Vol. 68: 7–15, 2005 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published December 30

Viral encephalopathy and retinopathy (VER) in Atlantic salmon *Salmo salar* after intraperitoneal challenge with a nodavirus from Atlantic halibut *Hippoglossus hippoglossus*

Kjetil Korsnes[1,3,*], Magnus Devold[2,4], Audun Helge Nerland[1], Are Nylund[2]

[1]Institute of Marine Research, PO Box 1870 Nordnes, 5817 Bergen, Norway
[2]Department of Biology, University of Bergen, 5020 Bergen, Norway
[3]Bodø University College, 8049 Bodø, Norway

[4]*Present address:* PatoGen Analyse AS, Larsgårdveien 4, 6009 Ålesund, Norway

ABSTRACT: Homogenate of tissue from juveniles of Atlantic halibut *Hippoglossus hippoglossus* suffering from viral encephalopathy and retinopathy (VER) was used to challenge smolt of Atlantic salmon *Salmo salar* with an initial average weight of 110 g. The nodavirus was administered in the form of an intraperitoneal injection, and the fish were kept for 134 d post challenge. Genotype characterisation of the nodavirus was performed by sequencing the RNA1 and RNA2 segments, and a quantitative real-time PCR (Q-PCR) assay was developed. Tissues from different organs were stained by immunohistochemistry (IHC). Samples were collected at random on Days 7, 25, 45, 69, 125 and 134 after challenge. Mortality, clinical signs and pathology of VER were observed only in the challenged group. The Q-PCR detected positive fish only in the challenged group, all of which were positive on all days of sampling. An increase in relative virus concentrations was observed from Day 7 to Day 25 post challenge. The increased level of virus concentration was maintained in the medulla oblongata throughout the experiment, suggesting persistence or slow elimination of the virus over time. The IHC detected positive cells on Days 34, 70 and 74. These results suggest that the nodavirus is transported to the medulla oblongata from the intraperitoneal injection site and is able to replicate in salmon. When injected, this nodavirus isolate caused mortality and established a persistent infection in the challenged salmon throughout the experiment. This susceptibility suggests that co-location of salmon and marine species should be avoided until further studies of possible transmission have been carried out.

KEY WORDS: Nodavirus · Atlantic salmon · Atlantic halibut · Experimental infection · Q-PCR · Immunohistochemistry · Viral encephalopathy · Retinopathy

INTRODUCTION

The fish nodaviruses (betanodavirus) are causative agents of viral encephalopathy and retinopathy (VER). They are a widespread group of pathogens that affect a large number of fish species (Munday et al. 2002). On the basis of their coat protein gene sequences (RNA2), fish nodaviruses all cluster within 1 distinct subtype (Nishizawa et al. 1997, Chi et al. 2001, Dalla Valle et al. 2001, Johnson et al. 2002, Gagne et al. 2004, Johansen et al. 2004b). It has been suggested that temperature is more important than host restrictions for the geographical distribution of fish nodavirus subtypes (Nishizawa et al. 1997, Chi et al. 2003). Low host-fish specificity, in which nodaviruses of different subtypes can infect a variety of fish species, has been suggested (Thiery et al. 2004). Susceptibility to nodavirus infections has been demonstrated in cultured cold-water species

*Email: kjetil.korsnes@hibo.no

such as cod *Gadus morhua* (Starkey et al. 2001, Johnson et al. 2002, Gagne et al. 2004), Atlantic halibut *Hippoglossus hippoglossus* (Grotmol et al. 1995, Grotmol et al. 1997b) and spotted wolffish *Anarhichas minor* (Johansen et al. 2003, Sommer et al. 2004). In Norway, Atlantic halibut hatcheries have suffered several outbreaks of VER during the past few years (Dannevig et al. 2000, Johansen et al. 2004a), suggesting the persistence of virus in cultured populations. Although VER has been considered to be a disease of juveniles, individuals weighing several kg have also shown clinical signs of VER (Aspehaug et al. 1999). This observed persistence of nodavirus in adult fish suggests that the virus could be present in farmed halibut for a long time and may represent a risk of spreading nodavirus with movement of persistently infected animals. Transportation of infected animals into areas with potential susceptible hosts may represent one important way of spreading diseases. In Norway, Atlantic salmon *Salmo salar* is the dominant farmed species, but farming of marine fish such as Atlantic cod and halibut is growing. Although VER is not reported in Atlantic salmon, the report of a nodavirus-like agent in heart tissue by Grotmol et al. (1997a) raises the question of potential susceptibility.

Nodaviruses have been regarded as pathogenic to marine fish species, but there have been a few reports on the susceptibility of freshwater species (Athanassopoulou et al. 2003, Chi et al. 2003, Hegde et al. 2003). Hence, the aim of this study was to ascertain whether a nodavirus isolate from a natural outbreak of VER in halibut juveniles would replicate in Atlantic salmon.

MATERIALS AND METHODS

The challenged salmon originated from a commercial hatchery in western Norway. Average weight at the start of the experiment was 110 g, and the fish were adapted to full seawater conditions. They were held for acclimatization to laboratory conditions for 2 wk at 12°C before the experiment, and were attended and fed daily with a commercial diet. Samples from the fish population were initially screened for the presence of known fish pathogenic viruses, including infectious pancreatic necrosis virus (IPNV), infectious salmon anaemia virus (ISAV), salmonid alphavirus (SAV) and nodavirus.

Virus preparation. The nodavirus (isolate AAG01/03) was collected from diseased juveniles of halibut from a hatchery in southern Norway. A suspension of virus was prepared by homogenizing 1 g of brain tissue from 7 individuals. The tissue was homogenized in a mortar in 3 ml Leibowitz's L15 cell medium without glutamine (Cambrex Bio Science). A further 7 ml of L15 cell medium was added after homogenization to make a 1:10 w/v dilution of the homogenate, which was centrifuged for 5 min at 1500 rpm (400 × *g*) to remove cell debris. The supernatant was subjected to a 2-stage filtering step, through a 0.45 µm and a 0.2 µm filter. The filtered 1:10 virus preparation was finally diluted to 1:200 v/v in L15 cell medium and stored at –80°C. This 1:200 dilution was used to infect the fish by intraperitoneal injection. As a control, 50 µl of the 1:10 dilution of virus preparation was tested by quantitative real-time PCR (Q-PCR) in order to detect the presence of nodavirus before injection.

