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## Identification and Characterization of a Piscine Neuropathy and Nodavirus from Juvenile Atlantic Cod from the Atlantic Coast of North America

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Abstract.—In 1999, disease outbreaks in juvenile Atlantic cod Gadus morhua that showed the classic signs of viral encephalopathy and retinopathy (VER) were reported in Nova Scotia. Brain and retinal tissues from moribund cod showed diffuse degenerative vacuolative encephalopathy and degenerative histiocytic retinitis. The affected brain and retinal tissues were observed to be positive for nodaviral antigens by means of immunohistochemical techniques. We partially characterized a nodavirus-like agent from brain and eye tissues and cell culture using reverse-transcriptase polymerase chain reaction and primer sets originally designed for amplification of white trevally Caranx dentex (also known as striped jack Pseudocaranx dentex) and Atlantic halibut Hippoglossus hippoglossus nervous necrosis virus coat (capsid) proteins. Sequencing of the T2 region of the coat protein revealed high similarities (>85% nucleotide identity) to the coat protein genes of other fish nodavirus strains, especially those of Atlantic halibut and barfin flounder Verasper moseri, which possessed 92% nucleotide identity. Based upon the sequence of its T4 region, this nodavirus is a member of the barfin flounder nervous necrosis virus clade. This is the first report of VER in Atlantic cod and the first report of this disease agent on the Atlantic coast of North America.

Viruses of the family Nodaviridae have been reported as the causative agents of disease in a wide variety of marine fish hosts throughout the world (Sweetman et al. 1996; Munday and Nakai 1997). Fish nodaviruses are responsible for the disease referred to as either viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER) (Office International des Epizooties 1997). The signs of this disease vary somewhat among species and life history stages but generally consist of loss of equilibrium, aberrant swimming behavior, and eventual death. Histological examination

of tissues from the central nervous system and the retina often reveals areas of conspicuous tissue vacuolation and necrosis. The causative agents are nonenveloped icosahedral viruses that are approximately 25-30 nm in diameter (Office International des Epizooties 1997). Their genome consists of two molecules of messenger sense RNA. RNA1 is approximately 3.1 kilo-base pairs (kb) in size and carries the gene that encodes for an RNA-dependent RNA polymerase referred to as protein A; RNA2 is approximately 1.4 kb in size and contains the open reading frame that encodes the capsid protein (Delsert et al. 1997). Based on similarities within the variable (T4) region of RNA2, fish nodaviruses have been categorized into four major clades: the barfin flounder Verasper moseri clade,

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the red-spotted grouper *Epinephelus akaara* clade, the white trevally *Caranx dentex* (also known as striped jack *Pseudocaranx dentex*) clade and the tiger puffer *Takifugu rubripes* clade (Nishizawa et al. 1997).

Diseases caused by nodaviruses have had major impacts on the culture of many species of marine fish (Munday and Nakai 1997). In Norway, incidences of high mortality in culture facilities for Atlantic halibut *Hippoglossus hippoglossus* have been reported since 1995, and this mortality has been attributed to nodavirus (Grotmol et al. 2000). With the development of culture techniques for other marine fish species, the potential problem of diseases caused by nodaviruses is well recognized.

In Atlantic Canada, Norway, and Scotland several research organizations and companies are developing techniques for rearing Atlantic cod *Gadus morhua* as an aquaculture species. At the Institute for Marine Biosciences in Halifax, Nova Scotia, we have been rearing from eggs small numbers of cod for experimental purposes. In 1999, we experienced a disease outbreak in our juvenile fish that resulted in high levels of morbidity and mortality. In this report we discuss the disease signs and the isolation and partial characterization of a nodavirus-like agent from these fish that we feel is the likely causative agent of this disease.

#### Methods

Clinical samples.—Moribund Atlantic cod juveniles were collected during a disease outbreak in early October 1999. Samples of juvenile haddock Melanogrammus aeglefinus that were being held in adjacent tanks but that showed no sign of disease were also collected. Additional samples of Atlantic cod and haddock juveniles were taken during another disease outbreak in another group of cod that occurred in November 1999. Samples for histology, virus isolation, and reverse-transcriptase polymerase chain reaction (RT-PCR) were collected.

Tissue and virus source.—Fish were euthanatized with a high dose of MS-222 (tricaine methanesulfonate) and necropsied under aseptic conditions. Brains from moribund Atlantic cod were divided midsagitally, and one-half was fixed for light and electron microscopy and the other half retained for virus assay by cell culture and RT-PCR. In addition, one eye from each fish was fixed for microscopy and the other eye was retained for virus assay by RT-PCR. Thirty brain samples from apparently healthy haddock were also collected for RT-PCR viral screening. Samples for RT-PCR were collected into sterile tubes containing RNA-

Later (Ambion), incubated at 4°C for 24 h, and then stored frozen at –80°C until analysis. Samples for assay using cell culture were collected into sterile vials and stored at 4°C or transferred to a–80°C environment for longer storage prior to assaying. At the start of the initial disease outbreak, samples of cod were also submitted for routine bacteriological, viral, and histological examination.

