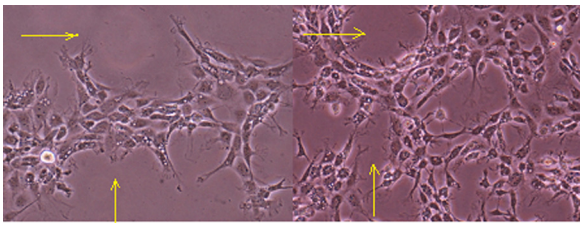

Fig. 17. CPEs in infected SSN-1 cells inoculated with brain and retina filtrate of *Poecilia retriculata* (Arrow keys).

intracytoplasmic vacuoles in the brain, optic nerve and nuclear layer of the retina. These histopathological findings are commonly reported lesions from other VNN mortalities worldwide (OIE, 2006; Maltese and Bovo, 2007; Hassan et al., 2008).

Histopathological findings in the brain of naturally infected grey mullet using light microscopy indicated vacuolation in the granular layer of the medulla oblongata. These results were similar to findings observed in the different species infected by VNN from earlier studies; the optic tectum was rarely affected in the diseased adult European sea bass (Le Breton et al., 1997). Lesions have also been described in the spinal ganglia in Japanese parrotfish (Yoshikoshi and Inoue, 1990).

In the retina, marked vacuolation was observed in the outer nuclear layer, inner nuclear layer and ganglion cell layer. Additionally, the presence of vacuoles and necrotized cells in the inner nuclear layer and ganglion cell layer were reported earlier (Tanaka et al., 2004). According to the mentioned findings, nerve cells in the olfactory lobe were most extensively necrotized with vacuolation, followed by infiltration of microglia and macrophages. Additionally, Purkinje cells and Golgi cells were extensively infected in the cerebellum. Megalo cells and small nerve-cell nuclei were also infected in the preoptic area, thalamus, medulla oblongata and spinal cord. Only a few small nerve cells were infected in the olfactory bulb and optic tectum. The retina of some diseased fishes displayed vacuolated bipolar cells in the inner nuclear layer and in the ganglion cell layer. These cells, nerve-infected by SJNNV, which is one of the four serotypes of the VNN virus, displayed viroplasmic inclusions that contained virions, vacuoles and myelin-like structures. In some severe cases, vacuolations were widespread in the spinal cord in the affected fishes (Nazari et al., 2011; Zorriehzahra et al., 2014).

The most remarkable clinical sign that was observed in the infected grey mullet was abnormality in swimming behaviour,

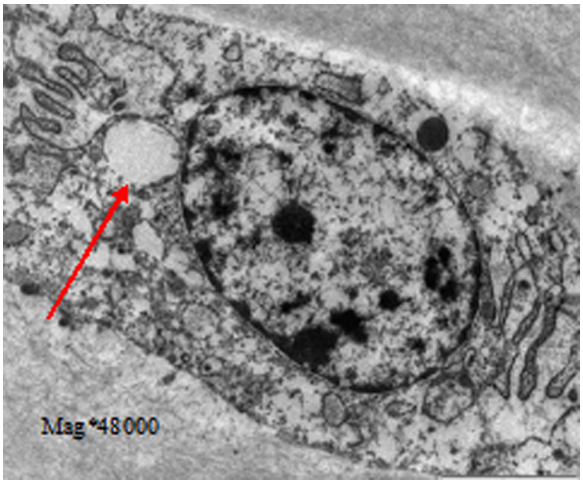

Fig. 18. TEM micrograph of retina cells at 20 dpi. The Infected cells showed intracytoplasmic vacuoles (Arrow key). Bar = 2 μm.

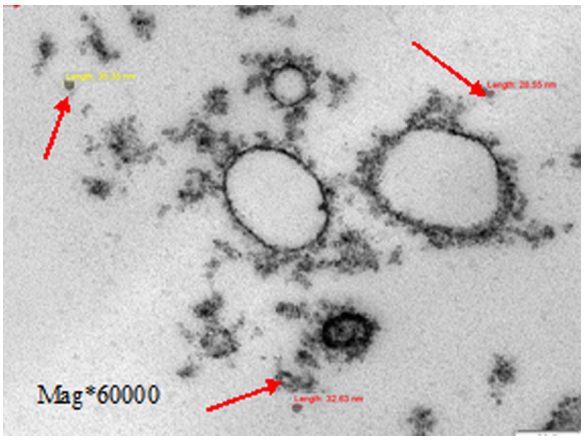

Fig. 19. TEM micrograph of retina cells at 20 dpi. The Infected cells showed intracytoplasmic 25–30 nm viral particles (Arrow keys).

characterized by darting, belly-up resting, swim bladder hyperinflation and exophthalmia. Fish buoyancy is controlled by the increase and decrease of air pressure in the swimbladder. The vacuoles and necrotized cells in the control centre of the swimbladder could result in abnormal or low swimbladder function. The medulla oblongata is the responsible part of the brain for control of the swimbladder function (Roberts, 1978). The presence of this vacuolation in the medulla can block this centre so that it is no longer able to send the neural impulses to the swimbladder. Thus, infected fishes continuously show abnormal swimming due to irregular air pressure in the swimbladder.

Abnormal swimming behaviour was one of typical clinical signs in affected mullet in the Caspian Sea. It could relate to the exophthalmia and retina lesions observed in diseased fishes. Exophthalmia could be due to inactive tissue fluid that leaked from capillaries into the eye orbit and body cavity or irregular oxygen secretion to the eye orbit from a network of capillaries in the choroid gland (Roberts, 1978; Hibiya, 1982). These abnormalities together with irregular air pressure in the swim bladder influences movement patterns changes.

