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Isolation, Characterization, and Sequencing of Nodavirus in Sturgeon *(Acipenser gueldestaedi* L.) Reared in Freshwater Facilities*

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Key words: *Acipenser* sp., nodavirus, isolation, characterization, sequencing

Abstract

The study demonstrates the presence of a nodavirus that affected sturgeon in fresh water, causing disease with neurological signs. The virus was isolated and inoculated onto SSN-1 (striped snakehead, *Channa striatus*) cell cultures where cytopathic effects (CPE) of the virus included vacuolation of the cells and degeneration of the monolayer. A 255 bp amplicon from nucleic acid preparations of brain tissue from infected sturgeon was detected by PCR (RT-PCR and nested-PCR) and compared with corresponding amino acid sequences of other infected species. The sequences from the sturgeon were similar to those of sea bass, red spotted grouper, and European eel, supporting the hypothesis that the virus originated in marine fish and was horizontally transmitted to freshwater sturgeon.

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* In loving memory of dear friend and colleague Alexandros Argyrokastritis.

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Introduction

Viral infections in cultured marine fish are increasingly being reported due to the rapid development of aquaculture. Viral encephalopathy and retinopathy (VER), caused by betanodaviruses, is one of the most devastating infections in a variety of marine fish (Bovo et al., 1999; Skliris et al., 2001). In the Mediterranean area, sea bass (*Dicentrarchus labrax* L.) is the most commonly affected cultured species but nodavirus has also been isolated from asymptomatic cultured sea bream carriers (Castric et al., 2001).

There are few reports concerning the presence of the virus in freshwater fish. Hedge et al. (2003) performed biochemical, genomic, and serological studies to characterize a virus obtained from a diseased guppy (*Poecilia reticulata*) kept in a freshwater aquarium. Based on cell culture, RT-PCR diagnosis, and biochemical and genomic characterization, they found that the disease was caused by a strain of nodavirus belonging to the red-spotted grouper nervous necrosis virus (RGNNV) type which was antigenically similar to marine nodavirus isolates. Using PCR, we detected a nodavirus from intensively reared freshwater sturgeon, *Acipenser gueldestaedi* L. (Athanassopoulou et al., 2004).

The sturgeon were produced in a commercial freshwater hatchery in Epirus, Greece. They were transported to a grow-out farm at 7-10 g where they were reared in an open-flow system using well water of 0‰ salinity and 7.8 pH. Following transfer, mortality was limited. About 18 months after transfer, in late summer when the water temperature was 25-28°C, an outbreak of nodavirus occurred in 80-g sea bass reared on the same farm and lasted one month. At that time, the sturgeon weighed approximately 100 g. There was sporadic mortality among the smallest sturgeon, but no clinical signs. The sturgeon mortality gradually increased and, when the fish reached 500 g, clinical signs of lethargy and sluggish swimming appeared. In contrast, the infected sea bass showed intense nervous signs (Athanassopoulou et al., 2003). The affected sturgeon were well developed and had no skeletal deformities. Later, they turned upside down in the tanks, were unable to eat, and appeared dead although they responded to stimulus and survived. There were no parasites or microbial infections in sturgeon with the above symptoms, but PCR tests for nodavirus detected a 262 bp amplicon from nucleic acid preparations of brain tissue from these fish (Athanassopoulou et al., 2003). In the present study, we isolated the nodavirus, inoculated it onto cell cultures, and sequenced it.

Materials and Methods

Fish sampling and clinical evaluation. In February 2004, ten sturgeon with clinical signs were sampled for bacteriology and parasitology. 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 killed fish were examined for the presence of parasites according to methods described by Roberts (1989). At the same time, ten brain samples from 500 g sturgeon with the above clinical signs were collected for tissue culture and PCR analysis.

Virus isolation. The brain material was homogenized in EMEM (Eagle's Medium Essential Medium) 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 solution was centrifuged at 1500 g for 15 min at 4ºC, then further diluted in medium to produce a 1:10 dilution of the original tissue sample. The dilution was filtered through 0.45 µm sterile filters (Santorius, Minisart, Germany) to eliminate bacterial contamination. The resultant tissue extracts were inoculated onto SSN-1 cells (striped snakehead, *Channa striatus* Bloch; Frerichs et al., 1996) in 24-well microplates (Nunc, Denmark) and incubated at 25ºC for at least 7-12 days. Cultures were checked daily for the presence of cytopathic effects (CPE)

indicating viral multiplication. CPE was identified by the appearance of round vacuoles in the cells. At least three passages were repeated per sample when no CPE were present before considering it negative.

