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First Incidence of Clinical Signs of Nodavirus Infection in Sea Bream, *Sparus auratus* L.

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Abstract

The causal agent of viral encephalopathy and retinopathy in fish is an RNA virus known as nodavirus, a member of the *Nodaviridae* family. According to published data, sea bream (*Sparus aurata* L.) that are infected with the virus show no clinical signs and are considered an asymptomatic carrier. In this paper we report for the first time a clinical case of nodavirus in commercially reared sea bream. Fish with clinical signs underwent necropsy and routine microbiological, parasitological, and histopathological examination. The virus was detected by RT-PCR and isolated in SSN-1 cell cultures. The results described in this study demonstrate the presence of a nodavirus agent that can infect sea bream in sea water, causing a disease similar to that observed in marine sea bass but with lower mortality and milder neurological signs. Histopathological lesions were also similar to those observed in sea bass infected with nodavirus.

Introduction

Due to the rapid development of global aquaculture, viral infections are increasingly being reported in commercially reared marine fish. Among these infections, viral encephalopathy and retinopathy (VER) caused by betano-daviruses (piscine nodavirus) is one of the most devastating. Nodavirus belongs to the *Nodaviridae* family (Mori et al., 1992). Together with the lymphocystis disease virus (Alonso et al., 2005), it is the only virus report-

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ed in cultured marine fish in the Mediterranean region (Bovo et al., 1999; Iwamoto et al., 1999; Skliris et al., 2001).

Nodavirus is a causal agent of severe mortality in marine fish such as the Mediterranean sea bass, *Dicentrarchus labrax* L., and the striped jack, *Pseudocaranx dentex* (Bloch and Schneider). It has been isolated from fish reared in fresh water such as golden grey mullet, *Liza auratus* (Zoriehzahra et al., pers. comm.), and sturgeon, *Acipenser gueldestaedi* L. (Athanassopoulou et al., 2004), as well as from cultured asymptomatic sea bream *Sparus aurata* L. carriers (Castric et al., 2001). There has been only one report of an experimental transmission of the virus to sea bream (Aranguren et al., 2002) and tilapia *Oreochromis mossambicus* Peters (Skliris and Richards, 1999) where diseased fish were observed. In the present paper we report on the first incidence of clinical signs in commercially reared sea bream in Greece infected by nodavirus. Until now, sea bream was thought to be only an asymptomatic carrier of this virus (Castric et al., 2001).

The sea bream (50-80 g) were reared in cages near sea bass rearing facilities on an island in the eastern Aegean Sea. One year before the present research, sea bass from the farm had shown nervous signs during an outbreak of clinical disease. Diagnosis of nodavirus was based on clinical signs, noda-positive RT-PCR results, and nodavirus isolation in SSN-1 cell cultures. At that time, no clinical signs were present in the sea bream population.

Clinical symptoms in the sea bream began at the end of August 2004 when the water temperature was 28°C and lasted approximately two months. Cumulative mortality during the two months was 19%. The sea bream showed clinical signs of lethargy, anorexia, sluggish swimming, and apathy. During the same period, sea bass in the nearby farm showed no clinical signs, although there was low mortality (approximately 5%) due to unspecified causes.

Materials and Methods

Collection and preparation of samples. During the outbreak, twenty fish from each cage of affected sea bream and sea bass were sampled for bacteriological and parasitological examination. Kidney and spleen samples were inoculated onto tryptone soy agar (TSA) and thiosulphate citrate bile salt agar (TCBS) for bacteriological examination according to methods described by Roberts and Shepherd (1997). Squash imprints of gill, skin, gall bladder, liver, spleen, kidney, muscle, brain, and gut tissue from freshly euthanized fish were examined for the presence of parasites according to methods described by Roberts (1989) and Athanassopoulou (1990).

