

## **Mortality of Wild Golden Grey Mullet (*Liza auratus*) in Iranian Waters of the Caspian Sea, Associated with Viral Nervous Necrosis-Like Agent**

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**Abstract:** An acute mortality caused by an uncertain agent occurred in wild golden grey mullet (*Liza auratus*) population in the Iranian side of the Caspian Sea in February, 2004. Clinical signs of the moribund fish were erratic behavior such as spiral swimming, belly-up at rest and over inflation of swim bladder. Histological examination of brain of dead fish revealed hyperaemia, degeneration and necrosis in external granular layers as well as focal extension in intestine. Severe leucopenia was

also observed. No putative causal factors were suggested by bacteriological, parasitological or environmental examinations. In virological examinations, however brains of affected golden grey mullet were positive in the reverse transcriptase-polymerase chain reaction test for piscine nodavirus (Nodaviridae, *Betanodavirus*), the causative agent of Encephalopathy and retinopathy (VER), otherwise known as viral nervous necrosis (VNN) in a variety of cultured marine fishes worldwide. Nucleotide sequence of the PCR amplicons is closely related with the coat protein gene of piscine nodaviruses, particularly redspotted grouper nervous necrosis virus (RGNNV). Although, it was not succeeded to isolate the virus, experimental infection with the brain homogenates of the affected fish in sevenband grouper (*Epinephelus septemfasciatus*), which is highly susceptible to RGNNV, produced neuro- logical abnormality followed by mortality. These results suggest that the mortality observed in golden grey mullet in the Caspian Sea might have been associated with piscine nodavirus infection.

**Keywords:** Golden grey mullet, VER, VNN, Nodavirus PCR, Caspian Sea, Iran

## Introduction

An unknown acute mortality occurred in wild golden grey mullet (*Liza auratus*) of the Caspian Sea in the Guilan province, Iran, during February, 2004. First announcement of the mortality was reported from Ziba-Kenar region of Rasht city, when water temperature was 10.4-10.8°C, in fish size of 15-20cm. A similar mortality, with severe losses was previously observed in *Liza auratus* living in Babolsar region of Mazandaran province in February, 2002. The increase of mortality rate during recent years, could be affected by ecological changes in the Caspian Sea. The affected fish had no gross pathological sign on the body surface except a significant swelling of the abdomen. They were also characterized by erratic swimming behavior which is could be often associated to viral encephalopathy and retinopathy (VER).

Viral encephalopathy and retinopathy (VER), otherwise known as viral nervous necrosis (VNN), caused by piscine nodavirus (Nodaviridae), is a worldwide

occurring disease except for Africa (Office International des Epizootices, 2003) affecting many marine fish species in which serious mortalities may be observed mainly in larvae and juveniles (Munday *et al.*, 1992; Nakai *et al.*, 1995; Chi *et al.*, 1997; Breton *et al.*, 1997; Munday *et al.*, 1997). Viral nervous necrosis was first described by Yoshikoshi and Inoue (1990) in Japanese parrotfish (*Opelegnathus fasciatus*) in Japan. It has been described in 30 or more marine fish species belonging to 14 families in the Indo-Pacific, the Mediterranean, Scandinavia and North America (Munday *et al.*, 2002).

In order to determine the cause of the observed mortality in the golden grey mullets in the Caspian Sea, laboratory investigation including environmental surveys, microbiological, hematological, molecular biology and histopathological examinations were carried out.

## Material & Methods

Moribund fish collected for examinations weighing 200-250g were examined clinically and submitted to the following investigations:

**Hematology:** Blood samples were taken from heart of moribund (n=20) and apparently healthy fish (n=20) as control group by sterile syringe and then very slowly dropped in tubes containing anti-coagulant solution (heparin). Erythrocyte count (RBC), leukocyte count (WBC), hemoglobin (Hb), packed cell volume (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and leukocyte differential count were measured. Furthermore, serum glutamicoxaloacetic transaminase (SGOT) and liver enzymes, such as ALP, LDH, SGPT, were also examined

**Histopathology:** Samples from liver, kidney, intestine, stomach, gill, skin and muscle, gall bladder, gonads and brain were removed and fixed in 10% buffered formalin. After dehydration and clearing, samples were embedded into paraffin wax using automatic tissue processor. Paraffinated blocks were further processed in

order to prepare 5 micron sections which then were stained by haematoxylin and eosin (H&E) staining method and observed by light microscope.