Histology and immunohistochemistry.—Tissues for examination by light microscopy were fixed in Davidson's solution or 10% buffered formaldehyde solution and dehydrated through to 100% alcohol. Tissues were embedded in wax, cut to a thickness of 5 µm, and stained with hematoxylin and eosin. Immunohistochemical examination of the wax-embedded samples was conducted by Renate Nilsen of the Veterinærinstituttet, Oslo, Norway, using several different polyclonal antisera against recombinant coat proteins of striped jack and Atlantic halibut nodaviruses (R. Nilsen, personal communication). It is important to note that no uninfected brain or eye tissues were examined. However, the polyclonal antisera used have been shown not to nonspecifically bind to the tissues of the central nervous system in other fish species, such as Atlantic salmon Salmo salar, turbot Scophthalmus maximus, and Atlantic halibut (Nilsen, personal communication).

Brain tissues from moribund individuals and cells from both uninfected cell cultures and those that were positive for cytopathic effect (CPE) were fixed for electron microscopy. Cell cultures and their media were transferred to Eppendorf tubes and spun at  $5,000 \times g$  for 5 min to obtain cell pellets. Brain tissue and cell pellets were fixed in 3% gluteraldehyde and 0.25 M sucrose in phosphate buffer (pH 7.2) for 1 h, postfixed in 2% osmium tetroxide and in blocked, and stained with 3% uranyl acetate aqueous overnight. These specimens were processed into Taab812-Araldite (Marivac, Montreal, Quebec) according to the manufacturer's protocol.

Reverse-transcriptase polymerase chain reaction was used to identify the virus in brain and eye tissue and to confirm its presence in cell cultures showing CPE. For the initial virus identification and to confirm its presence in cell cultures, total RNA from samples was extracted using TRIzol reagent (Gibco BRL, Burlington, Ontario) (cell culture) or QIAshredder columns (Qiagen, Mississauga, Ontario) and an RNAeasy Mini Kit (Qiagen) (tissue). RNA isolation and cDNA synthesis used either Ready-To-Go RT-PCR beads (Amer-

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TABLE 1.—Summary of primers and conditions used in polymerase chain reaction amplifications for Atlantic cod nodavirus coat protein.

	Primer		Final concentration			Cycling conditions			
Am- pli- con	Forward	Reverse				Denaturing	Annealing	Elongating	Num- ber of cycles
T2	F1, 5' GGATTTGGA CGTGCGAC CAA 3'a	R3, 5' CGAGTCAAC ACGGGTGA AGA 3'a	0.8	2.0	0.8	94°C for 30 s	61°C for 45 s	72°C for 90s	35
T4	F2, 5' CGTGTCAGT CATGTGTCG CT 3'a	R3, 5' CGAGTCAAC ACGGGTGA AGA 3'a	0.8	2.0	0.8	94°C for 30 s	61°C for 45 s	72°C for 90 s	35
	ANupper, 5' TATCCCATA GCCCCCAG TG 3'b	ANIower, 5' CTGAAGATA CATTCGCTC CAA 3'b	0.8	2.0	0.8	94°C for 2 min, 94°C for 30 s	55°C for 45 s	72°C for 90 s 72°C for 7 min	1 30 1

<sup>&</sup>lt;sup>a</sup> Primer sequences are given in Nishizawa et al. (1994)

sham Pharmacia Biotech, Baie d'Urfé, Quebec) or an RNAeasy Mini Kit (Qiagen) and a cDNA cycle kit (Invitrogen, Burlington, Ontario) and followed the manufacturer's protocols. For cDNA amplification, three sets of primers were used. These were the F1 and R3 and F2 and R3 as described in Nishizawa et al. (1994) and a set of primers based on the sequence given in Aspehaug et al. (1999) (Table 1). The conditions used in the PCR reactions are given in Table 1. PCR products were analyzed on 8% or 11% polyacrylamide gels or 2% agarose gels and visualized by ethidium bromide staining.

The RT-PCR products were either sequenced directly or cloned into pBluescript plasmid form by in vivo excision using reagents and protocols from Stratagene (La Jolla, California). Plasmid DNA was extracted and sequencing performed on an ABI 373 Automated Sequencer using the SK primer and PRISM Big Dye Terminator (PE Applied Biosystems, Foster City, California). The data were analyzed using Sequencher (Gene Codes, Ann Arbor, Michigan) and submitted for database searching at the National Center for Biotechnology and Information using BLAST (Altschul et al. 1997). Multiple alignments of deduced amino acid sequences were performed using DiAlign (Morgenstern 1999). Phylogenetic analysis was conducted using the DNAML Program of Web-PHYLIP (Lim and Zhang 1999). TreeView (version 1.6.6) was used to view the trees (Page 1996).