Cell culture. Stripe snakehead (SSN-1) cells were grown according to Dannevig et al. (2000) at 24°C to 60–80% confluency. The virus homogenate (isolate AAG01/03) was inoculated and incubated for 5 d and cells split into subcultures using trypsine-versene. Cells were sampled for nodavirus detection on Days 5 and 10 post inoculation. No CPE was observed, and Q-PCR detected a decrease in the virus quantity, with Ct values of 20.9 and 34.8 respectively. The virus thus did not propagate in SSN-1 cells, and a positive control was therefore prepared by spiking brain tissue from a non-challenged salmon with nodavirus isolate AH95NorA (GenBank accession number AJ401165). The spiked brain tissue also acted as an internal semi-quantitative positive control by the preparation of a 10-fold dilution series of cDNA to be used for Q-PCR detection.

Experimental design. Salmon were divided into a control group (n = 50) and a challenged group (n = 60), which were held in separate 0.15 m^3 tanks with running seawater at a constant temperature of 12°C throughout the experimental period. The challenged group was injected intraperitoneally with 0.2 ml of virus preparation (halibut origin), while the control group was not treated. The duration of the experimental period was 134 d, and all fish remaining at the terminal point were killed by a blow to the head.

Sampling protocol. On Days 7, 25, 45, 69 and 125 post challenge, tissue samples from 5 individuals from the challenged group and 3 individuals from the control group were sampled at random. Fish were killed by an overdose of metacain (Norsk Medisinaldepot). Length, weight and haematocrit (hct) were measured in all individuals sampled. Tissue samples from the medulla oblongata and heart ventricle were collected and stored at –80°C for virus detection. Samples from the medulla oblongata, eye, heart ventricle, spleen, kidney and liver were also sampled on Days 34 (2 challenged), 70 (2 challenged and 1 control), 74 (1 challenged) and 130 (2 challenged and 1 control) for immunohistochemistry and fixed in 4% phosphate-buffered formaldehyde. On Day 134 post challenge a brain sample from 1 challenged individual was exam-

ined by transmission electron microscopy. Tissues from the control group were always sampled first to reduce the risk of cross contamination.

RNA extraction. RNA from the collected samples was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions and stored at –80°C. Extracted RNA was adjusted to approximately 100 ng μl^{-1} by measurement of optical density (SmartSpec™ 3000, Bio-Rad). The RNA was transcribed into cDNA by using Moloney Murine Leukemia Virus reverse transcriptase (Perkin Elmer) according to the protocol of Grotmol et al. (2000). The cDNA was utilized as a template for both Q-PCR and PCR.

Characterization of RNA1 and RNA2 segments of isolate AAGO1/03. Partial sequencing of RNA1 (3.1 kb), encoding protein A (viral part of the RNA-dependent RNA polymerase), and RNA2 (1.3 kb), encoding the capsid protein and protein reviewed by Schneemann et al. (1998) were performed by utilizing 10 oligos for RNA1 and 4 oligos for RNA2 (Tables 1 & 2). Primers were designed by alignment of RNA1 and RNA2 sequences of Atlantic halibut deposited in the GenBank, using accession numbers AJ245641 and AF160473 for RNA2, and NOD401165 for RNA1.

RNA was extracted from 2 sources, i.e. initial virus preparation (halibut) and brain tissue from a single challenged individual (salmon) on Day 25 post injection. Extracted RNA was subjected to reverse transcription followed by PCR amplification of fragments of RNA1 and RNA2 segments as described by Devold et al. (2000). PCR products were prepared for sequencing using a PCR clean-up kit (Montage) according to the manufacturer's instructions. Sequencing was performed using the ABI Prism™ Big-Dye™ Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Applied Biosystems) and analysed on an ABI PRISM® 3700 DNA Analyzer (Applied Biosystems). Overlapping fragments within the RNA1 and RNA2 segments were assembled and aligned with ContigExpress in the Vector NTI™ Advance 9.0 computer package (Invitrogen). GenBank searches were performed using BLAST version 2.0. The RNA1 and RNA2 segments were submitted to GenBank (RNA1 AY962683 and RNA2 AY962682).

Phylogeny. Phylogenetic analysis of a selection of nodaviruses from fish, using the coat protein gene, was performed. In addition to the new sequence presented in this study, a large number of sequences were obtained from the GenBank (Accession nos.: Atlantic cod ACNNVa AF445800 and ACNNVb AY547548, Atlantic halibut AHNNVa AF160473 and AHNNVb NSP245641, barfin flounder BFNNV D38635, Chinese catfish CCNNV AY140794, Cobia CNNV AY140795, *Dicentrarchus labrax* DLNNVa Y08700, DLNNVb U39876 and DLNNVc AJ698094, dragon grouper DNNV AF-245004, *Epinephelus aeneus* EANNVa AY284968 and EANNVb AY284966, *Epinephelus coioides* ECNNV AF534998, *Epinephelus taurina* ETNNV AF318942, firespot snapper FSNNV AY140797, redspotted grouper RGNNV AY140801, guppy GuNNV AF499774, Japanese flounder JFNNVa D38527 and JFNNVb AB045980, *Lates calcarifer* LCNNVa AY284974 and LCNNVb AY284973, *Melanogrammus aeglefinus* MANNV AY547549, *Mugil cephalus* MCNNV AY284962, redspotted grouper RGNNVa AY744705 and RGNNVb D38636, striped jack SJNNVa D30814, SJNNVb AB056572 and SJNNVc NC-003449, tiger puffer TPNNV D38637, turbot TNNV AJ608266, yellow grouper YGNNV AF283554). The alignment was manually adjusted using the sequence alignment editor GeneDoc (available at: www.psc.edu/biomed/genedoc). Analyses were performed using TREE-PUZZLE 5.0 (available at: www.tree-puzzle.de). Phylogenetic trees were drawn using TreeView (Page 1996).