Fresh tissue samples (from brain, eyes, liver, spleen, stomach, intestine, heart, swim bladder, kidney, and gill) were collected for PCR and histopathological examination. Six affected fishes per species were processed histologically and sections were stained with hematoxylin and eosin according to methods described by Drury and Wallington (1980). Simultaneously, tissue samples from ten sea bream and ten sea bass were frozen at -20°C for subsequent PCR analysis.

Virus isolation. Brain and eye samples from each fish species were homogenized with about 1-2 ml of Eagle's Medium Essential Medium (EMEM) balanced salt solution supplemented with 2% FCS (foetal calf serum), 0.85% $NaHCO_3$, and the antibiotics penicillin (100 IU/ml), streptomycin (100 µg/ml), and amphotericin B (0.25 µg/ml). The tissue-medium suspension was centrifuged at 1500 g for 15 min at 4°C. The supernatant liquid was further diluted in EMEM to produce a 1:10 dilution of the original tissue sample and filtered through 0.45 µm sterile filters (Sartorius, Minisart, Germany) to eliminate bacterial contamination.

The resultant tissue extracts were inoculated in 24-well microplates (Nunc, Denmark) containing SSN-1 cells derived from striped snakehead, *Channa striatus* Bloch (Frerichs et al., 1996), and incubated at 25°C for at least 7-12 days. Cell cultures were checked daily by microscope observation for the presence of cytopathic effects (CPE) indicating viral replication. At least three passages were repeated per sample when no CPE were present before characterizing it as negative.

RNA extraction. RNA was extracted from tissue samples and cell cultures using a QIAamp Viral RNA Mini Spin Kit (Quiagen GmbH, Holden, Germany) according to the manufacturer's instructions, resulting in a final volume of 50 µl. RNA purity and concentrations were determined spectrophotometrically (A260 nm).

Primers. The initial PCR-primer used for amplification of a 725 bp highly conserved nervous necrosis virus (NNV) coat protein gene region was based on the published nucleotide sequence of the coat protein gene of SJNNV (Nishizawa et al., 1994). Primer F (5' GAATCTTCCAGCGATAC 3') consisted of 17 nucleotides complementary to nt 306-322 and primer R (5' CGAGTCAACACGGGT-GAAGA 3') of 20 nucleotides corresponding to nt 1011-1030 of the SJNNV coat protein. The sequences of the forward and reverse primers for the nested PCR amplifying a 255 bp fragment (Dalla Valle et al., 2001) were NF (5' AATGTGCCCCGCAAACAC 3') and NR (5' GACACGTTGACCACATCAGT 3').

Reverse transcription. RNA products were mixed with primer R at a ratio of 0.5 µg primer R per 1 µg RNA in a total volume of 11 µl DEPC-treated water, preheated at 70°C for 5 min and immediately placed on ice for 5 min. Reverse transcription was initiated by adding 20 units of AMV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) to a reaction mixture of 20 ml, containing 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, and 30 units of RNaseOUT inhibitor (Invitrogen). cDNA products were then incubated at 42°C for 90 min.

Polymerase chain reaction (PCR). PCR amplification of the NNV coat protein encoding the gene region of interest from cDNA was carried out using the primer sets F-R and NF-NR according to standard protocols (Sambrook et al., 1989). The master mixtures were preheated to 94°C for 3 min, then subjected to 40 thermal cycles, each cycle being 94°C for 30 sec, 50°C for 30 sec, and 72°C for 40 sec, followed by one final cycle at 72°C for 10 min. The resulting PCR products were analyzed on a 2% agarose gel stained with ethidium bromide (0.5 µg/ml), with a 100 bp DNA ladder (Gibco, BRL) serving as a size marker, and photographed under UV transillumination.

Results

No gross lesions or parasites were observed in the majority of the examined fish, except that 90% of the sea bream individuals had brain hemorrhages and gas accumulation that resulted in significant swim bladder distention. No bacterial infection was detected after inoculation of the samples in TSA and TCBS media.

