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Isolation and identification of *Betanodavirus* causing mass mortalities in golden grey mullet (*Liza auratus*) in the Caspian Sea

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Key Words:
Viral nervous necrosis; *Liza auratus*; Caspian Sea; nodavirus.

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Abstract

Outbreaks causing mass mortalities have been observed in the golden grey mullet (*Liza auratus*) population in southern coastal area of the Caspian Sea since 2002. The clinical signs include neurological abnormalities, such as erratic swimming behavior, spiral swimming, belly-up at rest and over inflation of the swim bladder. Three hundred and twelve samples of moribund fish with sizes that ranged from 15-26 cm showing abdominal enlargement were collected from 2006-2008. No pathogenic bacteria and parasite were found. The observed histopathological changes in 29 fish were necrosis and vacuolation of the brain, optic nerve and retina. Cytopathological effects of nodavirus characterized by vacuolation were observed in the SSN-1 cell line at 25ºC, 5 d after inoculation with the filtered supernatant of the brain and eye of four affected fish. The recovered virus from cell line exhibited 10^4 $TCID_{50}$ per mL when titrated. Indirect immunoflorescent antibody test showed nodavirus antigens in the retina and positive-CPE SSN-1 cells. Also, a RT-PCR product of approximately 289 bp was amplified from the brain and retina of the 23 samples, including the SSN-1 positive samples. This is the first report of the isolation and characterization of nodavirus from golden grey mullet from the Caspian Sea and, therefore, possible infections in other valuable species of the Caspian Sea warrant further studies.

Introduction

Viral nervous necrosis (VNN) causes viral encephalopathy and retinopathy (VER) in a variety of cultured and wild marine fish, including sea bass (*Lates calcalifer*), flounder (*Paralichthys olivaceus*), sea bream *(Sparus aurata),* turbot *(Scophthalmus maximus),* European sea bass *(Dicentrarcus labrax),* Atlantic halibut *(Hippoglossus hippoglossus),* groupers *(Epinephelus adara, E. fuscogutatus, E. malabaricus, E. moara, E. tauvina, E. coioides), jack (Pseudocaranx dentex), parrotfish (Oplegnathus fasciatus)*, flatfish (*Verasper moseri)* and sturgeon sp. in different parts of world (Munday *et al*., 2002; Athanassopoulon *et al*., 2004; OIE, 2006). Affected fish show a variety of neurological abnormalities, such as erratic swimming behavior, spiral swimming, whirling and belly-up at rest, resulting in severe morbidity and mortality (OIE, 2003). The most common histopathological changes observed are necrosis and vacuolation of the brain and retina (Mori *et al*., 1992; Comps *et al*., 1994; Chi *et al*., 1997, 2003). The causative agent of VNN disease is a non-enveloped bisegmented single-stranded positive sense RNA nodavirus with size from 25-30 nm (Mori *et al*., 1992). Golden grey mullet (*Liza auratus*) is one of the valuable commercial species in the Caspian Sea having about 50% of annual catching rate of bony fish in the Caspian Sea. This species has been faced with severe disease outbreaks causing high morbidity and mortality in the Caspian Sea since several years ago but no etiological agent has been reported so far (Soltani and Rehannandeh, 2002). This paper describes the isolation and characterization of a nodavirus from wild-caught moribund golden grey mullet studied during 2006-2008. Therefore, this is the first report of the isolation of nodavirus from golden grey mullet in the Caspian Sea.

Materials and Methods

Fish samples

Sample collection

Samples were collected from 312 wild-caught

moribund golden grey mullets from coastal waters in the southern part of the Caspian Sea from 2006-2008. Samples of gills, skin, fins, intestine, swimbladder, kidney, liver, brain and eye were processed for parasitological and bacteriological analyses, as well as virus isolation, reverse transcriptase polymerase chain reaction (RT-PCR) analysis, histological and serological studies.

Parasitological and bacteriological examinations

The external and internal organs, including the gills, skin, fins, intestine and swim bladder, underwent parasitological examinations under a light compound microscope (Olympus). Also, samples of kidney and liver were aseptically cultured on tryptic soy agar that was supplemented with 1.5% NaCl and incubated at 26°C for 72 h.

Histopathological analysis

Samples of brain, spinal cord, eyes and swim bladder from moribund fish were fixed in 10% buffered formalin and were processed using routine histological procedures before being sectioned into 5 µm-thick slices prior to staining with hematoxylin and eosin (H&E).