Table 1. Sequence and location on cDNA of primers used for sequencing the RNA1 segment of nodavirus isolate AAG01/03, accession number AY962683. The location on cDNA is given for the nodavirus isolate AH95Nor with accession number AJ401165

Primer	Sequence 5′–3′	Location on cDNA	Temp (°C)
nnvF1	CGCAAGGTTACCGTTTAAGC	36–55	68
nnvF2	TACTGACGATTCCGTTCACTACCG	624–647	68
nnvF3	GTAGTACATAAGCCTGGTGACCTGG	1222–1246	66
nnvF4	GCTGCCAGTACCACTGAGTTGG	1801–1822	68
nnvF5	GCTCCTCAAGCACATTGCG	2601–2619	67
nnvR1	TAGAGGAAGCCGCACAGAGC	797–778	67
nnvR2	AATCGTGGCACGCTCGTTGG	1392–1373	73
nnvR3	AGTTGTGAGTGCCGAACCGC	2034–2015	70
nnvR4	TCTCGTTCACCTGGACCACG	2871–2851	69
nnvR5	GCGTAAAGCTGACTAGGG	3084–3067	58

Table 2. Sequence and location on cDNA of primers used for sequencing the RNA2 segment of nodavirus isolate AAG01/03, accession number AY962682. The location on cDNA is given for the nodavirus isolate AH95NorA with accession number AJ245641

Primer	Sequence 5′–3′	Location on cDNA	Temp (°C)
VNNA	CTGATCCAACTGACAGCGACC	369–389	67
VNNC	ACTGTAGTCAATGGACAGCGGC	787–766	68
VNNRm	ACGGTAACCCAACAAGCCC	1323–1305	66
VNNFm	CTAAACCAGCGACCACAAAGG	12–32	66

Q-PCR protocol. Primers and probe for Q-PCR (Table 3) were designed aligning RNA2 segments from nodavirus deposited in the GenBank (accession nos.: AJ245641, AJ160473, D38635, AF445800, AY-547548, AY547549, AY547547, D30814 and D38527). The Q-PCR assay utilised a TaqMan® MGB probe (Applied Biosystems) and amplification was performed using ABI PRISM model 7700, under the following conditions: 2X TaqMan Universal PCR Master Mix, 900 nM of each primer, 250 nM TaqMan®MGB probe, 5 µl cDNA and DNAse free water to a final volume of 25 µl.

The thermocycler program consisted of a 2 min hold at 50°C, a 10 min hold at 95°C and 40 cycles consisting of 15 s at 95°C and 1 min at 60°C. Results were analyzed with the computer program Sequence Detector version 1.9 (Applied Biosystems). As an internal control of RNA and cDNA yield, elongation factor α was used according to the description of Moore et al. (2005). Heart ventricle tissue from 3 individuals (2 challenged and 1 control) from each sampling day were screened by Q-PCR, utilising specific probes and primers for detection of ISAV (Plarre et al. 2005), SAV (Hodneland et al. 2005) and IPNV (Watanabe et al. unpubl.). These viruses are frequently detected in commercial salmon farms in Norway.

Immunohistochemistry (IHC). Organ samples from brain, eye, heart, spleen, kidney and liver were collected for IHC from 7 challenged and 3 control fish. These samples consisted of 2 challenged fish on Day 34, 2 challenged and 1 control fish on Day 70, 1 challenged fish on Day 74 and 2 challenged and 1 control fish on Day 130. In addition, 1 control fish originating from a commercial fish farm was included to detect any non-specific binding of antibody. Tissue samples were dehydrated and embedded in paraffin and 3 µm section were cut on a microtome (Jung Biocut 2035) and placed on Superfrost slides. The primary antibody (Ø150) was used as described by Grotmol et al. (1999), with modifications by Sommerset & Nerland (2004).

Table 3. RNA2 primers and probe designed for detection of nodavirus by Q-PCR. The location on cDNA is given for nodavirus isolate AH95NorA with accession number AJ245641

	Sequence 5′–3′	Location on cDNA	Temp (°C)
Primer			
VERF	TTCCAGCGATACGCTGTTGA	278–297	66
VERR	CACCGCCCGTGTTTGC	347–332	67
Probe			
VER1	AAATTCAGCCAATGTGCCCC	312–331	66

Transmission electron microscopy (TEM). Tissue from the medulla oblongata from 1 challenged salmon was sampled at Day 134 post challenge and fixed in Karnovsky fixative. The fixed tissue was embedded in Epon 812 and historesin and sectioned for transmission electron microscopy as described by Nylund et al. (1995).

RESULTS

Mortality

During the experimental period mortality was first observed in the infected group on Day 32 (1 dead), followed by subsequent mortalities on Day 63 (4 dead and 2 moribund), 70 (1 dead), 76 (2 dead) and 125 (2 moribund). No mortality was observed in the control group. The experiment was terminated 134 d after challenge.

Clinical signs and gross pathology

The challenged group of fish gradually developed abnormal behavior. These fishes showed hyper-activity, and an abnormal, frequently erratic, pattern of swimming. The control group had a normal swimming pattern and showed no signs of hyperactivity in the course of the experiment. However, no differences in appetite were observed in either of the fish groups, and there were no significant differences in growth performance (Fig. 1).

Pathology was observed on Day 63 in moribund fish. The pathological signs consisted of haemorrhages in the brain, and on Days 70, 73, 76 and 125 the pathological signs were extensive, with erythema in the eye and fin base, petechiae in the liver, ascitic fluids in the swim-bladder, adherence in viscera and swollen spleen. However, the vacuolisation in the retina and brain are not as extensive as observed in Atlantic halibut suffering from VER (see Fig. 5). There were no significant differences in haematocrit between the challenged and non-challenged group during the experimental period (Fig. 2).