**Parasitology:** Gills and body surface were examined for ectoparasites and blood smears were stained by Giemsa solution and screened for protozoans.

**Bacteriology:** External surface of moribund fish was disinfected with 70% alcohols and in aseptic approach fish necropsy was conducted. Liver, kidney, intestine, stomach, brain, gill, gall bladder, gonads, skin and muscle, were removed, and inoculated on Tryptone Soya Agar (TSA), and Brain Heart Infusion Agar (BHIA), supplemented with 2% NaCl. All cultured plates were incubated at 23°C and kept for 3 days.

**Virology:** Necropsy was done in aseptic condition on fish frozen at -20°C. Tissues such as liver, kidney, spleen, eyes, and brain, were removed, homogenized with PBS, and centrifuged at 2,000 rpm for 10 minutes. The supernatant was passed through 0.45 µm membrane filter and aliquots were sent to Hiroshima University (O.I.E. Reference Laboratory for "Viral Encephalopathy and Retinopathy" or "Viral Nervous Necrosis"), Japan and to the National Taiwan University, where virological examinations targeting on piscine nodaviruses were carried out according to O.I.E. Manual of Diagnostic Tests for Aquatic Animals (O.I.E 2003) with some modifications. New primer sets (Nakai, unpublished) were used for reverse transcriptase-polymerase chain reaction (RT-PCR) and nested PCR. The sequence data obtained was compared with representative coat protein gene sequences of the four known fish nodavirus genogroups; striped jack nervous necrosis virus (SJNNV), tiger puffer nervous necrosis virus (TPNNV), barfin flounder nervous necrosis virus (BFNNV) and redspotted grouper nervous necrosis virus (RGNNV) (Nishizawa *et al.*, 1997; Iwamoto *et al.*, 2001). In order to investigate on the pathogenicity, sevenband grouper (*Epinephelus septemfasciatus*) weighing average 45g were challenged by intravitreal injection of the filtered homogenate obtained from pooled brains of affected golden grey mullet (water temperature 21°C).

**Ecological survey:** Sea water by Niskin Bottle Sampler (Hydro-Bioss Model), sediments by Grab Sediment Sampler (Van-Veen / Hydro -Bioss Model), biomass of Macro-Benthic fauna and nutrients (base on Standard Methods Reference-MOPAMP) in Laboratory, were examined.