Virus isolation

The samples of brain and eye tissues of moribund fish were homogenized in Eagle's minimum essential medium (EMEM) by grinding the tissues. The homogenates (10% w/v) were centrifuged at 4000 x g at 4°C for 15 min and the supernatants were filtered through a 0.45 µm filter. Virus isolation trials were performed by inoculation of the filtrates onto a confluent SSN-1 cell line monolayer (Frerichs *et al.*, 1991) in minimum essential medium with 10 % fetal bovine serum (FBS) and were incubated at 25°C for 10 days. If no CPE was observed, three blind passages were then undertaken after freezing the cells at -70°C and before thawing them.

Virus detection by RT-PCR

The total RNA from samples of brain, spinal cord, eye, swim bladder and the supernatant of SSN-1 cells inoculated with tissue filtrates were extracted using IQ2000™ RNA extraction solution (Farming Intelligene Tech. Corp., Taiwan). A reagent based upon a modification of the guanidium salt-phenol–chloroform method (Chomczynski and Sacchi, 1987) was used according to the instructions of the manufacturer instructions. Firstly, a volume of 500 µL RNA extraction solution containing phenol was added to 20 mg brain or eye sample. Samples were then ground and left at room temperature for 5 min. A 100 µl of chloroform was added, vortexed for 20 s and centrifuged for 15 min. Approximately 200 µL of the upper clear aqueous phase were transferred into new 0.5 mL tube containing 200 µL isopropanol. Samples were then centrifuged at 12,000 g for 10 min at 4°C, and the isopropanol was then decanted. The pellets were washed with 0.5 mL 75% ethanol and spun down at 7,500 g for 5 min at 4°C to recover the RNA pellet. The ethanol was decanted and the dried pellet was dissolved in 200 µL DEPC-treated water. RT- PCR and nested PCR were performed using IQ2000 ™ VNN detection kit (Farming Intelligene Tech. Corp., Taiwan). We added 8 µL of RT-PCR reaction mixture containing 7.0 µL RT-PCR pre-mix reagents, 0.5 µL IQzyme™ and 0.5 µL reverse transcriptase enzyme mixed with 2 µL of the extracted RNA sample or standard. The RT-PCR reaction was performed at 42ºC for 30 min, 94ºC for 2 min then continued for 15 cycles at 94ºC for 20 s, 62ºC for 20 s and 72ºC for 30 s using thermocycler (Biored). The final extension was at 72ºC for 30 s and the samples were kept at 20ºC for a further 30 s. For nested PCR reactions, a 15µL of nested PCR reaction mixture containing 14 µL nested PCR pre-mix and 1 µL IQzyme™ DNA polymerase was added to each tube containing RT-PCR product.

Indirect florescent antibody test (IFAT)

Serial tissue sections were deparaffinized and rehydrated to phosphate buffer saline (PBS) at pH 7.2. The sections were then treated with 0.1% trypsin in PBS at 37°C for 30 min in a humid chamber after washing with cold PBS and treated with a rabbit anti-nodavirus monoclonal antibody (Mab, Aquatic Diagnostics Ltd., Scotland) at 37°C for 30 min in a humid chamber. Samples were then rinsed four times with PBS-Tween 80 (PBST). The sections were then treated with a commercially available fluorescein isothiocyanate–conjugated anti-rabbit Ig antibody at 37°C for 30 min, rinsed with PBST, and were then examined under a fluorescent microscope. Additionally, the supernatant of brain and eye tissues were inoculated into the SSN-1 cell line in 96-well plates, which were fixed after 7 d with cold acetone, incubated with 2% Triton-X 100 for several minutes, washed with PBST (PBS containing 0.05% Tween 80), and then treated with the antibody as mentioned above.

Results

Macroscopic observations

During the fishing period, the affected fish with body weights that ranged from 80250 g exhibited prominent clinical signs, including lethargy, dark coloration of the skin, abnormal swimming behavior, belly-up positioning, and whirling movements. No gross lesions were observed in necropsy except for the enlargement of the swim bladder (Figure 1).

Parasitological and bacteriological studies

No pathogenic bacteria and parasites were observed in the affected fish.

