TERMINATION REPORT

Project Code: 03-17

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supported by 2002-38500-12056

Project Title: "Development of diagnostic and management techniques to select cod broodstocks and hatchery stocks free from nodavirus"

Reporting Period: November 2003 – October 2006

Funding Level: $124,612

Participants:

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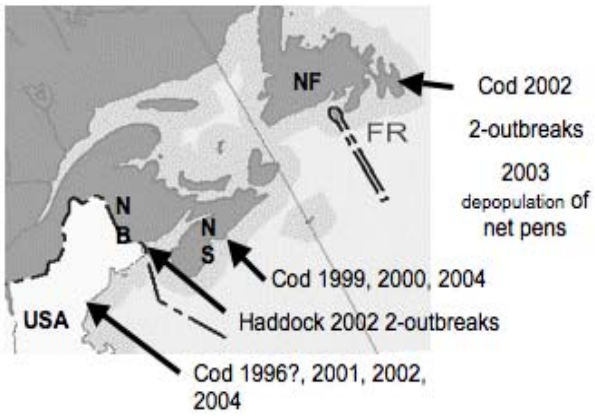
Reason for Termination: End of Project

Project Objectives:

1. Continue the development and refinement of a Real-time PCR assay for the detection of cod nodavirus in cod broodstocks, gametes, fertilized eggs and larvae.
2. Validate the use of the Real-time PCR assay for detecting nodavirus in infected tissues (brain, eyes, eggs, larvae and juveniles) and for the non-lethal detection of nodavirus in cod broodstock (via blood and/or gametes).
3. Screen captive broodstocks and progeny in New England to test the Real-time PCR assay in an industry setting and explore the development of a reagent kit.
4. Develop egg disinfection methods that effectively eliminate nodavirus on the surface of eggs and possible vertical transmission.
5. Develop extension and outreach materials and presentations for nodavirus detection and disinfection.

Background

In the last ten years, nodavirus infections that cause viral encephalopathy and retinopathy (VER, and also sometimes known as viral nervous necrosis (VNN)) have become a major disease concern for many cultured marine fish species. Nodaviruses are small (25-34 nm) icosahedral viruses that infect the central nervous system (CNS) of the fish. The genome of this virus is composed of two single stranded, positive-sense RNA molecules, RNA1 and RNA2. Outbreaks of cod nodavirus in Atlantic Canada and New Hampshire have caused catastrophic mortality at the hatchery and nursery stages (Figure 1). In order to prevent additional set-backs to New England's developing marine fish aquaculture industry, the current research project focused on obtaining a better understanding of this epidemic and means to properly manage it.



Anticipated Benefits

One of the primary benefits of the completed research is a real-time PCR assay for nodavirus that can be used to accurately quantify levels of the deadly virus. In addition, the assay can be used to detect nodavirus through non-lethal means. The diagnostic activities carried out as part of this project in combination with our egg disinfection trials contributed to the understanding that nodavirus is prevalent in the environment. The products developed as part or this project will lay the groundwork for future projects characterizing nodavirus etiology and exploring vaccine development.

Principal Accomplishments

Development of a real-time quantitative RT-PCR assay for nodavirus in cod was completed during the first year of the project. Real-time PCR monitors in actual time the fluorescence emitted during the PCR reaction as an indicator of amplicon production. In Real-time, the amplicon is produced by a pair of unlabelled primers. Interior to these primers is a dual-labeled oligonucleotide probe that is 20-30 bp with a Tm value of 10 ^{o}C higher than the adjacent primers. The probe contains a fluorescent on the 5' base, and a quenching dye on the 3' base. When the polymerase replicates a template on which a probe is bound, its 5' exonuclease activity cleaves the probe emitting fluorescence that increases in each cycle proportional to the rate of probe cleavage. Accumulation of the amplicon is then detected automatically by monitoring the increase in fluorescence of the reporter dye. Using retinal tissue from a nodavirus infected cod (provided from Stewart Johnson; Institute for Marine Bioscience, National Research Council, Canada) this assay has been rigorously validated. The technical details of the assay can be found below in Part II.

The sensitivity of this molecular assay facilitated the first non-lethal detection of Atlantic cod nodavirus. As nodavirus targets the central nervous system, customarily the brain is removed for diagnostic testing. During a nodavirus outbreak at a regional hatchery we were able to sample blood and detect circulating nodavirus. Water samples were also analyzed using the assay and nodavirus was detected. These data indicate that assay developed as part of this project would be a valuable tool to understand the nodavirus's life history and environmental factors influencing outbreaks.

Working with aquaculture industry was a major focus of the project and we worked with several commercial and non-commercial facilities (Figure 2). While most of the samples were from Atlantic cod, we did spend time on assays for nodavirus effecting flounder, haddock, pompano, and barramundi. Over 250 individual Atlantic cod samples were tested for nodavirus. Most of the samples were from eggs (62), blood (39), brains (36), and whole larvae (27). Other samples included gonads, livers, and milt. As part of the diagnostic activities, an experiment was set up to determine if Atlantic cod nodavirus could infect haddock juveniles. Following one month of exposure from preserved cod larvae severely infected with nodavirus, no haddock tested positive. Water samples taken from the exposure tank were positive for Atlantic cod nodavirus. One of the most significant impacts of the assay development was accurately diagnosing the samples from a commercial hatchery to be free from nodavirus, which saved valuable broodstock from being unnecessarily destroyed (see next section: Impacts)