Cell culture of nodavirus.—Ten moribund Atlantic cod were processed to form two viral pools

per individual. One pool contained eye and brain tissues, the other spleen, kidney, pyloric caeca, and gill tissues. Initial virus screening using the chinook salmon Oncorhynchus tshawytscha embryo (CHSE) and epithelioma papillosum cyprini (EPC) cell lines followed standard protocols (Anonymous 1984). Subsequent viral isolation was done on the SSN-1 cell line (European Collection of Cell Cultures [ECACC] 96082808). These cells were grown and maintained in Leibovitz's L-15 medium (Gibco BRL) supplemented with 5% feral bovine serum (FBS) at 25°C in 75-cm<sup>2</sup> culture flasks. For virus isolation, cells were seeded into 24-well tissue culture plates to give a confluency of 80-100% after a 24-48-h incubation at 25°C. Cod brains that had been stored frozen at -80°C were thawed, weighed, diluted fivefold in Hanks' balanced salt solution (HBSS; ICN Biomedicals, Costa Mesa, California), and homogenized at high speed for 1 min in a Stomacher 80 laboratory blender (Seward, Mississauga, Ontario). A 10-fold dilution, prepared for each homogenate in HBSS, was centrifuged  $1,200 \times g$  in a GS-6R centrifuge (Beckman Instruments, Fullerton, California), followed by filtration using 0.45-µm Acrodisc syringe filters (Pall Gelman Science, Ann Arbor, Michigan). Monolayers of SSN-1 cells in 24-well culture plates (80–100% confluency), which had been prepared 24-48 h earlier and sealed with pressure-sensitive film (Becton Dickinson, Mississauga, Ontario), were washed with 1 mL of HBSS and inoculated with filtrates (0.1 mL per well). The inoculated plates were sealed with pres-

<sup>&</sup>lt;sup>b</sup> Primer suggested by Trine Ranheim (Veterinærinstituttet, Oslo, Norway) from sequence described by Aspehaug et al. 1999 (AF160473).

sure-sensitive film, incubated at 20°C for 1 h, and then topped with 1 mL Leibovitz's L-15 medium (Gibco BRL) supplemented with 5% FBS and 1× antibiotic—antimycotic liquid (Gibco BRL). The plates were incubated at 20°C for at least 21 d and monitored for cytopathic effects at least once a week. Cultures showing cytopathic effects were harvested for RT-PCR and electron microscopy study following the methods described above.

#### Results

## Disease History

The disease problem was first recognized in early October 1999. The infected Atlantic cod were hatched from egg batches obtained from wild broodstock that had been collected in Nova Scotian waters. The cod had an average weight of approximately 3.0 g, and they were held in two tanks under the same conditions and with the same water supply as similarly sized haddock, which suffered no disease. Stocking density was low (<500 g/m³), and the tanks were operated as flowthrough systems using filtered and UV-treated seawater at a temperature of 16–17°C. Oxygen levels were maintained at or slightly above saturation levels.

Disease signs included a loss of equilibrium (i.e., a period of swimming on their sides) followed by a period of lethargy during which the fish lay on the bottom of the tank until disturbed. When disturbed, the fish would exhibit abnormal swimming behavior (e.g., looping and spiraling) for a short period of time. No other external signs of disease were evident. These fish were destroyed 35 d after the first signs of the disease, when cumulative mortality in the two tanks had reached 28% and 34%, respectively.

A second outbreak of disease occurred in November 1999. The affected fish were from the same egg source and were being reared in a different building that uses the same water source. At the time of this disease outbreak, these fish had an average ( $\pm$ SD) weight of 19.4  $\pm$  3.8 g; the water temperature in the tank was approximately 12°C, and oxygen levels were at or slightly above saturation. Mortality rates were not monitored, as these fish were destroyed immediately upon the first signs of disease. Throughout this time no evidence of disease was seen in haddock stocks that had been hatched and reared to the juvenile stage within these facilities and that were maintained on the same water supply. In neither of these disease outbreaks was there evidence that the observed

morbidity and mortality were related to environmental conditions or toxic materials within the culture system.

Histological, bacteriological, and viral sampling for culture and RT-PCR screening were carried out to determine whether there was any evidence of an infectious etiological agent. Except for the presence of bloody fluid within the brain cavity of most of the dissected Atlantic cod, there were no obvious gross signs of disease.

