Vacuolating encephalopathy and retinopathy associated with a nodavirus-like agent: a probable cause of mass mortality of wild Golden grey mullet (*Liza aurata*) and Sharpnose grey mullet (*Liza saliens*) in Iranian waters of the Caspian Sea

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Abstract Mulletts are dominant fishes in the catch composition in the southern coasts of the Caspian Sea and after (*Rutilus frisii kutum* Kamensky, 1901) have a worthy role in production of marine proteins and incomings of north provinces of Iran. Mulletts stocks decreased dramatically in recent decades in the Caspian Sea and catch amount reached from 6446 MT on 2002 to 2151 MT in 2012. Mysterious mortalities occurred in wild mullet (*Liza aurata*) and (*Liza saliens*) in Iranian waters of Caspian Sea in recent years. Regarding to investigation of causative agent of mentioned outbreak about 322 suspected samples were collected from coastal capture sites of Iranian north

provinces in 2008 till 2011. Moribund fish revealed skin darkening, erratic swimming, belly-up at rest and high distension of swim bladder. Target tissues such as brain and eye were removed and then fixed for histopathology and TEM assay. Widespread and massive vacuolation were observed in brain, spinal cord, retina and optical nerve and intracytoplasmic vacuoles and virus particles in retina. So concerning to clinical signs, histopathological and TEM findings, it could be concluded that nodavirus-like agent could be probable cause of mass mortality of wild mullet in Iranian waters of the Caspian Sea.

Keywords Histopathology · TEM · Nodavirus-like · Caspian Sea · Iran

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Introduction

The Caspian Sea is the biggest enclosed body of water on Earth. The number of endemic aquatic taxa living there are over 400 species. There are 115 fish species of which a number are anadromous and migrate from the Caspian Sea up the rivers to spawn. The best known of these are seven Sturgeon species and subspecies, which have been a valuable economic resource for over a century. The Caspian fishes can be divided into 4 groups according to the ecological peculiarities. (A) Sea fishes such as Mullet (*Liza aurata* and *Liza saliens*) and Caspian kilka (*Clupeonella delicatula caspia*). (B) Anadromous fishes such as Caspian salmon (Salmonidae). (C) Semi-migratory fishes such as Kutum (*Rutilus frisii kutum*). (D) River fishes such as the pile (*Esox lucius*) and the tench (*Tinca tinca*) [1]. Mulletts stocks decreased dramatically in recent decades in the Caspian Sea and its catch amount reached from 6446 MT on 2002 to 2151 MT in 2012. Encephalopathy and

retinopathy (VER), otherwise known as viral nervous necrosis (VNN), caused by piscine nodavirus (Nodaviridae), is a worldwide occurring disease except for Africa [2]. The disease was first described in Japanese parrot fish (*Oplegathu fasciatus*) [3]. Agent of VNN is a virus belongs on genus of Betanodavirus and family of Nodaviridae. The main target tissues are nervous tissues especially CNS and retina. To date, the disease has been reported in more than 50 fish species, mainly marine with the greatest impact being in striped jack, European sea bass (*Dicentrarchus labrax*), groupers, and flatfishes [4, 5]. A few outbreaks have also been documented in freshwater farms [6, 7]. Recently, the disease has been considered as the most serious viral threat affecting marine farmed species in the Mediterranean region, thus representing one of the bottlenecks for further development of the aquaculture industry [8]. The characteristic lesions of VNN are necrosis and vacuolation of central nervous tissues and retina of the affected larvae and juvenile fishes showing abnormal swimming behavior [9]. In Iran the first announcement of the mortality was reported in Caspian Sea in 2004 [1]. Unknown acute mortality occurred in wild golden grey mullet (*L. aurata*) of the Caspian Sea in the Ziba-Kenar region from Guilan province during February, 2003 when water temperature was 10.4–10.8 °C, in fish size of 15–20 cm. The affected fish exhibited loss of appetite, lethargy, abnormal swimming behavior, belly up at rest and distention and enlargement of swim bladder. So far mortality had occurred continuously in wild mullet population in the Caspian Sea since 2003. According to OIE protocol presumptive diagnosis of VER or VNN can be made on the basis of the appearance of vacuoles in the brain, spinal cord and/or retina as seen by light microscopy [2]. So this study was conducted in order to investigate the histopathology and EM lesions occurring in wild Golden grey mullet (*L. aurata*) and Sharpnose grey mullet (*L. saliens*) affected by VER.