Characterisation of isolate AAG01/03

Using primers designed from published sequences, followed by sequencing of the products, 3041 nucleotides (nt) of RNA1 and 1296 nt of RNA2 were obtained by RT-PCR. Sequence comparison revealed high similarities with other nodaviruses isolated from

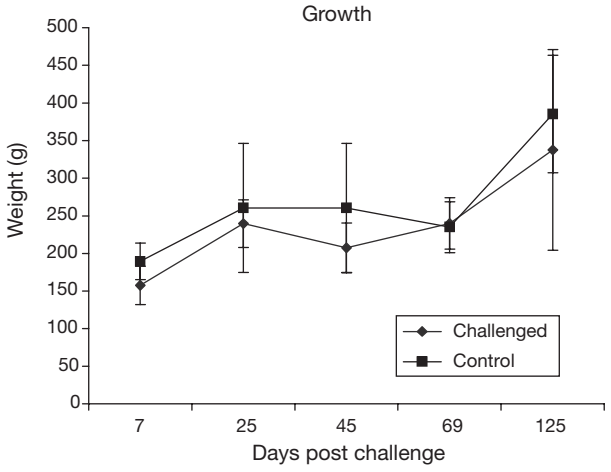

Fig. 1. *Salmo salar*. Growth performance of challenged and control group at sampling Days 7, 25, 45, 69 and 125, where n = 5 for each sampling day

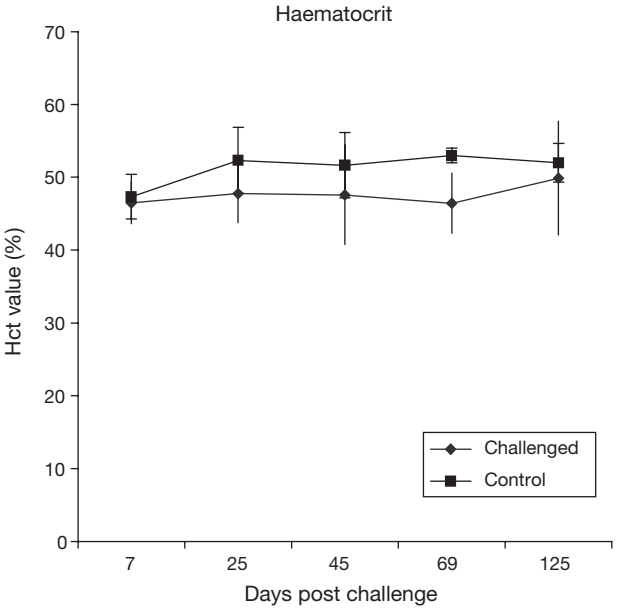

Fig. 2. *Salmo salar*. Haematocrit (hct) in challenged and control groups on sampling Days 7, 25, 45, 69 and 125, where n = 5 for each sampling day

Atlantic halibut (Aspehaug et al. 1999, Grotmol et al. 2000, Sommerset & Nerland 2004). Within the encoding part of RNA1 only 13 out of 2943 nt were different between AAG01/03 and AH95NorA (GenBank accession no. NOD401165), and only 1 of these substitutions causes a change of the encoded amino acid. The sequence obtained did not include the ends of the RNA2 segment. Therefore, 44 nt at the beginning of the encoding part of RNA2 remained unknown. However, for the remaining 970 nt belonging to the encoding area, only 7 nucleotides were different between AAG01/03 and AH95NorA (GenBank accession no. NSP245641), and 3 of these cause coding of different amino acids.

Phylogeny

Phylogenetic analysis of AAG01/03 using the coat protein gene (AY962682) in an alignment of related sequences from GenBank showed that the nodavirus is closely related to other nodaviruses from Atlantic halibut and fish from cold waters (Fig. 3).

Q-PCR results

Q-PCR testing of the 1:10 dilution of virus preparation resulted in a Ct value of 15.63, demonstrating a high level of nodavirus in the homogenate used to challenge the salmon. Comparison of sequenced RNA1 segments from the RNA extractions of virus preparation (halibut) and tissue of medulla oblongata from 1 individual on Day 25 post challenge (salmon), gave sequences identical to the AAG01/03 isolate.

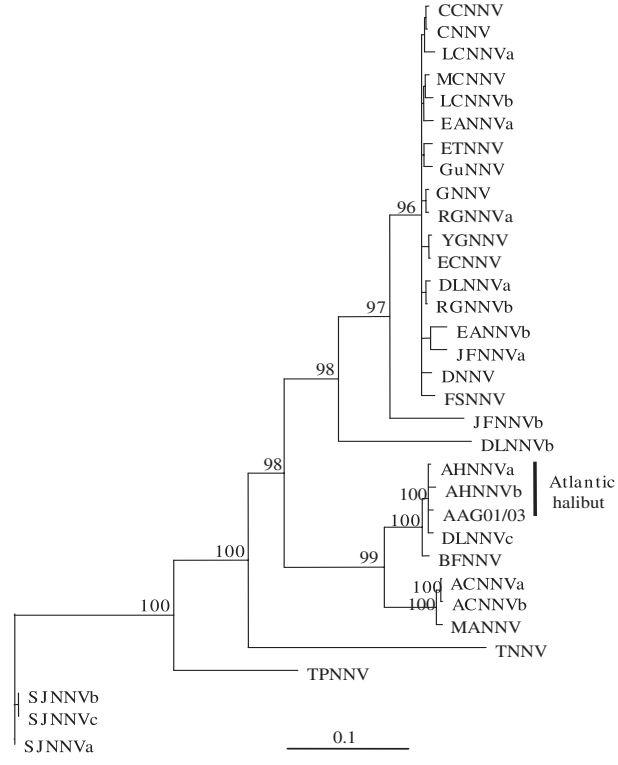

Fig. 3. Phylogenetic position of nodavirus AAG01/03 from Atlantic halibut compared to a selection of other nodaviruses from fish. The evolutionary relationship is presented as a maximum likelihood tree based on an alignment of sequences of segment 2, the coat protein gene. Three nodaviruses from striped jack (SJNNV) were used as outgroup. Branch lengths represent relative phylogenetic distances according to maximum likeli-hood estimates