## Histopathology

Histopathologic evaluation of samples from 11 moribund Atlantic cod collected in early October just after the first signs of disease were evident revealed low-frequency renal intratubular myxosporidians and mild to marked degenerative changes to the brain and retinal tissues. Specifically, the brain and retinal tissues showed diffuse degenerative vacuolative encephalopathy and degenerative histiocytic retinitis (Figure 1a, b). Subsequent examination of matching tissues using immunohistochemistry revealed positive staining for nodavirus antigens within affected neurons and retinal ganglia cells, primarily within the external nuclear layer (Figure 1c, d).

Using electron microscopy, we were unable to detect virus in the brain tissue from moribund Atlantic cod from either disease outbreak. However, examination of infected SSN-1 cell cultures by electron microscopy revealed a general disruption of cellular structure and the presence of numerous membrane-bound aggregates of viral particles 20–30 nm in diameter within the cells (Figure 2). Examination of uninfected SSN-1 cell cultures revealed intact cells and no evidence of virus aggregates.

#### Cell Culture

No CPE were detected on CHSE and EPC cell cultures inoculated with pooled tissue samples collected from individual moribund Atlantic cod. The development of CPE was evident in cultures of SSN-1 cells within 7 d of inoculation with homogenates made from the brains of moribund cod that had been stored frozen at—80°C. To confirm the presence of nodavirus in CPE-positive cultures, RT-PCR was conducted using a primer set that was designed using the published sequence for Atlantic halibut nodavirus (GenBank accession number AF160473) given in Aspehaug et al. (1999; Table 1). With this primer set a single product of 264 bp could be amplified from CPE-positive cultures. When sequenced, this product was

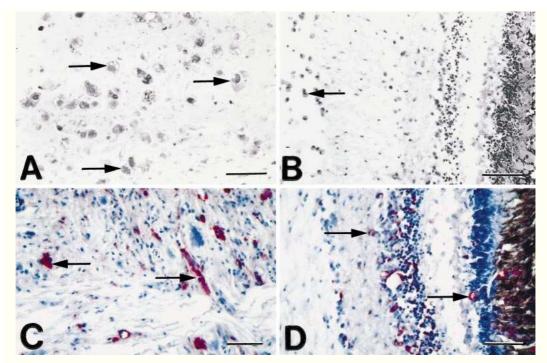


FIGURE 1.—Light micrographs of the neuropil and retina of juvenile Atlantic cod infected with nodavirus. Panel (A) shows the neuropil of an infected host, with arrows indicating vacuolated neurons within the tissue (hematoxylin and eosin staining). Panel (B) shows retinal tissue from an infected host, with the arrow indicating the presence of histiocytes on the surface of the nerve fiber layer (hematoxylin and eosin staining). Panel (C) shows immunostaining of brain tissue. The red stain and arrows indicate antibody-reactive cells. Panel (D) shows immunostaining of retinal tissue. The red stain and arrows indicate antibody-reactive cells within multiple layers of retina. The scale bar =  $50 \mu m$  in all panels.

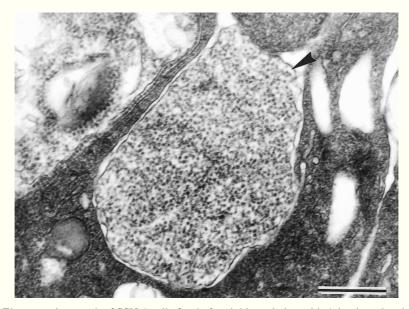


FIGURE 2.—Electron micrograph of SSN-1 cells fixed after 4 d inoculation with Atlantic cod nodavirus. Sections were stained with uranyl acetate. Note the presence of virus particles within a membrane-bound structure (arrowhead). Scale bar = 500 nm.

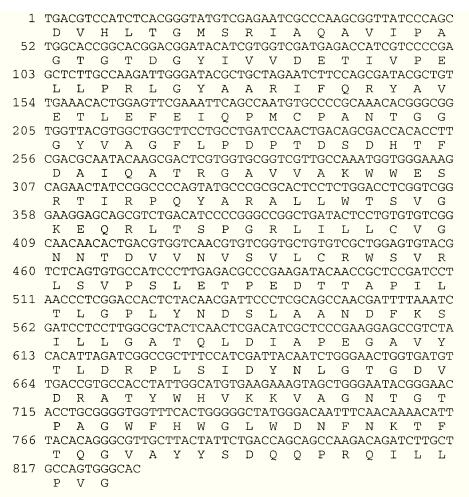


FIGURE 3.—Regions T2 and T4 of the coat protein of Atlantic cod nodavirus cDNA nucleotide and deduced amino acid sequence. The GenBank accession number is AF445800. Numbers are the nucleotide positions from the start of the T2 region.

found to be highly similar to those of nodavirus strains from other fish species, notably barfin flounder and Atlantic halibut.