Materials and methods

Fish

About 322 moribund mullet samples were collected from three coastline provinces namely the Gilan, Mazandaran and Golestan in the south of Caspian Sea from October 2008 to May 2011. The average weight of each sample was (136 ± 10) g and the average of TBL was (19 ± 2) cm.

Histopathology

Brain and eyes obtained from clinically affected fish were maintained in Bouin's solution for 1 week at 4 °C and then

soaked in 15 % trichloroacetic acid for 1 week at 4 °C. The fixed tissues were processed using an automatic tissue processor (SHANDON CITADEL 1000, USA) following standard method [10]. Processed tissues then were embedded in Paraffin wax and 5 µm sections were prepared using a rotary microtome (JUNG MULTICUT 2045, LEICA INSTRUMENTS, GERMANY). After that, the sections were stained using routine Haematoxylin and Eosin (H&E) and examined under compound light microscope (NIKON 100, TOKYO JAPON) [11].

Electron microscope

Method of TRANSMISSION ELECTRON MICROSCOPY (TEM) was used for evaluation of probably virus particles inside the cells and samples prepared base on the method recommended [12]. First fish brain and eye samples were kept in glutaraldehyde 2/5 % in PBS 0/1 M with pH = 7/2 for 2 h, then three times washed with phosphate buffer or 1 % sodium cacodylate. The second fixation phase was performed by adding cool 1 % Osmium tetroxide about 1.5 h and then placed in the resin system and squeeze the tissue by Microtom and ultra thin slices were prepared. The sections were placed on the copper grids and then stained by uranyl acetate 2 % and imaging was done by electron microscope device (Hitachi Model HT7700, Japan).

Pathogenicity test

Ninety adult male and female guppies were obtained with mean weight 0.47 ± 0.09 g. The fishes were acclimatized in clean aquaria for 2 weeks before the challenge trials and fed with commercial ornamental fish pellet twice daily. The temperature was maintained at 25 °C and the aquaria were aerated. During the 2 weeks, mortality wasn't observed. The challenge virus used in this study was obtained from SSN-1 cell culture inoculated with brain and eye homogenized tissues originating from naturally infected Golden grey mullet. The cell monolayer with marked CPE was scraped from the flasks, and fresh EMEM media (SIGMA) was added. Then, the scraped cells and media were dispensed into centrifuge tube and centrifuged at 1,500 rpm, for 10 min at 4 °C. The cell pellet was collected and re-suspended in EMEM medium. This new supernatant was freeze-thawed twice at 70 °C to break the cells and permit the virus release. The supernatant was centrifuged again at 4,000 rpm for 20 min at 4 °C to separate the cell associated viral particles from the cell debris.

Titration of the virus was performed in SSN-1 cells in 96 well-plates and the TCID₅₀/mL⁻¹ calculated according to standard method. Three experimental groups each consisting of 30 specimens was established, i.e. test group 1, test group 2 and control group. Fish were immersed in 6L

Fig. 1 **a** Distension of swimming bladder (*arrow head*) **b** erratic swimming behaviour such as spiral and belly-up at rest and **c** greenish gallbladder distention (*head arrow*), swollen intestine (*In*), white patches (*thin arrow*) and melanosis (*small arrow*) observed in the digestive system of infected *Liza aurata*

of water added with 50 mL of infected supernatant (TCID₅₀/ml = $10^{4.25}$) respectively for 2 h in test group 1 and 4 h in test group 2. The control group was immersed for 4 h in 6L of water containing 50 mL cell culture media (EMEM). All the aquaria were aerated accordingly.

Results

Clinical signs

Affected fish revealed skin darkening, erratic swimming behavior such as spiral and belly-up at rest and high distention of swimming bladder (Fig. 1a, b). In addition fish were weak with poor body condition. Digestive system showed distended gallbladder filled up with bile and the intestine was swollen (Fig. 1c). Melanosis were observed in the abdomen cavity. White patches were observed on the surface of intestine. Also hyperemia was observed on the lips in mouth area and in abdomen ventricular.

Histopathological and EM findings

CNS

Following H&E staining of the brain, vacuoles were seen in granular layer in medulla oblongata and optic nerve (Fig. 2a, b).