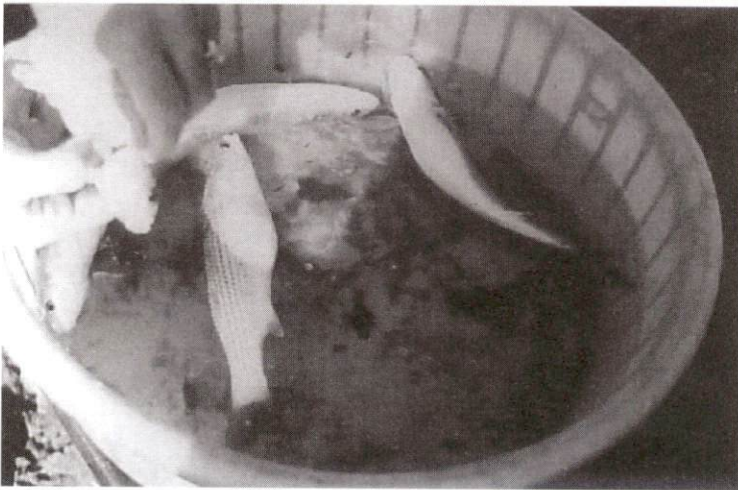
## **Results**

### **Clinical signs and macroscopic observations**

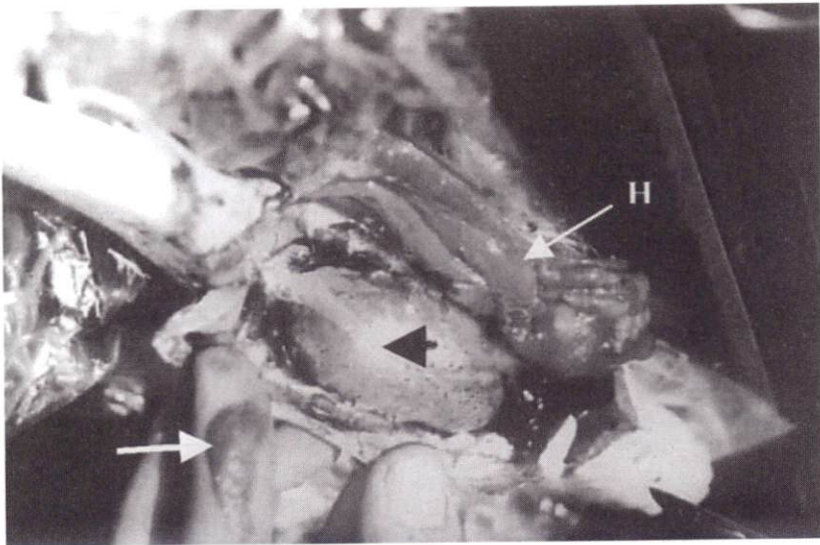
Clinical signs of moribund fish were represented by erratic swimming behavior such as spiral and belly-up at rest, lethargic appearance without any surface erosion. The gross pathological changes were characterized by gas accumulation and significant over inflation of the swim bladder, yellowish liver, and presence of excess micro sands accumulation in caecum with hyperaemia of intestine (Figures 1, 2, 3).



**Figure 1:** External clinical signs including over inflation, due to gas accumulation in swim bladder in moribund fishes



**Figure 2:** Erratic swimming behaviour such as spiral and belly-up at rest



**Figure 3:** Clinical and gross pathological signs of moribund fishes including gas accumulation and significant over inflation of the swim bladder (arrow head), and presence of excess micro sands accumulation in caecum (arrow) with hyperaemia of intestine (H)

### Hematological and biochemical findings

As shown in table 1, the amount of erythrocyte (RBC), leukocyte (WBC), hemoglobin (Hb) and packed cell volume (Hct), of affected fish are significantly ( $P < 0.05$ ) decreased when compared to the control group.

In the other hand, amount of blood index including mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) did not changed. Comparison of liver enzymes only showed increase of SGOT.

**Table 1:** Hematological comparison of healthy and affected golden grey mullet collected in the Caspian Sea

| Fish               | RBC × 10<br>/mm <sup>3</sup> | Hct<br>(%) | Hb<br>(g/100 <sup>cc</sup> ) | WBC<br>(No./mm <sup>3</sup> ) | MCV<br>(fl) | MCH<br>(pg) | MCHC<br>(%) |
|--------------------|------------------------------|------------|------------------------------|-------------------------------|-------------|-------------|-------------|
| Normal<br>(n=20)   | 406.8±21.5                   | 45.5±1.3   | 11±0.49                      | 48033.3±4591                  | 115.2±517   | 27.9±115    | 24±0.516    |
| Affected<br>(n=20) | 298.3*±24.9                  | 33.1*±28   | 9.1*±0.97                    | 19375*±2156                   | 111±4.1     | 30.5±2      | 27.2±1.08   |

**p < 0.05**

### Histopathological findings:

Examination of brain sections revealed hyperemia, degeneration and necrosis in external granular layers as well as focal vacuolation. However, these damages in the brain were not severe. Hyperemia was observed in the swim bladder. There were no significant pathological changes in other organs.

### Bacteriological and parasitological findings:

No predominant bacteria were isolated from any internal organs of the affected fish. Neither ectoparasites were found on the body surface, nor were any protozoans observed in the blood smears.

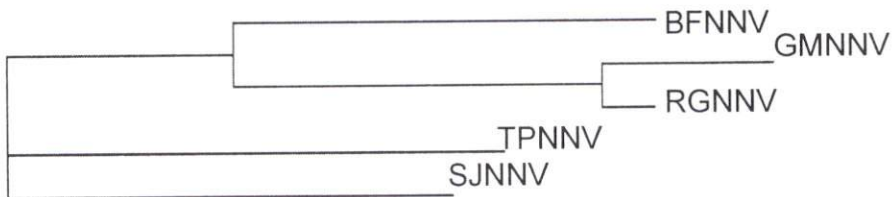
### Examinations for piscine nodavirus:

The coat protein gene of piscine nodavirus was detected by RT-PCR and nested PCR, in all the 8 brain obtained from affected golden grey mullet, though the RT-PCR amplicons weakly appeared in the agarose gel electrophoresis. The sequence analyses on the nested PCR products (177 bases) suggest the presence of a viral



agent closely related to piscine nodaviruses. Experimental infection induced 100% mortality in sevenband grouper following intravitreal injection with pooled brain homogenates. Fish lost balance and died 4-6 days after injection. The brains of the dead sevenband grouper were strongly positive by RT-PCR test. The sequence analysis of the RT-PCR amplicon (345 bases) revealed, again, genetic relatedness between the present virus and RGNNV (Table 2, Fig. 4).

However, nodavirus antigens were not demonstrated by indirect fluorescent antibody technique (IFAT) using rabbit polyclonal antibodies (anti-SJNNV) in the brains of the affected golden grey mullet or sevenband grouper. Moreover, trials to isolate viruses using E-11 cells (Iwamoto *et al.*, 2000) have not succeeded.



**Figure 4:** Molecular phylogenetic tree deduced from analysis of the nucleotide sequences of known betanodaviruses and GMNNV

**Table 2:** Nucleotide sequence similarities of the coat protein genes of known betanodaviruses and GMNNV\*

|       | GMNNV | BFNNV | RGNNV | SJNNV | TPNNV |
|-------|-------|-------|-------|-------|-------|
| GMNNV | 100   | 72    | 93    | 64    | 62    |
| BFNNV |       | 100   | 77    | 64    | 69    |
| RGNNV |       |       | 100   | 66    | 66    |
| SJNNV |       |       |       | 100   | 73    |
| TPNNV |       |       |       |       | 100   |

\*present virus from diseased golden grey mullet

### Ecological findings:

The average biomass found in the region was  $2.124 \text{ gm}^2$  which was very similar to the results observed previously in 1992 (less than  $4 \text{ g/m}^2$ ) (Table 3), furthermore no significant differences in physico-chemical parameters have been observed between pre- and post-mortality outbreak (Table 4).

**Table 3:** Specification of biomass in Ziba-Kenar beach (Feb.2004)

| Specimen name | No./ $\text{m}^2$ | Weight/ $\text{m}^2$ |
|---------------|-------------------|----------------------|
| Nereidae      | 50                | 2.075                |
| Tubificidae   | 25                | 0.033                |
| Amphipoda     | 8                 | 0.016                |
| Totals        | 83                | 2.124                |

Table 4: Physico-chemical analysis of the Caspian Sea water in Zibakenar beach

| Date weather time          | Station        | Transparency (cm) | Air Temperature (°C) | Water Temperature (°C) | CO <sub>2</sub> | CO <sub>3</sub> | EC Ms/cm | Salinity (ppt) | pH   | SiO <sub>2</sub> | PO <sub>4</sub> | NH <sub>4</sub> | NO <sub>2</sub> | NO <sub>3</sub> | O <sub>2</sub> |
|----------------------------|----------------|-------------------|----------------------|------------------------|-----------------|-----------------|----------|----------------|------|------------------|-----------------|-----------------|-----------------|-----------------|----------------|
| Jan. 26, 2004 cloudy 16:15 | Surface        | 250               | 15                   | 11.4                   | -               | 12              | 13.90    | 11.10          | 8.51 | 0.462            | 0.053           | 0.033           | 0.005           | 0.024           | 11.04          |
|                            | 5 meter depth  | 250               | 15                   | 10.2                   | -               | 12              | 14       | 11.20          | 8.51 | 0.381            | 0.082           | 0.025           | 0.003           | 0.012           | 10.09          |
|                            | 12 meter depth | 250               | 15                   | 10.4                   | -               | 12              | 14.60    | 11.58          | 8.51 | 0.088            | 0.059           | 0.026           | 0.001           | 0.020           | 10.70          |
| Feb. 3 2004 sunny 10:45    | Surface        | 200               | 11                   | 10.4                   | -               | 5               | 14.70    | 11.05          | 8.45 | 0.370            | 0.023           | 0.021           | 0.003           | 0.041           | 10.54          |
|                            | 18 meter depth | 200               | 11                   | 10.8                   | -               | 8               | 15.62    | 11.84          | 8.50 | 0.084            | 0.022           | 0.018           | 0.002           | 0.023           | 10.54          |