## RT-PCR Amplified Products

Two other sets of primers were used successfully for RT-PCR amplification of nodavirus RNA2 from Atlantic cod tissues (Table 1). Using the primer set for the T2 region (F1-R3; Nishizawa et al. 1994), a single product of 828 bp was amplified from the eye and brain tissues of moribund fish from the October and November disease outbreaks (Figure 3). With this primer set, there was no amplification of an 828-bp product in 30 brain samples from apparently healthy haddock and 5 samples each of brain and eye tissues from mor-

ibund haddock collected after the November disease outbreak. However, there was amplification of varying degrees of intensity in all haddock samples of a single product of approximately 220 bp. Sequencing this product revealed no similarities to known sequences.

Comparison of the nucleotide sequence of the T2 region with nodavirus data for other fish species indicated a 92% nucleotide identity with barfin flounder and Atlantic halibut nervous necrosis virus (NNV) coat protein genes (GenBank accession numbers D38635 and AJ245641, respectively) and a more than 85% nucleotide identity with other nodavirus coat protein sequences. The deduced amino acid sequence identity was more than 80% similar to that of other nodavirus sequences (Fig-

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```
ACNNV
              Atlantic cod NNV coat protein (AF445800)
BFNNV
              Barfin flounder NNV coat protein (D38635) 96% AA identity
AHNNV
              Atlantic Halibut NNV coat protein (AJ245641) 94% AA identity
PCNNV
              Pacific cod NNV (Nishizawa et al. 1997)
JFNNV
              Japanese flounder NNV coat protein (D38527) 88% AA identity
SJNNV
              Striped jack NNV coat protein (D30814) 81% AA identity
Note: only uppercase letters are considered to be aligned.
               -----DV HLTGMSRIAQ AVIPAGTGTD GYIVVDETIV PELLPRLGYA
BFNNV
               -----DV HLTGMSRIAQ AVIPAGTGTD GYIVVDETIV PELLPRLGEA
AHNNV
         40
               TGFGRGTNDV HLTGMSRIAQ AVIPAGTGTD GYIVVDETIV PELLPRLG<mark>e</mark>A
               -----
PCNNV
          1
               -----DV HLSGMSRISQ AVLPAGTGTD GYVVVDATIV PDLLPRLGHA
TGFGRATNDV HISGMSRIAQ AVVPAGTGTD GKIVVDSTIV PELLPRLGHA
JFNNV
         1
SJNNV
         51
               ARIFORYAVE TLEFEIQPMC PANTGGGYVA GFLPDPTDSD HTFDAIQATR
ACNNV
         43
        43
               ARIFQRYAVE TLEFEIQPMC PANTGGGYVA GFLPDPTDSD HTFDAIQATR
BENNV
AHNNV
               ARIFQRYAVE TLEFEIQPMC PANTGGGYVA GFLPDPTDSD HTFDAIQATR
PCNNV
         1
               ARIFQRYAVE TLEFEIQPMC PANTGGGYVA GFLPDPTDMD HTFDALQATR ARIFQRYAVE TLEFEIQPMC PANTGGGYVA GFLPDPTDMD HTFDALQATR
JFNNV 43
SJNNV 101
ACNNV 93
               GAVVAKWWES RTIRPQYARA LLWTSVGKEQ RLTSPGRLIL LCVGNNTDVV
BFNNV 93
               GAVVAKWWES RTIRPQYARA LLWTSVGKEQ RLTSPGRLIL LCVGNNTDVV
AHNNV 140
               GAVVAKWWES RTIRPOHARA LLWTSVGKEQ RLTSPGRLVL LCAGNNTDVV
PCNNV
         1
               GAVVAKWWES RTURPQYTRT LLWTSSCKEQ RLTSPGRLIL LCVGNNTDVV GAVVAKWWES RTURPQYTRT LLWTSTCKEQ RLTSPGRLUL LCVGSNTDVV
JFNNV 93
SJNNV 151
ACNNV 143
               NVSVLCRWSV RLSVPSLETP EDTTAPILTL GPLYNDSLAA ND--FKSILL
               NVSVLCRWSV RLSVPSLETP EDTFAPILTL GPLYNDSLAA ND--FKSILL NVSVLCRWSV RLSVPSLETP EDTFAPILTL GPLYNDSLAP ND--FKSILL
BFNNV 143
AHNNV 190
PCNNV 1
               ----SV RLSVPSLETP EDTFAPILTL GPLYNDSLAA ND--FKSILL
               NVSVLCRWSV RLSVPSLETP EBITAPINTO GPLYNDSLST ND--FKSILL NVSVMCRWSV RLSVPSLETP EDTTAPITTO APLHNDSIN GYTGFRSILL
JFNNV 143
SJNNV 201
ACNNV 191
               GATQLDIAPE GAVYTLDRPL SIDYNLGTGD VDRATYWHVK KVAGNTGTPA
               GSTQLDIAPE GAVYSLDRPL SIDYSLGTGD VDRAVYWHVK KVAGNVGAPA
GSTQLDIAPD GAVYSLDRPL SIDYSLGTGD VDRAVYWHVK KVAGNVGAPA
GSTQLDIAPD GAVYSLDRPL SIDYSLGTGD VDRAVYWHVK KVAGNVGTPA
GSTELDIAPD GAIFQLDRPL SIDYSLGTGD VDRAVYWHLK KYAGNTGTPA
GSTQLDIAPA NAVFVEDKPL PIDYNLGVGD VDRAVYWHLQ KKAGDTQVPA
BFNNV 191
AHNNV 238
PCNNV 41
JFNNV 191
SJNNV 251
ACNNV 241
               GWFHWGLWDN FNKTFTQGVA YYSDQQPRQI LLPVG---- ----
               GWFHWGLWDN FNKTFTQGVA YYSDAQPRQI LLPVGT---- -----
BFNNV 241
               GWFHWGLWDN FNKTFTQGAA YYSDAQPRQI LLPVGTLFTR ADSgn
AHNNV 288
               GWFHWGLWDN FNKTFTQGVA YYSDAQPRQI LLPVGT---- ----
PCNNV 91