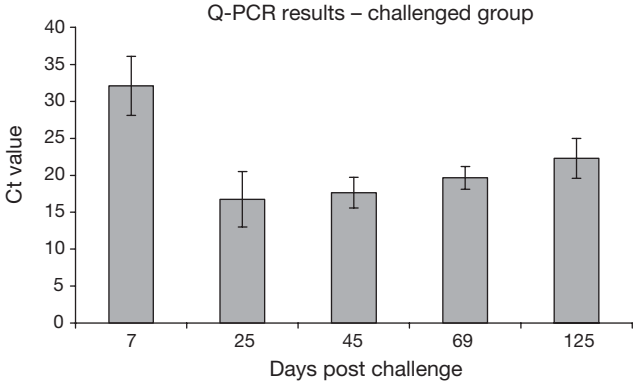

Fig. 4. *Salmo salar*. Average Ct values detected in brain tissue of experimentally challenged salmon on sampling Days 7, 25, 45, 69 and 125 post challenge. Each sampling day consisted of 5 individuals (except Day 7, when n = 4). A decrease in the Ct value represents an increase in the virus concentration. The control group was negative throughout the experiment

Prior to the experimental challenge, the fish population was screened by Q-PCR for common viruses in Norwegian aquaculture, including ISAV, SAV, IPNV and nodavirus. RNA was extracted from the heart ventricle, cDNA synthesis was performed, and used as template for Q-PCR. No fish were positive for these viruses. Amplification efficiency of the Q-PCR was calculated by establishing a 10-fold dilution curve of cDNA from nodavirus isolate AH95norA spiked brain tissue. This 10-fold dilution series could be represented as the simple linear function $f(x) = 3.44x + 16.65$, with the regression coefficient $R^2 = 0.99$. This equation gives a 10-fold difference in relative concentration of samples compared, when a 3.44 discrepancy in Ct value is observed.

Detection of elongation factor α by Q-PCR was used as a control of RNA extraction efficiency and cDNA synthesis. This Q-PCR generated an average Ct value of 21.74, with a mean deviation of ±1.63.

There was no detection of nodavirus in the control group throughout the experiment. On Day 7 post challenge, all tissues of medulla oblongata examined from the challenged group were positive. This shows that there is a transportation of virus from the intraperitoneal injection site to the medulla oblongata. The decrease in Ct values observed on Day 25 post challenge demonstrates a proliferation of virus in the brain tissue, representing an approximately 10^3 to 10^4 increase in relative virus concentration as compared to Day 7. The virus infection was maintained in the medulla oblongata of challenged fish throughout the experimental period (Fig. 4).

Heart ventricle tissues in fish from the challenged group were screened for presence of ISAV, IPNV and SAV. A total of 15 samples from each sampling day were examined. A single fish sample tested positive for SAV on Day 7 post challenge, generating a Ct value of 38.50. No fish were positive for ISAV or IPNV. Screening of heart ventricle tissues of dead and moribund fish (n = 4) gave negative results for ISAV, SAV and IPN. Dead and moribund fish were positive for nodavirus in both heart ventricle and the medulla oblongata, generating mean deviation Ct values of 34.91 ± 1.54 and 19.5 ± 0.69 respectively.

Immunohistochemistry

On Days 34, 70 and 74 post challenge, all samples from challenged fish were positive for nodavirus by IHC in the medulla oblongata. Positive cells in other tissues than brain were only found in 1 challenged fish on Day 70; these cells were in kidney, eye and spleen. No positive cells were found in challenged fish on Day 130 or in any of the negative controls examined. The negative control originating from a commercial farmed salmon suggests that there is no non-specific binding of the antibody used. Generally, only few cells were positive, and these were scattered throughout the parenchyma (Fig. 5).

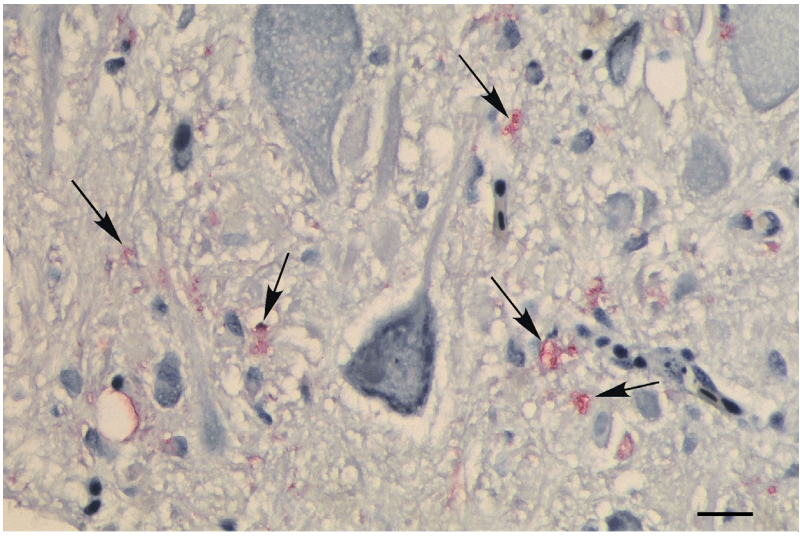

Fig. 5. *Salmo salar*. Light microscopy observations in nodavirus-challenged salmon. Immunolabelled section of the medulla oblongata 34 d after challenge, where the positive cells (arrows) are scattered in the parenchyma. Scale bar = 20 µm

Transmission electron microscopy (TEM)

Nodavirus-like particles were observed by TEM in the medulla oblongata in a challenged salmon after 134 d (Fig. 6). The virus particles were observed in membrane bound vacuoles in brain cells, with a particle size between 20 and 30 nm. The observed virus particles match the size of nodavirus.