Histological examination

Histological analysis revealed the presence of vacuolations in the brain, spinal cord, optic nerve and granular layer of the retina (Figures 2, 3, 4, and 5). Most of the examined fish had degenerative and vacuolated lesions in the retina. The extent of vacuolation of the retinal and brain tissue varied between fish. In the brain and spinal cord, vacuolation was mostly observed in the grey matter. In some cases, the optic nerve showed extensive vacuolation.

Virus isolation by cell culture

Six days after inoculation of tissue samples on the SSN-1 cell line at 25°C, a cytopathic effect (CPE) developed slowly in some areas of cell line. Rounded, granular and vacuolar cells developed, and this effect spread to the other areas of the cell sheet, but not to the entire sheet (Figures 6 and 7). More CPE were observed in the first and second passages (Figures 8 and 9).

Electron microscopy examination

Electron microscopy analysis revealed vacuolation and aggregation of some virus particles with a size that ranged from 25-30 nm in the cytoplasm of the affected nerve cells of the retinal layer (Figure 10).

RT-PCR analysis

Gel electrophoresis of the nested RT-PCR products of the deproteinized nucleic acids extracted from the naturally infected fish and the CPE positive cells revealed a band that was 289 bp in size in samples collected from the eye and brain tissues (Figure 11). Also, two bands with sizes of 289 and 479 bp were seen in the PCR products of some eye and brain samples (Figure 12).

IFAT

Using IFAT, a clear fluorescence was detected in the retina of some affected fish samples that were positive for nested RT-PCR amplicons and histopathological examinations (Figure 12). Also, viral proteins were initially detected in infected SSN-1 cells 7 d after inoculation (Figure 13).

Discussion

The annual catching rate of grey mullet from the Caspian Sea was above 6000 tm in 2002 and, it has decreased to below 3000 tm in 2008 (Department of Fish Stock Assessment Management, Iranian Fisheries Organization). This indicates that the level of mullet production has reduced by 50% (Figure 14). The present study describes the causative agent associated with the mass mortality of wild golden grey mullet, which is one of the most economically important bony fish species in the Caspian Sea. The most remarkable clinical signs observed in the affected fish were erratic swimming behavior, belly-up at rest, hyperinflation of the swim bladder and abdominal distention. Isolation of a nodavirus from the moribund fish was achieved using the SSN-1 cell line. The isolated virus was identified as a fish nodavirus based on the results of histopathological, IFAT, RT-PCR and electron microscopy examinations performed on both naturally infected brain and eye tissues, and the CPE of cell cultures. Histopathological investigations in the naturally infected fish revealed severe vacuolation, necrosis and degeneration of nerve cells in the retina and brain.

The clinical signs and histopathological changes, particularly lesions of vacuolation and necrosis in the brain, spinal cord and retina, were consistent with those that have been described in other VNN-affected fish species (Fukuda *et al*., 1996; Nakai *et al*., 1995; Munday *et al*., 1997; Munday, 2002). Remarkable histopathological signs were seen in retina and brain tissues of these affected fish species. Nopadon *et al*. (2009) recently described a range of histopathological signs caused by nodavirus infection, including hyperplasia and necrotic changes in the gas glandular epithelium of the swim bladder of affected spotted coral groupers (*Plectropomus maculates*). However, in this current study, we could not see any histopathological changes in the swimbladder of affected *Liza auratus.*

Extensive CPE characterized by vacuolation was observed in SSN-1 cell cultures 5 d after inoculation with filtered homogenates from the brain and eye of moribund fish. CPE was also seen in the further passages. CPE developed initially as some areas of rounded granular cells and slowly spread to other areas. Electron microscopy examination of the CPE-positive cell cultures revealed vacuolation and aggregation of some virus particles with a size of between 25-30 nm in the cytoplasm of nerve cells of the retinal layer. The size of viral particles detected in infected cells in this study were in agreement with those described for sea bass nodavirus cultured in SSN-1 cells (Frerichs *et al*., 1996) and Atlantic halibut (Dannevig *et al*., 2000). Recovered viral particles exhibited 4 log $TCID_{50}$ mL^{-1} when titrated in the SSN-1 cell line. In this study, the cultivation of *L. auratus* nodavirus at 20°C was not performed. However, Dannevig *et al*. (2000) were able to cultivate Atlantic halibut nodavirus in SSN-1 cells at 20°C. It is difficult to determine whether temperatures below 25°C are optimal for viral growth because the SSN-1 cells grow poorly at temperatures less than 20°C (Dannevig *et al*., 2000). However, natural infection of *L. auraus* occurred within the range of 11-18°C, and other researchers have also reported the isolation of nodavirus from other species at water temperature of 12.5-26°C (Frerichs *et al*., 1996; Thiery *et al*., 1999;