Histologically, lesions were observed in the brain, spinal cord, and eyes of all examined sea bream. No lesions were observed in any organ of any of the examined sea bass. Affected brains had pathological changes in the mesencephalon, medulla oblongata, and cerebellum. Brain and spinal cord lesions included vacuolation, mainly extended into the grey matter (Fig. 1). Varying degrees of gliosis were seen in a few cases of both vacuolated and non-vacuolated areas of the central nervous system. Vacuolating retinopathy was also observed. There were large vacuoles in the granular layers of the retina (Fig. 2) and extended hemorrhages in the grey matter (Fig. 3). No further lesions were observed in any other organ of the examined sea bream.

Virus isolation. The nodavirus cytopathic effect (CPE) is characterized by the appearance of round-shaped vacuoles in the susceptible SSN-1 cells (Frerichs et al., 1996). All sea bream isolates induced CPE at least 10 days after inoculation by creating small intracytoplasmatic vacuoles in the SSN-1 cells and disrupting the monolayer within 2-4 days by forming a mesh of degenerating cells (Fig. 4). No CPE were observed in inoculations made from sea bass samples.

RT-PCR and nested-PCR results. All samples were negative when tested by RT-PCR, probably because the quantity of virus was less than the technique required. In contrast, a 255 bp amplicon was constantly detected by nested-PCR in nucleic acid preparations of brain tissue from infected sea bream as well as in the supernatants of SSN-1 cell culture isolations and this was compared to sea bass
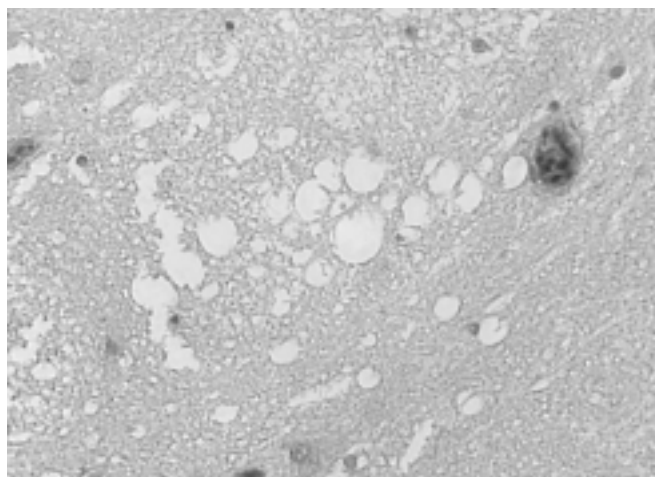
Fig. 1. Vacuolation in the brain of sea bream infected by nodavirus. Histological section: H+E, x400.

Fig. 2. Vacuolation in the retina of diseased sea bream. Histological section: H+E, x200.

infected by nodavirus from other marine farms. Nested-PCR is a more sensitive method than RT-PCR.

Discussion

We hereby report a clinical case of nodavirus in commercially reared sea bream for the first time. Infected sea bream demonstrated anorexia, sluggish swimming, and apathy while mortality was low.