Retina

Severe vacuolation in the outer nuclear layer, inner nuclear layer and ganglion cell layer of retina observed in fish sampled in Gilan province in 2009 (Fig. 2c, d).

In order to confirmation of causative agent of mortality in region, trial infection was done on Guppy fish as pathogenicity test of disease. Same tissue changes such as vacuolation was observed in CNS and retina. Tissue changes after 15 dpi in Guppy were revealed (Fig. 3a, b).

Electron Microscope

Virus particles were observed in very thin sections of retina tissue under the electron microscope, and with a regular arrangement and focus within the vacuole walls. Approximate size of the viral particles were measured 25–30 nm (Fig. 4a).

Also, in trial infection that was done on Guppy fish as pathogenicity test of disease, Electron Microscopy samples results showed the virus components in retina cells at 20 dpi. The infected cells showed intracytoplasmic vacuoles (Fig. 4b).

Discussion

Pathogenesis and clinical signs of VNN are related to the neuroinvasive nature of the virus and the subsequent effect

Fig. 2 **a** Vacuolation in the granular layer of medulla oblongata in the brain of naturally VNN infected Grey mullet. H&E. Mag, $\times 100$. **b** Cross section of optic nerve in naturally VNN infected *L. aurata* shows vacuolation (arrows). H&E. Mag, $\times 100$. **c** Severe vacuolations (arrows) in the retina of naturally VNN infected Grey mullet. **PE**

Pigment epithelium, **ON** outer nuclear layer, **IN** inner nuclear layer, **IP** inner plexiform layer, **GC** ganglion cell layer. H&E. Mag, $\times 40$. **d** Severe vacuolations (arrows) in the retina of naturally VNN infected Grey mullet

Fig. 3 Vacuolation in the olfactory bulb of telencephalon region in brain tissue of infected Guppy at 15 dpi (arrows). **S** septal area. **V** ventral olfactory area, H&E. **a** $\times 50$, **b** $\times 400$

on tissues such as brain and retina [13]. In general, histopathology of VNN includes cellular vacuolation and neuronal degeneration, usually in retina, brain, spinal cord and ganglia in the peripheral nervous system [4]. Typical

histopathological lesions include severe widespread vacuolation throughout the CNS and all retinal layers. The bipolar and ganglion cell retinal layers exhibit the most obvious vacuolation. Gliosis is also a common finding in

Fig. 4 **a** TEM micrograph of retina cells at 20 dpi. The infected cells showed intracytoplasmic vacuoles (Arrow key), **b** TEM micrograph showing the vacuoles in the cytoplasm of the retina cell of Guppy fish, (arrows) Scale bar 0.2 μm

the CNS. Neuronophagia occasionally were seen by accumulation of microglia around degenerated or necrotic neurons. Vacuolation is usually greater in the grey matter of the optic tectum and cerebellum and there is often purkinje cells involved. Vacuolation can also be seen in the white matter, adjacent to the ventricles [14]. In moribund fish in mentioned outbreak in the Caspian Sea, vacuolation was severe and widespread in the grey and white matter of the brain compared to the nerve cells of the spinal cord.

In this outbreak *L. aurata* and *L. saliens* displayed clear abnormal swimming behavior such as darting and circling and some taking a vertical position and resting on the water surface. This clinical signs were characteristic of VNNV disease. Uncoordinated swimming, i.e. spiral swimming and darting, although flatfish tend to have a looping swimming pattern and to rest belly up were reported by [3, 15–21]. Similar clinical signs were reported during serious mortalities affecting Grey mullet in Iranian coastal water of the Caspian Sea [1, 22].

The marked clinical signs in infected mullets in this study were swimbladder distention. Swimbladder hyperinflation was reported previously in barramundi, European sea bass and striped jack infected with VNNV [4, 17, 19]. Also swimbladder hyperinflation was reported in infected Grey mullet [1].

Gross clinical signs, i.e. lethargy and poor body condition, gallbladder distention, exophthalmia, intestine swollen, white spots on the surface of intestine and hyperemia in the abdomen and lips were other clinical signs observed in naturally infected grey mullets. Reports from some fish species infected by VNNV also confirmed anorexia, exophthalmia and hyperemia, especially in larvae, as marked clinical signs of VNN disease [4, 23]. Moreover, observation of exophthalmia, abdominal distention and hemorrhage in the skin represent viral infection in fishes [10].