Isolation of nodavirus RNA. RNA extractions from infected material and tissue cultures were carried out using the QIAamp Viral RNA Mini Spin Kit (Quiagen GmbH, Holden, Germany) resulting in a final sample volume of 50 µl. RNA purity and concentrations were determined spectrophotometrically (A 260nm).

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

Reverse transcription. RNA samples were mixed with primer R at a ratio of 0.5 µg primer per µg RNA in a total volume of 11 µl DEPC-treated water, preheated at 70°C for 5 min, and 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). Samples were then incubated at 42°C for 90 min.

Polymerase chain reaction (PCR). PCR amplification of the *A. gueldestaedi* nervous necrosis virus (AGNNV) 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 reaction mixtures were preheated to 94°C for 3 min and 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 2% agarose gel stained with ethidium bromide (0.5 µg/ml) along a 100 bp DNA ladder (Gibco, BRL) serving as a size marker, and photographed under UV transillumination.

Cloning. Nested PCR products were extracted from agarose gels and purified using the Nucleo Spin Extract kit (Macherey-Nagel), ligated into Topo-TA (Invitrogen, Carlsbad, CA, USA) vector and used to transform highly efficient One Shot Chemically Competent *E. coli* (Invitrogen).

Sequence determination of capsid encoding gene region. Recombinant plasmids were purified using the Plasmid Mini Prep kit (Roche) and sequenced by the dideoxy method on a Li-Cor 4200 automated sequencer (Li-Cor Inc., Lincoln, NE, USA), using the M13 forward (-20) (5' GTAAAAC-GACGGCCAG 3') and M13 reverse (5' CAGGAAACAGCTATGAC 3') sequencing primers. The obtained sequences were analyzed using BLAST (Altschul et al., 1990; www.ncbi.nlm.nih.gov/BLAST).

Results

Clinical evaluation. No internal lesions or parasites were observed in the examined samples and no bacterial agents were obtained by culture methods.

Virus isolation. All isolates induced CPE characterized by vacuolation and initial degeneration of the monolayer of the SSN-1 cells 4-7 days after inoculation (Fig. 1). Small intracytoplasmatic vacuoles appeared which, after a few days, affected all of the monolayer by forming a mesh of degenerating cells. This progressed over the next 3-4 days leading to complete destruction of the monolayer.

RNA extraction, reverse transcription, and PCR amplification. A 255 bp amplicon from the nested PCR was consistently detected from nucleic acid preparations of brain tissue from infected sturgeon and compared to nodavirus-infected samples from marine fish (Fig. 2).

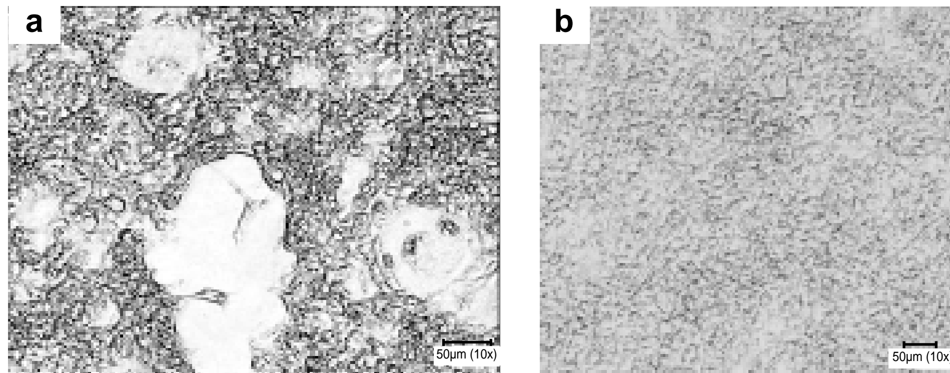

Fig. 1. (a) SSN-1 cells, inoculated with *Acipenser gueldestaedi* nervous necrosis virus (AGNNV), showing intracytoplasmatic vacuolation and initial degeneration of the monolayer; (b) normal SSN-1cells.

DNA sequencing and similarity. The nucleotide sequence of the 255 bp PCR fragment of the AGNNV coat protein encoding gene was compared to sequences in the Genbank. It had 99% homology with RGNNV (red-spotted grouper NNV; accession number AY7444705; Huang et al., unpubl.), 90% with SJNNV (striped jack NNV; AB056572; Iwamoto et al., 2001), 90% with TPNNV (tiger puffer NNV; D38637; Nishizawa et al., 1997), and 89% with BFNNV (barfin flounder; D38635; Nishizawa et al., 1997). A phylogenetic tree was constructed using PHYLIP to illustrate the relationship of AGNNV with other known forms of nervous necrosis virus (Fig. 3). The analysis shows that AGNNV is closely related to RGNNV and can be placed on the same branch.