Fish nodaviruses generally have a strong pathogenicity to larvae and juveniles of several marine fish. A disease pattern with similar symptoms, epizootiology, and lesions of nervous tissue has been reported in many cultured marine teleost species (Yoshikoshi and
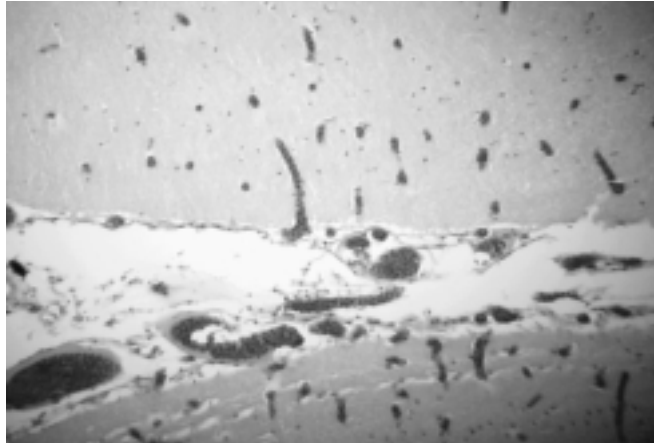
Fig. 3. Extended hemorrhages in the grey matter of sea bream. Histological section: H+E, x300.
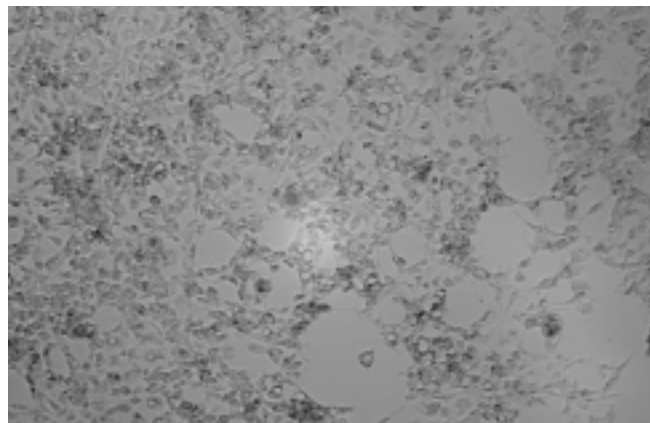

Fig. 4. Vacuolation in the monolayer of SSN-1 cells six days after inoculation with organ extracts from sea bream displaying typical signs of nodavirus infection.

Inoue, 1990; Grotmol et al., 1997). This viral infection is usually described as viral nervous necrosis (VNN; Yoshikoshi and Inoue, 1990), viral fish encephalitis (Breuil et al., 2001), or viral encephalopathy and retinopathy (VER; Munday et al., 1992).

Histologically, the disease is characterized by vacuolating encephalomyelopathy and retinopathy. There are constant histopathological findings associated with the virus in the brain, grey matter, spinal cord, and granular layer of the retina. Additionally, the virus can replicate and be distributed in other organs such as the gonads and digestive tract under stress conditions (Castric, 1997). In our case, histological sections revealed the presence of

lesions that are similar to those in the standard pattern of the disease in sea bass (Le Breton et al., 1997).

VNN, long thought to be limited to marine fish species, was recently reported in reared freshwater species such as the European eel (*Anguilla anguilla*) and the Chinese catfish (*Parasilurus asotus*), as well as in sea bass (Athanassopoulou et al., 2004), indicating that salinity is not a limiting factor in VNN transmission (Chi et al., 2003). Clinical signs and mortality rates vary depending on fish species and virus strain. Molecular and cross infection studies indicate that some nodavirus strains/species are not host specific and that some hosts are susceptible to not only one strain/species (Munday et al., 2002).

The presence of nodavirus was detected by molecular and histopathological methods. Castric et al. (2001) reported that the virus can be transmitted from sea bass to sea bream and vice versa during cohabitation, demonstrating horizontal transmission of the disease, and that sea bream do not seem to suffer from the disease, even if they are raised in the vicinity of an epizootic of the disease in sea bass. Castric et al. (2001) reported that sea bream are susceptible to nodavirus but refractory to the disease and experimentally demonstrated the role of sea bream as a reservoir of nodavirus to infect susceptible fish species.

The present study demonstrates a case of a nodavirus agent infecting sea bream in sea water causing clinical signs accompanied by histopathological lesions similar to those observed in sea bass in the marine environment. Sea bass reared in the vicinity of the sea bream cages were not infected with the virus although infected sea bass stocks a year earlier in the farm caused an acute summer outbreak. This fact may indicate that some conditions of the physical infection differed, increasing the pathogenicity of the virus to sea bream. It is also possible that the nodavirus adapted in the sea bream while the sea bass stock became resistant to the infection. This is an unusual case and therefore further research and investigation is underway. It is very important for the management of commercial farms to understand more about the pathogenicity of nodavirus in sea bream, especially when different fish species are reared in the vicinity.

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