DISCUSSION

In this study Atlantic salmon *Salmo salar* with an initial average weight of 110 g were successfully challenged by intraperitoneal injection of nodavirus. A persistent infection was established, which was confirmed by virus detection throughout the experimental period by means of Q-PCR and immunohistochemistry. Challenged fish showed clinical signs of VER, pathology and mortality, and these signs were only observed in the challenged group.

Sequencing the virus isolate used for the challenge (AAG01/03) revealed over 99% identities of RNA1 and RNA2 with previously sequenced genomes of nodavirus isolated from Atlantic halibut, both at the nucleotide and amino acid level. This is also reflected in the phylogenetic analysis, where this strain reveals a close relationship with other halibut and cold-water genotypes. Sequences were obtained from brain tissue of challenged salmon, and the dominating nodavirus in the brain had a sequence identical to the dominant virus in the homogenate of halibut used for the challenge. Consequently, no detection of possible host adaptation was observed in the virus obtained from challenged salmon.

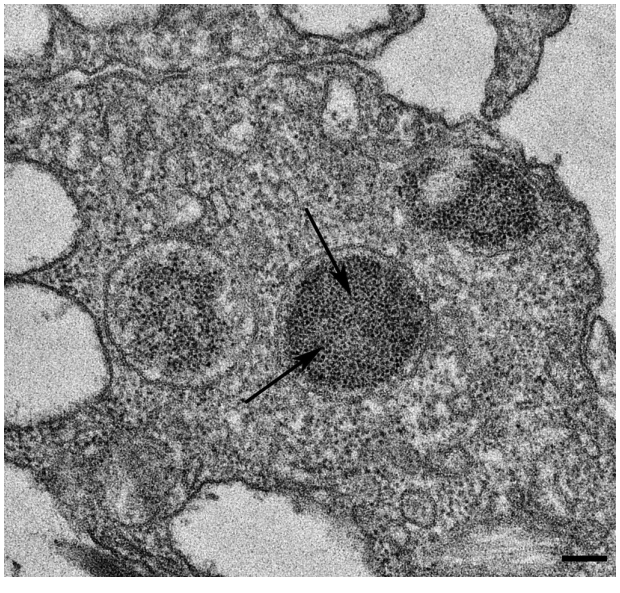

Fig. 6. *Salmo salar*. Ultra thin section of the medulla oblongata of a salmon 134 d after challenge. Nodavirus-like particles (arrows) are present in membrane bound vacuoles within an unidentified brain cell. Scale bar = 0.2 µm

The virus AAG01/03 failed to propagate in the SSN-1 cell line, which may be due to the source, juvenile Atlantic halibut, suffering from VER in a commercial hatchery. This is in contrast to other reports on halibut nodavirus genotypes (Dannevig et al. 2000, Grove et al. 2003). However, there has also been a report of failure of a turbot nodavirus from a natural VER outbreak to propagate in this cell line (Johansen et al. 2004b), indicating a possible sub-optimality of the SSN-1 cell line.

Intraperitoneal injection of nodavirus has been successfully used to challenge adult seabass *Dicentrarchus labrax* of 140 g, in which clinical signs and mortality were observed (Skliris & Richards 1999a). In Atlantic halibut *Hippoglossus hippoglossus* of 20 g, no clinical signs of mortality were observed, but Q-PCR detection revealed an increase in virus in brain tissue post injection (Grove et al. 2003). In similar studies in spotted wolffish *Anarhichas minor*, a nodavirus originating from Atlantic halibut caused mortality in 10 g fish after intraperitoneal and intramuscular injections as well as in 0.7 g juveniles after bath-challenge (Johansen et al. 2003, Sommer et al. 2004). However, reports on juvenile sea bream *Sparus aurata* suggest that there are differences in host susceptibility, as intraperitoneal injection failed to reproduce clinical signs (Aranguren et al. 2002). Low mortality in juvenile sea bass has been reported following intraperitoneal challenge compared to intramuscular, bath and cohabitant exposures (Peducasse et al. 1999). By injecting nodavirus intraperitoneally, this challenge experiment does not take into account the role of important parts of the immune system of salmon. However, the finding of proliferation of the injected virus in target cells in brain shows a state of susceptibility in the salmon host. Hence, this study demonstrates that nodavirus that is able to infect target cells in the salmon host, may replicate and cause disease. However, this study does not show transmission of nodavirus between halibut and salmon but the existence of susceptibility in the salmon host to the particular nodavirus isolate. Consequently, further studies addressing possible transmission should be carried out.

In this study nodavirus was detected in brain tissue of challenged salmon 7 d post intraperitoneal injection, demonstrating a transportation of virus particles from the injection site to the brain. A peak in virus concentration was reached on Day 25, and the subsequent samplings may suggest a slow decrease of virus during the experimental period. This demonstrates that virus particles are able to propagate and establish a persis-

tent infection. The detection of nodavirus by Q-PCR is supported by immunohistochemistry, which revealed positive cells in the medulla oblongata. In contrast to reports of nodavirus infections in halibut and spotted wolffish (Grotmol et al. 2000, Johansen et al. 2002, Johansen et al. 2003), the positive cells in brain tissue of salmon were scattered rather than focally aggregated. On Day 130 no positive cells were detected, and this demonstrates the lower sensitivity of the immunohistochemistry method. However, the examination of brain tissue from a challenged individual on Day 134 by TEM revealed nodavirus-like particles in vacuoles in brain cells. This supports the Q-PCR detections and confirms the presence of virus at the terminal point of the experiment. Examination by Q-PCR of heart tissue of dead and moribund salmon in the challenged group revealed the presence of nodavirus, supporting a previous finding of nodavirus-like particles in salmon suffering from cardiac myopathy syndrome (Grotmol et al. 1997a). Nodavirus distribution in affected hosts appears to be systemic, and the presence of virus in different tissues has been reported in several studies (Mushiake et al. 1994, Nguyen et al. 1997, Breuil et al. 2000). In this study only 1 fish out of 7 examined by IHC was positive in other tissues (spleen, eye and kidney) than brain. However, these tissues were not examined by Q-PCR. A comparison of the results from the Q-PCR and IHC methods demonstrates the difference in sensitivity. The Q-PCR developed may represent a sensitive and specific method for detecting noda-virus in cold-water fish species.