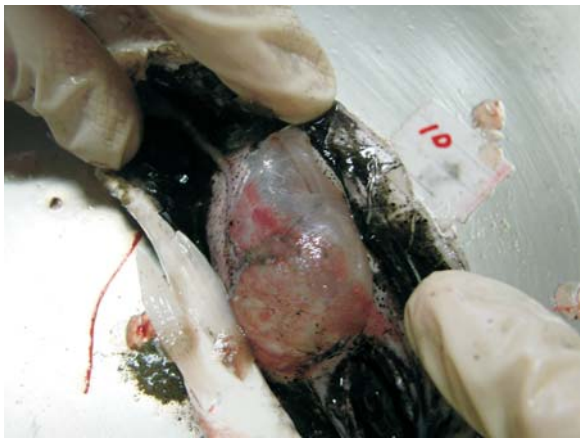


Figure 1: Swim bladder enlargement in grey mullet obtained from the Caspian Sea.

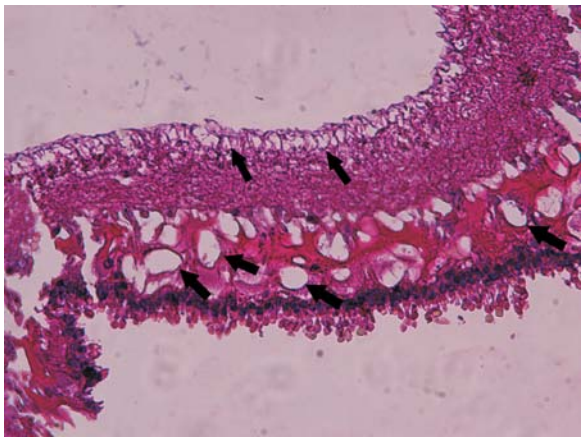


Figure 4: Section of eye tissue of the affected *L. auratus* showing vacuolation in retina (arrow; H&E, x40).

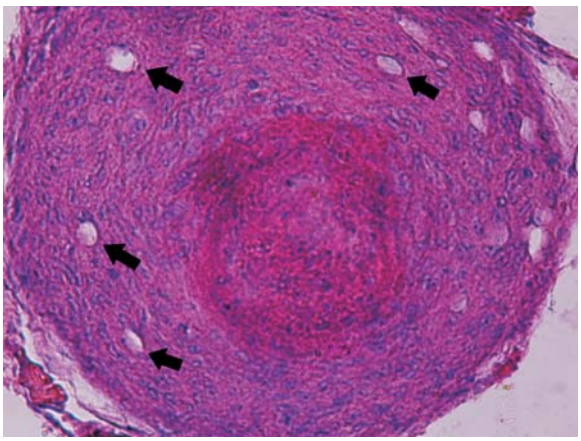


Figure 2: Section of spinal cord of the affected *L. auratus* showing vacuolation (arrow; H&E, x100).

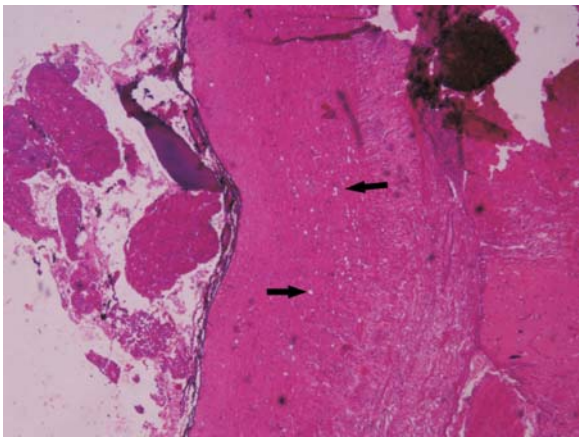


Figure 5: Lower magnification of brain tissue of the *L. auratus* showing vacuolation (arrow; H&E, x40).