Greenish gallbladder filled up by bile and swollen intestine indicated that fish are not eating for long time. These findings can approve some lethargy, weakness and emaciated body in affected fish of recent outbreaks.

Histopathology findings in brain of naturally infected Grey mullet using light microscopy indicated vacuolation in granular layer in medulla oblongata. These results were similar to findings observed in different species infected by VNNV from earlier studies which characterized by vacuolation and necrosis of the central nervous system. They are remarkably consistent between the various species [4]. In general, the anterior brain was more severely affected than the posterior brain and spinal cord [4]. Also optic tectum was rarely affected in the diseased adult European sea bass [24]. Lesions have also been described in the spinal ganglia in Japanese parrotfish [3]. The most characteristic lesion is the presence of vacuoles in the grey matter of the brain which appeared to be intracytoplasmic, but their exact position cannot always be determined [4].

Cross and longitudinal sections in the optic nerve and retina of diseased Grey mullets displayed vacuolation. In the retina, marked vacuolation were observed in the outer nuclear layer, inner nuclear layer and ganglion cell layer. Also presence of vacuoles and necrotized cells in the inner nuclear layer and ganglion cell layer were reported [25]. According to mentioned findings, nerve cells in the olfactory lobe were most extensively necrotized with vacuolation followed by infiltration of microglia and macrophages. Also, Purkinje cells and Golgi cells were extensively infected in the cerebellum. Megalocells 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 fish displayed vacuolated bipolar cells of the inner nuclear layer and in the

ganglion cell layer. These SGNNV-infected nerve cells displayed viroplasmic inclusions containing virions, vacuoles and myelin-like structures. In some severe cases vacuolations were widespread till spinal cord in affected fish [26].

The most remarkable clinical signs observed in infected Grey mullet were abnormality in swimming behavior which is characterized by darting and belly up at rest. Moreover, infected fish also showed swimbladder hyperinflation and exophthalmia. The Fish buoyancy is controlled by the rise and decreases of air pressure in the swim bladder. Occurrence of vacuoles and necrotized cells in control center of swimbladder could result in abnormal or low swimbladder function. The Medulla oblongata is responsible part of brain for control the swimbladder function [27]. The presence of vacuolation in medulla oblongata of the infected Grey mullet (Fig. 2a), causes this center would not be able to send the neural impulses to the swim bladder. Thus, infected fish showed abnormal swimming continuously due to irregular air pressure in the swim bladder.

Abnormal swimming behavior also could relate to the exophthalmia and retina lesions observed in diseased fish. 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 [27, 28]. These abnormalities together with irregular air pressure in the swimbladder would have influence on movement patterns changes.

Hyperemia observed in naturally infected fish could be due to inflammatory factors of VNN. Similar congestions were observed in our recent investigation about new mortality in *Liza klunzingeri* in Persian Gulf and Oman Sea. The affected fish showed hemorrhage in lateral and abdominal cavities. Also, hemorrhages were seen in the operculum and thoracic and caudal fins [29].

Lethargy, emaciation, gallbladder filled with bile and swollen intestine indicated that the fish was not feeding since long time which could due to different reasons. As mentioned in introduction, Grey mullets fed on benthos. Infected fish could have inability to see food because of exophthalmia and retina lesions and this could be the reason of lethargy in infected fish. Changes in environmental conditions or low available food could be the reason of fish emaciation [1].

In conclusion, the marked clinical signs, histopathology and TEM findings seen in the current investigation lead to possibility of a viral disease agent infection as the cause of morbidity and mortality in Grey mullet in Iranian coastal water of Caspian Sea.

Moreover, virus particles were also visualized in infected brain and retina using positive staining in TEM. Therefore, considering observed clinical signs, type of

vacuoles, their positions and presence of inclusions in the infected tissues which seen in TEM micrograph, it was concluded that observed inclusions in sampled fish were similar to VNNV particles that was reported earlier [23].

Therefore clinical signs, histopathology and EM finding could be considered as valuable and complimentary tools for primary diagnosis and confirmation of other findings. Also, regarding to variation of aquatic biodiversity in the Caspian Sea, susceptibility and sensitivity of some economic fish to VNN virus, it could be concluded that VNNV should be considered as new threat in the aquatic ecosystem of the Caspian Sea.

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