Mortality in the challenged group was observed from Day 34 onwards. Due to the nature of the sampling in this study, a mortality rate could not be established. However, the observation of dead and moribund smolts exceeding 200 g suggests a potentially high virulence of the nodavirus isolate AAG01/03. Although virulence and host-specificity have not been widely studied, some reports have been published. One study did not find the Atlantic halibut nodavirus isolate AH95NorA to be virulent in striped jack *Pseudocaranx dentex* (Totland et al. 1999). However, another report suggests low host-fish specificity, as a cold-water clade nodavirus was isolated from sea bass *Dicentrarchus labrax* during an outbreak of VER at low temperature (Thiery et al. 2004). The same conclusion was reached by a study of nodavirus infections in Israeli mariculture (Ucko et al. 2004). The low host-fish specificity view is also supported by our study of Atlantic salmon. The various isolate clusterings may rather be a result of geographic distribution or establishment in particular host populations. Further, susceptibility of fish in freshwater (Hegde et al. 2003), and fish reared in freshwater has also been reported (Skliris & Richards 1999b, Athanassopoulou et al. 2003).

In conclusion, this study has demonstrated that the nodavirus isolate AAG01/03 was neuroinvasive and proliferated in brain of salmon after intraperitoneal injection. The presence of clinical signs of VER and mortality demonstrate that the salmon is susceptible to this nodavirus isolate after challenge. This raises the question about potential risk for transmission of nodavirus from Atlantic halibut to Atlantic salmon and vice versa. Hence, future studies should address this question, in order to study possible transmission in cultures of salmonid and marine species. Since nodavirus is able to propagate in farmed salmon, co-location with marine species should be avoided until further studies have been carried out.

Acknowledgements. The Institute of Marine Research and the Fish Disease Group of the Department of Biology at the University of Bergen financially supported this study. The authors thank K. F. Ottem, M. Karlsen, F. Fridell, A. G. Eriksen, I. U. Fiksdal, Dr. C. Skår, Dr. Ø. Bergh and K. Watanabe for valuable advice and technical assistance.

LITERATURE CITED

Aranguren R, Tafalla C, Novoa B, Figueras A (2002) Experimental transmission of encephalopathy and retinopathy induced by nodavirus to sea bream, *Sparus aurata* L., using different infection models. J Fish Dis 25:317–324

Aspehaug V, Devold M, Nylund A (1999) The phylogenetic relationship of nervous necrosis virus from halibut (*Hippoglossus hippoglossus*). Bull Eur Assoc Fish Pathol 19: 196–202

Athanassopoulou F, Billinis C, Psychas V, Karipoglou K (2003) Viral encephalopathy and retinopathy of *Dicentrarchus labrax* (L.) farmed in fresh water in Greece. J Fish Dis 26: 361–365

Breuil G, Pepin JF, Castric J, Fauvel C, Thiery R (2000) Detection of serum antibodies against nodavirus in wild and farmed adult sea bass: Application to the screening of broodstock in sea bass hatcheries. Bull Eur Assoc Fish Pathol 20:95–100

Chi SC, Lo BJ, Lin SC (2001) Characterization of grouper nervous necrosis virus (GNNV). J Fish Dis 24:3–13

Chi SC, Shieh JR, Lin SJ (2003) Genetic and antigenic analysis of betanodaviruses isolated from aquatic organisms in Taiwan. Dis Aquat Org 55:221–228

Dalla Valle L, Negrisolo E, Patarnello P, Zanella L, Maltese C, Bovo G, Colombo L (2001) Sequence comparison and phylogenetic analysis of fish nodaviruses based on the coat protein gene. Arch Virol 146:1125–1137

Dannevig BH, Nilsen R, Modahl I, Jankowska M, Taksdal T, Press CM (2000) Isolation in cell culture of nodavirus from farmed Atlantic halibut *Hippoglossus hippoglossus* in Norway. Dis Aquat Org 43:183–189

Devold M, Krossøy B, Aspehaug V, Nylund A (2000) Use of RT-PCR for diagnosis of infectious salmon anaemia virus (ISAV) in carrier sea trout *Salmo trutta* after experimental infection. Dis Aquat Org 40:9–18

Gagne N, Johnson SC, Cook-Versloot M, MacKinnon AM, Olivier G (2004) Molecular detection and characterization of nodavirus in several marine fish species from the northeastern Atlantic. Dis Aquat Org 62:181–189

Grotmol S, Totland GK, Kryvi H (1997a) Detection of a nodavirus-like agent in heart tissue from reared Atlantic salmon *Salmo salar* suffering from cardiac myopathy syndrome (CMS). Dis Aquat Org 29:79–84

Grotmol S, Totland GK, Thorud K, Hjeltnes BK (1997b) Vacuolating encephalopathy and retinopathy associated with a nodavirus-like agent: A probable cause of mass mortality of cultured larval and juvenile Atlantic halibut *Hippoglossus hippoglossus*. Dis Aquat Org 29:85–97

Grotmol S, Bergh O, Totland GK (1999) Transmission of viral encephalopathy and retinopathy (VER) to yolk-sac larvae of the Atlantic halibut *Hippoglossus hippoglossus*: occurrence of nodavirus in various organs and a possible route of infection. Dis Aquat Org 36:95–106

Grotmol S, Nerland AH, Biering E, Totland GK, Nishizawa T (2000) Characterisation of the capsid protein gene from a nodavirus strain affecting the Atlantic halibut *Hippoglossus hippoglossus* and design of an optimal reverse-transcriptase polymerase chain reaction (RT-PCR) detection assay. Dis Aquat Org 39:79–88

Grotmol S, Totland GK, Kvellestad A, Fjell K, Olsen AB (1995) Mass mortality of larval and juvenile hatchery-reared halibut (*Hippoglossus hippoglossus*) associated with the presence of virus-like particles in the central nervous system and retina. Bull Eur Assoc Fish Pathol 15: 176–180