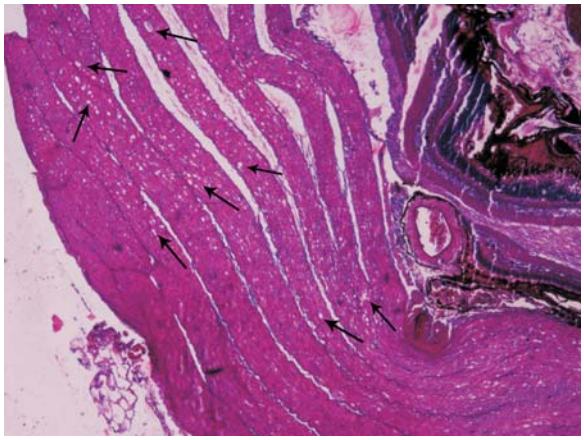


Figure 3: Lower magnification of optic nerve tissue of the *L. auratus* showing extensive vacuolation (arrow; H&E, x40)

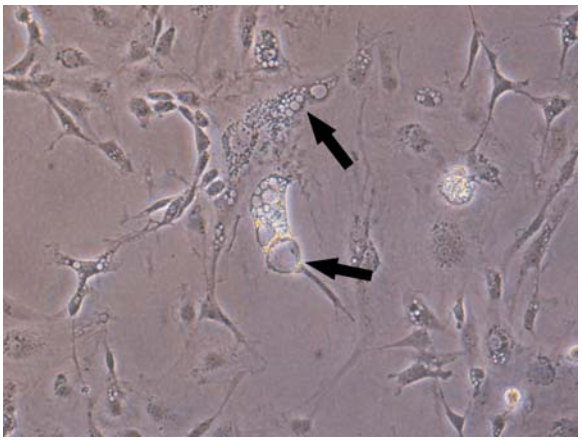


Figure 6: Vacuolation in the SSN1 cell line 6 d after inoculation of filtrated supernatant from the eye tissue of affected *L. auratus* (x200).

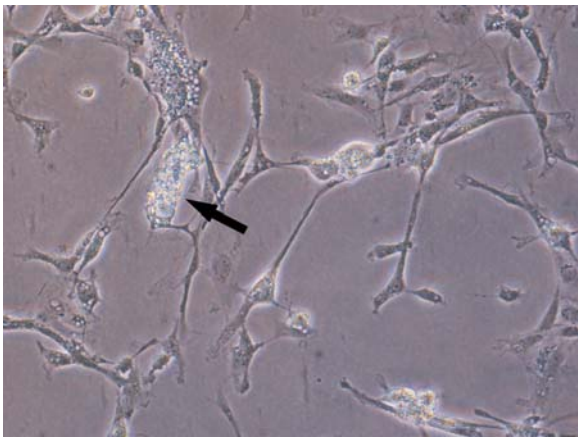


Figure 7: Vacuolation in the SSN1 cell line 7 d after inoculation of filtrated supernatant of eye tissue of affected *L. auratus* (x200).

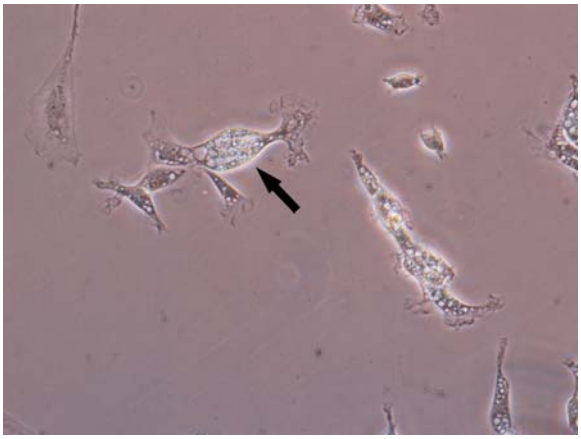


Figure 8: Vacuolation in the SSN1 cell line 5 d after the first subcultivation of filtrated supernatant of eye tissue from affected *L. auratus* (x200).

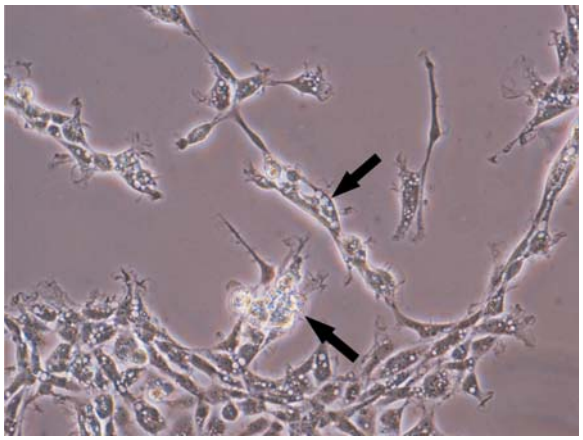


Figure 9: Vacuolation in the SSN1 cell line 5 d after the second subcultivation of filtrated supernatant of eye tissue from affected *L. auratus* (x200).