Additionally, hyperaemia observed in naturally infected fishes could be due to the inflammatory factors of VNN. Similar congestion was observed in *L. klunzingeri* in the Persian Gulf and Oman Sea. The affected fishes showed haemorrhaging in the lateral and abdominal cavities. Additionally, haemorrhaging was observed in the operculum and thoracic and caudal fins (Koohkan et al., 2012).

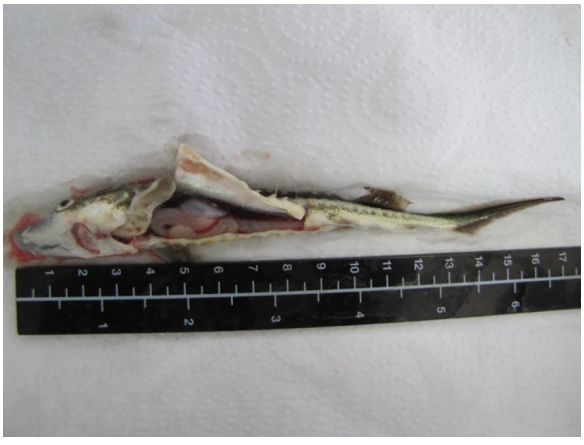

Fig. 20. Necropsy of affected sturgeon (*Acipenser persicus*) after death.

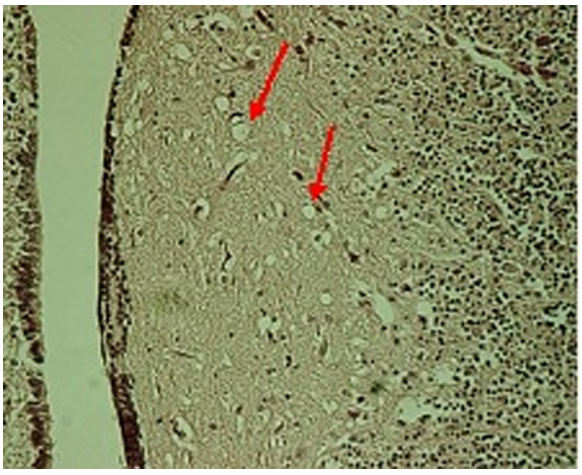

Fig. 21. Widespread vacuolation in brain and spinal cord of *Acipenser persicus* (Arrow keys).

Furthermore, in this study, the SSN-1 cell line, which is the specific monolayer for identification of VNN, was inoculated with sterile filtrate of the brain and eye. Marked specific CPEs were visualized after two passages. This method, recognized as the gold standard for characterizing viral agents, herein highlights for the first time the presence of VNN in the Caspian region (OIE, 2013).

Furthermore, the EM observations of negative staining of infected SSN-1 cells showed an intracytoplasmic icosahedral-shaped viral particle measuring 25–30 nm in diameter, which is similar to the *Betanovirus* described by other researchers. Indeed, OIE described VNN as non-enveloped and icosahedral, both intra- and extracellular and varying in size from 22 to 34 nm.

Moreover, the virus particles visualized by TEM in infected brain and retina using positive staining concludes that the infected tissue may contain viral particles similar to VNN (Munday and Nakai, 1997).

Additionally, RT-PCR testing showed that the brain and eye of 21 naturally infected wild mullets from the Guilan coastline were positive for VNN infection.

IFAT was performed on slides with suspicious tissues, which consisted of brain and eye tissues with severe vacuolation, and on slides with control tissue and smears from the supernatant of infected cells from culture and the control group. All infected tissues revealed positive results. According to the IFAT results, all the IHC examinations, performed on 170 slides from infected brain and eye tissues revealed positive results compared to the 10 control slides. These results were similar to previous results reported; Iwamoto et al. (2000).

Furthermore, in an experimental trial, guppy fish and sturgeon fry, which are more sensitive and easier to handle, were used instead of the natural hosts, who could not adapt to captive conditions and quickly died. Thus, after 15 dpi, guppy bathed in VNN-infected tissue culture with 10^{-4} $TCID_{50}$ showed clinical signs similar to naturally infected fishes, and the mortality rate reached 100% in 30 dpi. When target organs were examined by cell culture isolation, the IFAT, IHC and histopathology revealed the presence of virus in the guppy tissues. Meanwhile, symptoms such as abdominal distension, imbalanced swimming and death were observed 21 dpi in the virus to the sturgeon treatment groups. This experimental trial used Koch's postulates to confirm the obtained causative agent.

The haematology findings revealed that the fishes examined showed a decrease in MCHC and an increase in MCV, which characterises macrocytic hyperchromic anaemia. These results were similar to previous reported results; Binaii et al. (2010). Additionally, the infected fishes suffered from severe protein catabolism or dystrophy. The elevated levels of AST and ALP in the current study may be related to the histopathological change and hepatocyte damage in infected fish. Similar results were reported in infected fish to VNN disease (Binaii et al., 2010).

Therefore, according to clinical signs, haematological findings and biochemical studies, a type of infectious dystrophic and chronic disease can be concluded to have occurred in wild mullets in recent years in the Iranian southern Caspian Sea, with a new feature of VNN disease being revealed for fish in the Caspian region.

In conclusion, based on the results obtained in this study from pathogenic and clinical signs; histopathological, virological and bacteriological results; molecular analysis; TEM; IHC; IFAT and haematological findings in naturally-infected wild mullets and experimentally infected guppy and sturgeon, VNN virus should be considered as the main causative agent for the disease outbreak in wild mullet along the southern coastline of the Caspian Sea.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2016.04.023.

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