               GWFRWGLWDD FNKTFTDGVA YYSDDOPROI LLPVGT---- -----
GWFDWGLWDD FNKTFTVGAF YYSDQQPRQI LLPAGTLFTR VDSen
JFNNV 241
SJNNV 301
```

FIGURE 4.—Comparison of the derived amino acid sequence of regions T2 and T4 of the coat protein of an Atlantic cod nodavirus (ACNNV) with other highly similar sequences. Shaded boxes denote differences from the ACNNV. The start of region T4 is indicated by an arrow. Sequences were aligned using DiAlign (Morgenstern 1999).

ure 4). At position 22 there is an aspartic acid residue, a feature that is common to all nodaviruses. Using the primer set for the T4 region (F2-R3; Nishizawa et al. 1994), a single product of 422 bp was amplified from the brain tissues of moribund Atlantic cod. The nucleotide sequence

of this region showed identities of 87, 87, and 86% with Pacific cod *Gadus macrocephalus*, Atlantic halibut, and barfin flounder nodavirus strains, respectively, and more than 77% similarity to other nodavirus sequences. A molecular phylogeny was constructed using the T4 region, and Atlantic cod

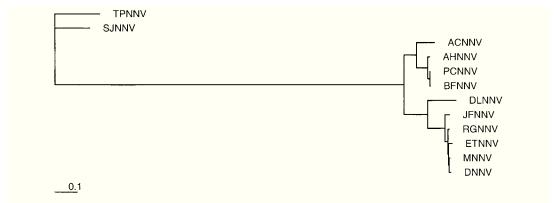


FIGURE 5.—Unanchored phylogenetic tree based on nucleotide sequences of region T4 (379 base pairs) of the coat protein of Atlantic cod nodavirus and other isolates of fish nodaviruses. Nodavirus isolate abbreviations (with GenBank accession numbers in parentheses) are as follows: ACNNV, Atlantic cod (AF445800); AHNNV, Atlantic halibut (AJ245641); BFNNV, barfin flounder (D38635); DLNNV, European seabass *Dicentrarchus labrax* (U39876); DNNV, dragon grouper *Epinephelus laceolatus* (AF245004); ETNNV, greasy grouper *Epinephelus tauvina* (AF318942); JFNNV, Japanese flounder *Paralichthys olivaceus* (D38527); MNNV, Malabar grouper *Epinephelus malabaricus* (AF245003); PCNNV, Pacific cod (Nishizawa et al. 1997); RGNNV, red-spotted grouper (D38636); SJNNV, Striped jack (D30814); and TPNNV, tiger puffer (D38637). The tree was built by means of maximum likelihood criteria using the DNAML program of WebPHYLIP (Lim and Zhang 1999). The length of the horizontal branches is proportional to the number of nucleotide substitutions; the bar = 0.1 nucleotide replacement.

nodavirus was found to cluster with nodaviruses from Atlantic halibut, Pacific cod, and barfin flounder (Figure 5).

#### Discussion

This paper describes the first report of VER in Atlantic cod and the first report of nodavirus on the Atlantic coast of North America. The clinical signs of this disease in cod included dark coloration, loss of equilibrium, uncoordinated swimming patterns, and the presence in some specimens of bloody fluids within the brain case. The behavioral changes noted were consistent with the classical clinical signs reported for this disease in other finfish species (Office International des Epizooties 1997). Brain and retinal tissues from moribund cod showed diffuse degenerative vacuolative encephalopathy and degenerative histiocytic retinitis. The pathological changes observed in infected cod were similar to those reported by other authors for naturally infected fish (Boonyaratpalin et al. 1996; Le Breton et al. 1997; Starkey et al. 2000). The vacuolation of the nervous and eye tissues reported in our study was, however, generally less severe than that reported in other species. We were unable to detect the presence of nodavirus in infected brain tissue by electron microscopy. This is not surprising due to the small size of this virus, which makes it difficult to identify from other components of the cytoplasm. We did not see any evidence of virus aggregates within the cells or the

intracellular spaces, as has been reported for Atlantic halibut (Grotmol et al. 2000). Numerous membrane-bound aggregates of viral particles similar in size (20-30 nm) to that reported for other nodaviruses were evident within the cytoplasm of SSN-1 cells from cell cultures showing CPE. We cannot state with complete certainty that these viral aggregates were nodavirus, as the SSN-1 cell line is spontaneously infected by a C-type retrovirus (Iwamoto et al. 2000). However, these aggregates were not present in uninfected SSN-1 cells, and the C-type retrovirus appears in transmission electron microscopy sections as 90-nmdiameter particles that are not membrane bound and that are located within the extracellular spaces (Iwamoto et al. 2000). Brain and retinal tissues from moribund cod were also observed to be positive for viral antigens by immunohistochemistry using a polyclonal anti-recombinant Atlantic halibut NNV coat protein serum encoded by the T2 region.