## Discussion

The present study was carried out in order to investigate the cause of severe mortality occurred in golden grey mullet in the Caspian Sea in Iran. The disease was characterized by erratic swimming behavior and hyperinflation of swim bladder; no other clinical signs were observed. These signs are different from abdominal swelling, water belly, dropsy, gas bubble disease or swim bladder stress syndrome (Woo & Bruno, 1999) and have not previously reported from wild fish of the Caspian Sea. *Sphaerospora* sp. may be responsible for swim bladder distention (Lom & Dykova, 1992) but this parasite was not found in blood smears of the affected fish.

Hematological examinations indicated that the number of erythrocyte count, hemoglobin and packed cell volume were significantly ( $p < 0.05$ ) decreased in diseased fish, while the MCV, MCH and MCHC were not suggesting that the diseased fish faced a normocytic normochromic anemia. The significant decrease of leukocyte number could suggest a recent acute viral infection (Lom & Dykova, 1992).

The feed organisms in the region consist of *Ostracoda*, *Mysidae*, *Balanus*, *bivalva* and larvae of *Nematod* and *Gastropoda* revealed that the diets of golden grey mullet were normal without any quantitative changes. Water analysis records also showed no changes in comparison with the previous sampling. It was concluded that no environmental parameters could be responsible for the incidence of the disease.

At present, the positivity to VER/VNN detected by PCR from the brains of affected fish suggests piscine nodavirus could be regarded as the causative agent of the observed mortality in golden grey mullet, though viral etiology was not fully demonstrated. Piscine nodavirus is the causative agent of the viral encephalopathy and retinopathy (VER) or viral nervous necrosis (VNN) in several marine fish species worldwide (Munday & Nakai, 1997; Munday *et al.*, 2002; O.I.E, 2003). In

addition to the similarity of clinical signs observed with those described during the viral encephalopathy and retinopathy outbreaks, RT-PCR carried out using specific primers to piscine nodaviruses identified the coat protein gene in the brains of the affected golden grey mullet; furthermore the nucleotide sequence analysis revealed that the mullet virus is genetically most related to RGNNV among four known betanodavirus genogroups. However, the amino acid sequence similarity with RGNNV was only 80% suggesting that the suspected virus belongs to a new deferent genogroup. This difference in the amino acid sequence probably leads to antigenic difference of the coat protein since the brains from the affected fish had negative reaction in IFAT test using anti-SJNNV rabbit serum, which reacts with all known betanodaviruses (Mori *et al.*, 2003). Meanwhile, the brain homogenate from diseased golden grey mullet produced mortality in sevenband grouper. Therefore, it is concluded that a new betanodavirus RGNNV-like is associated with severe mortality of the recent golden grey mullet in the Iranian side of the Caspian Sea. To fulfill Koch's postulates, experimental infection in golden grey mullet should be investigated and efforts should be made to maintain this difficult species under experimental conditions.

RGNNV-genotype betanodaviruses have been frequently isolated from warm water fish species and show higher optimum growth temperature *in vitro* and *in vivo* (25-30°C) (Iwamoto *et al.*, 2000; Chi *et al.*, 2003). Although the mortality in grey mullet occurred when water temperature was low (10-11°C), more severe mortality could be expected in the Caspian Sea during summer, if betanodavirus infection is the causative agent. VER/VNN has long been limited to marine fish species but recently has been reported in some reared freshwater species like, European eel (*Anguilla anguilla*) and Chinese catfish (*Silurus asotus*) indicating that salinity is obviously not a limiting factor in VNN transmission (Chi *et al.*, 2003). Therefore, VNN outbreaks in the Caspian Sea could be a potential hazard to freshwater and marine fish including sturgeons of the northern Iran.

In conclusion, Virological examination suggested that the mortality affecting golden grey mullet of the Caspian Sea is associated with viral encephalopathy and retinopathy (VER) / viral nervous necrosis (VNN) caused by piscine nodavirus (Nodaviridae).

Based on the results of this study, it is very important to monitor the spread of VNN in the Caspian Sea carefully, because the disease may cause very high mortalities and severe economic losses. Intensive investigation must be undertaken for all fish species in Caspian Sea in order to prevent further spread.

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