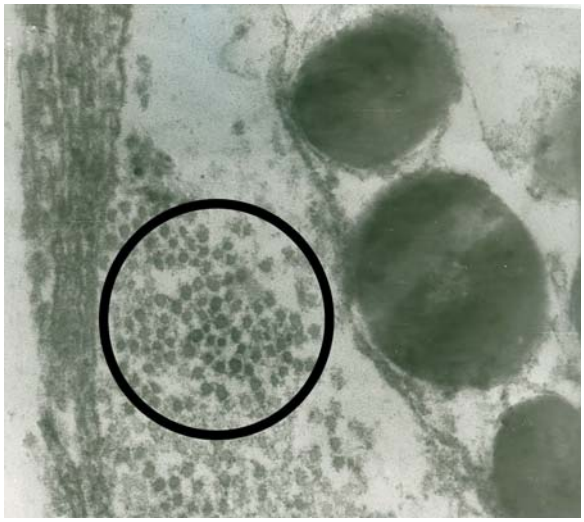


Figure 10: Transmission electron micrograph of the section of eye tissue of the affected *L. auratus* showing nodavirus particles (25-30 nm in size) in the retina (arrow; x 7,650).

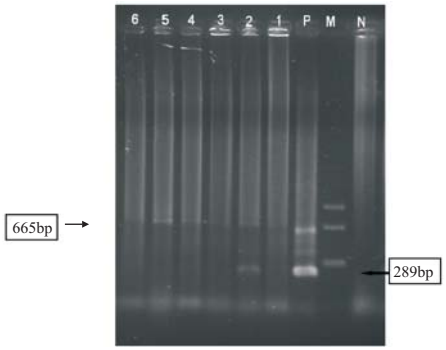


Figure 11: Agarose gel electrophoresis of a nested RT-PCR product showing 289 bp for the amplicon of the test sample. Lane M = molecular weight marker; N = negative control (no template); P = positive control; Lanes 1, 3 and 5 = brain samples; Lanes 2, 4 and 6 = eye samples. The template cDNA was prepared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

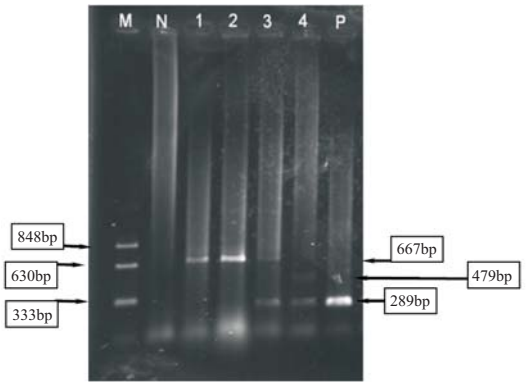
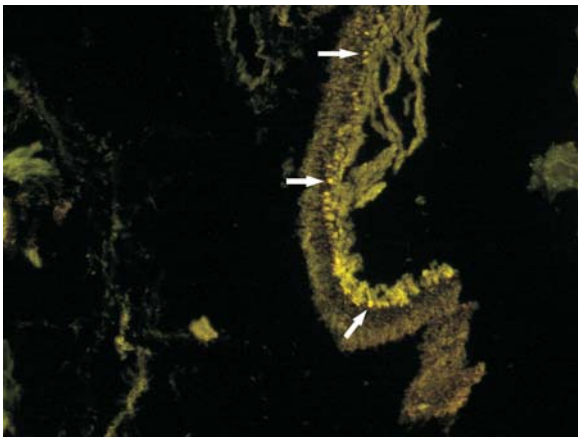


Figure 12: Agarose gel electrophoresis of the nested RT-PCR product. Lane M = molecular weight marker; N = negative control (no template); P = positive control; Lanes 1 and 2 = non-infected SSN-1 cells showing 665 bp products; Lane 3 = infected SSN-1 cells showing 289 bp and 665 bp products; Lane 4 = infected SSN-1 cells showing 289 bp and 479 bp products.