Our initial attempt at virus culture using CHSE and EPC cell lines was unsuccessful. We were able, however, to successfully culture virus using the SSN-1 cell line. This cell line has in the past been used for the culture of 17 different nodavirus strains from marine fish (Iwamoto et al. 1999). Virus could be identified in tissue and CPE-positive cell cultures by RT-PCR amplification using primers designed for the coat protein of nodavirus strains from striped

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jack and Atlantic halibut. Specific primers will need to be developed for this Atlantic cod strain of no-davirus, especially if RT-PCR is going to be used as a confirmation for cell culture results or a detection method without cell culture.

The high (>85%) nucleotide identity of the T2 region of the coat protein sequence with other nodavirus coat protein sequences confirms our characterization of this virus as a strain of nodavirus. Based on the variable T4 region of the coat protein sequence, this virus was found to be highly similar (≥86% identity) to the barfin flounder, Atlantic halibut, and Pacific cod nodavirus strains and to be a member of the barfin flounder clade. This clade includes nodavirus strains isolated from Pacific cod, Atlantic halibut, and barfin flounder, all coldwater hosts (Nishizawa et al. 1997; Aspehaug et al. 1999; Grotmol et al. 2000).

During the disease outbreaks, we were unable (using RT-PCR) to detect nodavirus in moribund and apparently healthy haddock juveniles that were being reared within the same facilities. With primers specific for the Atlantic halibut nodavirus, the lower detection limit for RT-PCR was determined to be 100-1,000 copies of in vitro transcribed RNA per sample (Grotmol et al. 2000). It is possible that our RT-PCR technique failed to detect low levels of nodavirus infection in haddock, especially since we were using primers based on sequence data for nodavirus strains from other species. Furthermore, because we do not know the source of the nodavirus infection, we cannot be sure that the haddock were exposed to this viral agent. Nodavirus is thought to be transmitted vertically (Munday and Nakai 1997; Office International des Epizooties 1997). It is possible that the wild Atlantic cod broodstock were infected with nodavirus and that this agent was passed to the progeny. However, we did not observe any obvious clinical signs of nodavirus infection within the larval and juvenile stages of the cod prior to the outbreak of the disease. Although the water used in the facilities within which the disease outbreaks occurred is UV treated, the effectiveness of UV treatment in destroying nodavirus is unknown. It is possible that the virus was present within the water supply. If this is the case, then our inability to identify nodavirus-positive haddock may be due to the fact that this strain is not pathogenic to haddock. Evidence suggests that strains of nodavirus may be highly species specific with respect to their pathogenicity. Totland et al. (1999) reported that the Japanese strain of the striped jack NNV (SJ93Nag; Nishizawa et al. 1997) did not replicate in Atlantic halibut larvae and that the Atlantic halibut NNV strain (AH95NorA; Grotmol et al. 2000) did not replicate in striped jack larvae. Phylogenetic analysis of coat protein sequences suggests that these two strains are quite distantly related (Aspehaug et al. 1999; Grotmol et al. 2000). Studies are required to determine whether the strain isolated from Atlantic cod is pathogenic to other host species within the barfin flounder clade, such as Atlantic halibut. Challenge studies should also be carried out to determine whether this species is pathogenic to haddock and other gadid fishes. Further work is also required to design an optimal RT-PCR detection assay for nodavirus in Atlantic cod.