Grove S, Johansen R, Dannevig BH, Reitan LJ, Ranheim T (2003) Experimental infection of Atlantic halibut *Hippoglossus hippoglossus* with nodavirus: tissue distribution and immune response. Dis Aquat Org 53:211–221

Hegde A, Teh HC, Lam TJ, Sin YM (2003) Nodavirus infection in freshwater ornamental fish, guppy, *Poicelia reticulata* — comparative characterization and pathogenicity studies. Arch Virol 148:575–586

Hodneland K, Bratland A, Christie KE, Andresen C, Nylund A (2005) New subtype of salmonid alphavirus (SAV), togaviridae, from Atlantic salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss* in Norway. Dis Aquat Org 66: 113–120

Johansen R, Ranheim T, Hansen MK, Taksdal T, Totland GK (2002) Pathological changes in juvenile Atlantic halibut *Hippoglossus hippoglossus* persistently infected with nodavirus. Dis Aquat Org 50:161–169

Johansen R, Amundsen M, Dannevig BH, Sommer AI (2003) Acute and persistent experimental nodavirus infection in spotted wolffish *Anarhichas minor*. Dis Aquat Org 57: 35–41

Johansen R, Grove S, Svendsen AK, Modahl I, Dannevig B (2004a) A sequential study of pathological findings in Atlantic halibut, *Hippoglossus hippoglossus* (L), throughout one year after an acute outbreak of viral encephalopathy and retinopathy. J Fish Dis 27:327–341

Johansen R, Sommerset I, Torud B, Korsnes K, Hjortaas MJ, Nilsen F, Nerland AH, Dannevig BH (2004b) Characterization of nodavirus and viral encephalopathy and retinopathy in farmed turbot, *Scophthalmus maximus* (L.). J Fish Dis 27:591–601

Johnson SC, Sperker SA, Leggiadro CT, Groman DB, Griffiths SG, Ritchie RJ, Cook MD, Cusack RR (2002) Identification and characterization of a piscine neuropathy and nodavirus from juvenile Atlantic cod from the Atlantic coast of North America. J Aquat Anim Health 14:124–133

Moore LJ, Somamoto T, Lie KK, Dijkstra JM, Hordvik I (2005) Characterisation of salmon and trout CD8 alpha and CD8 beta. Mol Immunol 42:1225–1234

Munday BL, Kwang J, Moody N (2002) Betanodavirus infections of teleost fish: a review. J Fish Dis 25:127–142

Mushiake K, Nishizawa T, Nakai T, Furusawa I, Muroga K (1994) Control of VNN in striped jack — selection of spawners based on the detection of SJNNV gene by Polymerase Chain-Reaction (PCR). Fish Pathol 29:177–182

Nguyen HD, Mushiake K, Nakai T, Muroga K (1997) Tissue distribution of striped jack nervous necrosis virus (SJNNV) in adult striped jack. Dis Aquat Org 28:87–91

Nishizawa T, Furuhashi M, Nagai T, Nakai T, Muroga K (1997) Genomic classification of fish nodaviruses by molecular phylogenetic analysis of the coat protein gene. Appl Environ Microbiol 63:1633–1636

Nylund A, Hovland T, Watanabe K, Endresen C (1995) Presence of infectious Salmon Anemia Virus (Isav) in tissues of Atlantic Salmon, *Salmo salar* L., collected during 3 separate outbreaks of the disease. J Fish Dis 18:135–145

Page RDM (1996) TreeView: An application to display phylogenetic trees on personal computers. Comput Appl Biosci 12:357–358

Peducasse S, Castric J, Thiery R, Jeffroy J, Le Ven A, Laurencin FB (1999) Comparative study of viral encephalopathy and retinopathy in juvenile sea bass *Dicentrarchus labrax* infected in different ways. Dis Aquat Org 36:11–20

Plarre H, Devold M, Snow M, Nylund A (2005) Prevalence of infectious salmon anemia virus (ISAV) in wild salmonids in Western Norway. Dis Aquat Org 66:71–79

Schneemann A, Reddy V, Johnson JE (1998) The structure and function of nodavirus particles: A paradigm for understanding chemical biology. Adv Virus Res 50:381–446

Skliris GP, Richards RH (1999a) Induction of nodavirus disease in seabass, *Dicentrarchus labrax*, using different infection models. Virus Res 63:85–93

Skliris GP, Richards RH (1999b) Nodavirus isolated from experimentally infected tilapia, *Oreochromis mossambicus* (Peters). J Fish Dis 22:315–318

Sommer AI, Strand MA, Rasmussen E, Mennen S (2004) Susceptibility of spotted wolffish *Anarhichas minor* to experimental infection with nodavirus and infectious pancreatic necrosis virus. Dis Aquat Org 59:101–108

Sommerset I, Nerland AH (2004) Complete sequence of RNA1 and subgenomic RNA3 of Atlantic halibut nodavirus (AHNV). Dis Aquat Org 58:117–125

Starkey WG, Ireland JH, Muir KF, Jenkins ME, Roy WJ, Richards RH, Ferguson HW (2001) Nodavirus infection in Atlantic cod and Dover sole in the UK. Vet Rec 149: 179–181

Thiery R, Cozien J, de Boisseson C, Kerbart-Boscher S, Nevarez L (2004) Genomic classification of new betanodavirus isolates by phylogenetic analysis of the coat protein gene suggests a low host-fish species specificity. J Gen Virol 85:3079–3087

Totland GK, Grotmol S, Morita Y, Nishioka T, Nakai T (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. Dis Aquat Org 38:169–175

Ucko M, Colorni A, Diamant A (2004) Nodavirus infections in Israeli mariculture. J Fish Dis 27:459–469

Editorial responsibility: Carey Cunningham, Aberdeen, UK

Submitted: May 24, 2005; Accepted: September 30, 2005
Proofs received from author(s): December 23, 2005