Discussion

Nishizawa et al. (1997) proposed a classification of fish nodaviruses based on analysis of nucleotide sequences of the coat protein gene from 25 distinct isolates from Japanese, Thai, Australian, and Italian fish farms. In the resulting phylogenetic tree, fish nodaviruses were divided into four groups: tiger puffer nervous necrosis virus (TPNNV), striped jack NNV (SJNNV), barfin flounder NNV (BFNNV), and red-spotted grouper NNV (RGNNV). Dalla Valle et al. (2001) reported that all Mediterranean isolates are clustered within RGNNV.

In contrast to these findings, Thiery et al. (1999) demonstrated two isolates of the virus (one from the Atlantic Ocean coast and one from the Mediterranean Sea) that cause similar signs in infected sea bass. These isolates have distinct genomes and therefore, they argued, the virus may have no species barrier. This was also shown by Ucko et al. (2004) in sequences from Israel where no host specificity was evident.

Horizontal transmission of the disease has been demonstrated in sea bream and sea bass (Castric et al., 2001). In addition, molecular and cross infection studies indicate that some nodavirus strains/species are not host specific, while some hosts are susceptible to more than one strain/species (Munday et al., 2002).

Athanassopoulou et al. (2003) reported on the presence of a nodavirus agent that infected sturgeon in fresh water, causing disease with neurological signs. They also reported that sea bass showed intense clinical signs eight months after introduction to fresh water. Although nodavirus held in fresh water in laboratory conditions lost stability and no viable virus was detected after six months in storage (Frerichs et al., 2000), in our case, fish developed clinical signs of disease after eight

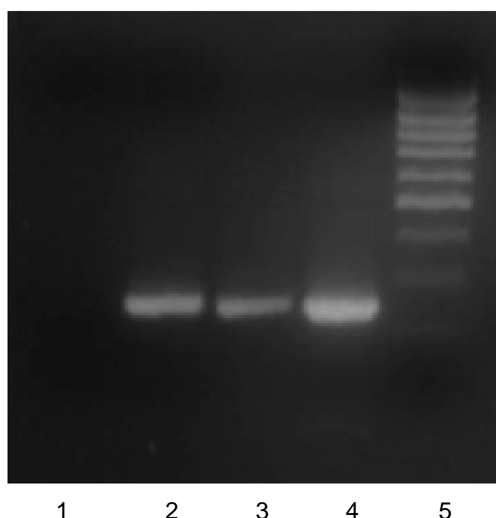

Fig. 2. Nested PCR from fish brain. 1: healthy fish (negative control); 2-4: Nested PCR fragments of nodavirus isolates from sturgeon brains, 5: 100 bp ladder molecular weight marker (1000-200 bp).

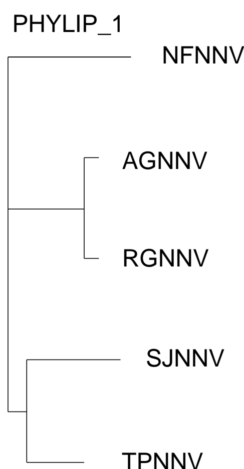

Fig. 3. Phylogenetic tree of nodaviruses, constructed from the 255 bp PCR fragment of the Greek strain of sturgeon, *Acipenser gueldestaedi*, nervous necrosis virus (AGNNV) and the corresponding regions in NNV from barfin (BFNNV; Genbank accession number D38635), red-spotted grouper (RGNNV; Genbank AY7444705), striped jack (SJNNV; Genbank AB056572), and tiger puffer (TPNNV; Genbank D38637). AGNNV has greatest similarity with RGNNV (96%), forming a separate branch. Horizontal branch lengths are drawn to scale.

months and the virus remained infective even longer, clinically infecting "new" susceptible hosts. The virus may have passed horizontally from the marine sea bass to the freshwater sturgeon where it continued to multiply. More research is needed to determine the salinity tolerance of nodavirus transmission in fresh water.

The genomic sequence of the sturgeon isolate was very similar to that of sea bass from marine cages in the region, supporting the hypothesis that the virus was initially introduced by marine fish and subsequently transmitted to the sturgeon (Athanassopoulou et al., 2003, 2004). Further investigation is required to determine genotypic and phenotypic features of betanodaviruses detected in freshwater conditions.

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