In summary, juvenile Atlantic cod are susceptible to infection and can undergo disease outbreaks caused by nodavirus. The clinical signs of this disease are similar to those seen in other fish species. Characterization of this nodavirus strain using a partial sequence of its coat protein revealed a very close similarity to nodavirus strains from barfin flounder, Pacific cod, and Atlantic halibut. Based on experiences with rearing other marine finfish, it is possible that disease caused by nodavirus could become a major problem in the development of commercial-scale Atlantic cod production.

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### References

Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25:3389-3402.

Anonymous. 1984. Fish health protection regulations. Manual of compliance. Canadian Special Publication of Fisheries and Aquatic Sciences 31.

Aspehaug, V., M. Devold, and A. Nylund. 1999. The

- phylogenetic relationship of nervous necrosis virus from halibut (*Hippoglossus hippoglossus*). Bulletin of the European Association of Fish Pathologists 19:196–202.
- Boonyaratpalin, S., K. Supamattaya, J. Kasornchandra, and R. W. Hoffmann. 1996. Picorna-like virus associated with mortality and a spongious encephalopathy in grouper *Epinephelus malabaricus*. Diseases of Aquatic Organisms 26:75–80.
- Delsert, C., N. Morin, and M. Comps. 1997. A fish encephalitis virus that differs from other nodaviruses by its capsid protein processing. Archives of Virology 142:2359–2371.
- Grotmol, S., A. H. Nerland, E. Biering, G. K. Totland, and T. Nishizawa. 2000. Characterisation of the capsid protein gene from a nodavirus strain affecting the Atlantic halibut *Hippoglossus hippoglossus* and the design of an optimal reverse-transcriptase polymerase chain reaction (RT-PCR) detection assay. Diseases of Aquatic Organisms 39:79–88.
- Iwamoto, T., K. Mori, A. Arimoto, and T. Nakai. 1999. High permissivity of the cell line SSN-1 for piscine nodaviruses. Diseases of Aquatic Organisms 39:37– 47.
- Iwamoto, T., T. Nakai, K. Mori, M. Arimoto, and I. Furusawa. 2000. Cloning of the fish cell line SSN-1 for piscine nodaviruses. Diseases of Aquatic Organisms 43:81–89.
- Le Breton, A., L. Grisez, J. Sweetman, and F. Ollevier. 1997. Viral nervous necrosis (VNN) associated with mass mortalities in cage-reared sea bass, *Di*centrarchus labrax (L.). Journal of Fish Diseases 20:145–151.
- Lim, A., and L. Zhang. 1999. WebPHYLIP: a web interface to PHYLIP. Bioinformatics 15:1068–1069.
- Morgenstern, B. 1999. DIALIGN2: improvement of the segment-to-segment approach to multiple sequence alignment. Bioinformatics 15:211–218.

- Munday, B. L., and T. Nakai. 1997. Special topic review: nodaviruses as pathogens in larval and juvenile marine finfish. World Journal of Microbiology and Biotechnology 13:375–381.
- Nishizawa, T., M. Furuhashi, T. Nakai, and K. Muroga. 1997. Genomic classification of fish nodaviruses by molecular phylogenetic analysis of the coat protein gene. Applied and Environmental Microbiology 63: 1633–1636.
- Nishizawa, T., K. Mori, T. Nakai, I. Furusawa, and K. Muroga. 1994. Polymerase chain reaction (PCR) amplification of RNA of striped jack nervous necrosis virus (SJNNV). Diseases of Aquatic Organisms 18:103–107.
- Office International des Epizooties. 1997. Viral encephalopathy and retinopathy. Pages 99–107 in Diagnostic manual for aquatic animal diseases. Office International des Epizooties, Paris.
- Page, R. D. M. 1996. TREEVIEW: an application to display phylogenetic trees on personal computers. Computer Applications in the Biosciences 12:357– 358.
- Starkey, W. G., J. H. Ireland, K. F. Muir, A. P. Shinn, R. H. Richards, and H. W. Ferguson. 2000. Isolation of nodavirus from Scottish farmed halibut, *Hippoglossus hippoglossus* (L). Journal of Fish Diseases 23:419–422.
- Sweetman, E., J. Sweetman, A. Le Breton, and L. Grisez. 1996. Nodavirus: a review of the findings of the XIV/NODA/95 investigation. Pages 87–101 in Seabass and seabream culture: problems and prospects. European Aquaculture Society, Oostende, Belgium.
- Totland, G. K., S. Grotmol, Y. Morita, T. Nishioka, and T. Nakai. 1999. Pathogenicity of nodavirus strains from striped jack *Pseudocaranx dentex* and Atlantic halibut *Hippoglossus hippoglossus*, studied by waterborne challenge of yolk-sac larvae of both teleost species. Diseases of Aquatic Organisms 38:169– 175.