13a



13b

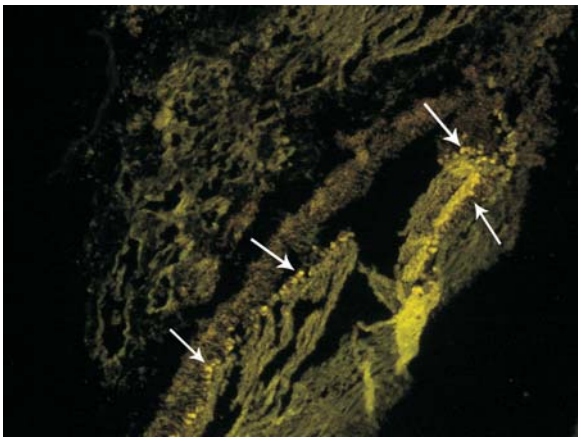


Figure 13: IFAT showing fluorescent nodavirus particles (arrow) in the retina of affected *L. auratus* (IFAT, x 40).

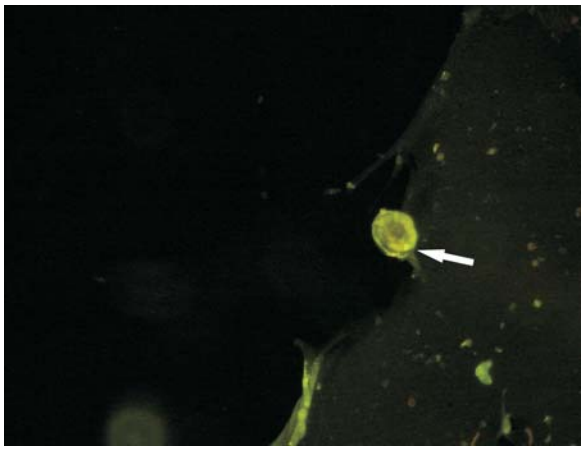
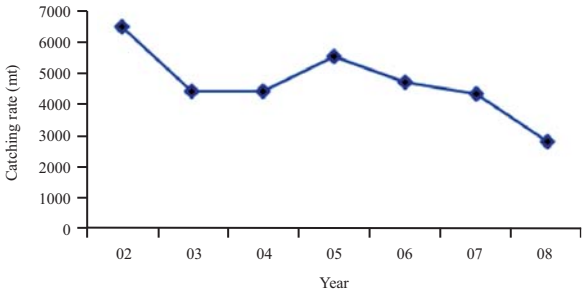


Figure 14. IFAT showing nodaviral antigens in the SSN1 cell line 6 d post-inoculation (x 400).

Figure 15: Annual catching rate of grey mullet from the Caspian Sea, (Department of Fish Stock Assessment Management, Iranian Fisheries Organization, 2009).



Dannevig *et al*., 2000). The water temperature of the Caspian Sea at the time of sample collection was in the range of 11-18°C, which indicates that the viral particles are able to infect the host at a wide range of water temperatures. In vitro studies also support evidence for viral resistance to temperature fluctuations; for example, in a study by Chi *et al*. (1999), the GNNV isolates of the virus were resistant to heating at 56°C, which caused CPE at 24-32°C.

In a study by Aspehaug *et al*. (1999), VNN was reported from some cold water species of fish. Therefore, the available data on the effect of temperature on nodavirus infections under in vitro and in vivo conditions may display some variations among different viral isolates in marine and freshwater environments (Furusava *et al*., 2006; Jithendran *et al*., 2006). The virulence level of nodavirus in *L. auratus* is not clear and requires further investigations. Also, it is not clear whether this nodavirus is able to affect other Caspian Sea species, particularly the highly valuable species of sturgeons. Further studies are in progress to demonstrate the virulence level of this isolated betanodavirus in some species of the Caspian Sea, including mullet and sturgeon.

In conclusion, our histopathological, virological and seromolecular findings confirm that VNNV is the cause of mass mortality in grey mullet in the Caspian Sea. However, more investigations are required to assess the virulence level of the isolated virus in this species of fish. Additionally, because of wide range of susceptible hosts, wide geographical distribution and high losses reported in other species, further investigations are required to study the epizootiology and genotyping of this isolated Betanodavirus in the Caspian Sea. Preliminary data from these studies show that both sturgeons and guppy are susceptible to the virus under *in vitro* conditions (data not shown).

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