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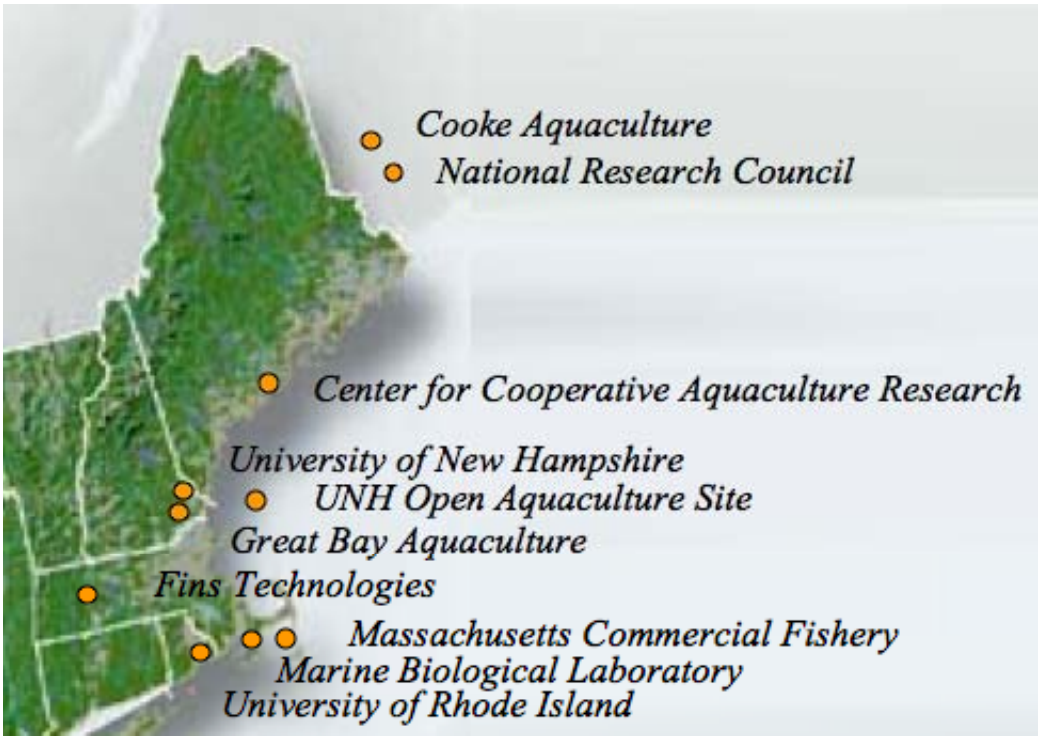

Figure 2. Map showing the hatcheries and facilitates that provided samples for diagnostic analysis.

Egg disinfection technology was another component of the grant. Several agents were used as disinfectants on cod eggs from captive cod. Some eggs were obtained from experimentally infected broodstock. Iodine was one agent used with no effect on survival of the treatment concentration at any of the treatment times tested (up to 20 minutes at 100ppm). Ozone treatment also had no effect on survival (2ppm up to 4 minutes). A novel disinfection agent, RNAse A, used during our research.

RNAses are enzymes that specifically degrade RNA. RNAse disinfection trials were carried out (0.1ppm and 1.0 ppm for 1 minute) without negative effects on survival. No cod eggs were positive for nodavirus. It is not known whether nodavirus can be consistently transmitted from infected broodstock to the offspring. It was determined that Atlantic cod eggs can withstand high levels of disinfection and the ozone levels used were sufficient to kill nodavirus based on research by other groups. The figure at the bottom of the page shows an example of the disinfection trial set-up.
While not associated with the original objectives of this project, limited effort was placed into exploring commercial use of a vaccine. Specifically recombinant protein was produced that corresponds to the coat protein of the cod nodavirus. This protein was produced in a bacterial system (pBAD; Invitrogen) and purified using column chromatography (Figure 3). The long-term goal of such an effort would be to expose cod larvae to this synthetic protein in the hopes that their immune system would be better prepared to handle a nodavirus.

Outreach and extension has been integral to the success of this project. This involved regular communication with hatcheries and production facilities to determine the status of any outbreaks and their need for diagnostics. Regular communication with GBA, allowed us to be present during one such outbreak when we were able to obtain a comprehensive sample set.

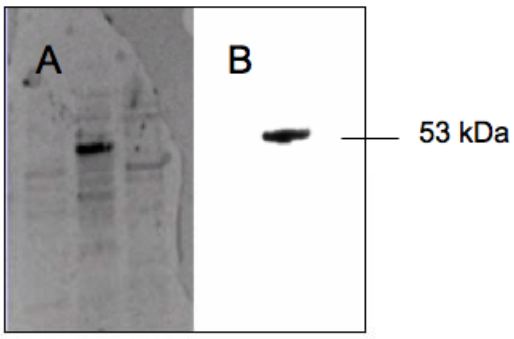

Figure 3. Images of Coomassie stained gel (A) and Western antibody was used for western analysis, corre blot (B) of recombinant nodavirus protein. An AntiV5 antibody was used for western analysis, corresponding to a V5 epitope incorporated into the construct.

Providing information to the public has also been very important. In order to effectively carry out this goal a website was developed (http://www.mbl.edu/aquaculture/nrac/) for technology transfer and dissemination of techniques for disease diagnostics and egg disinfection methods. In December 2004 our research was presented at the Nodavirus II workshop in Portsmouth, New Hampshire. The proceedings of this workshop, including presentations, are also available on site. The website also contains several presentations and technical reports. The current termination report will be made available on the website which will be maintained into the future. There has been a significant international and regional feedback as a result of the website.

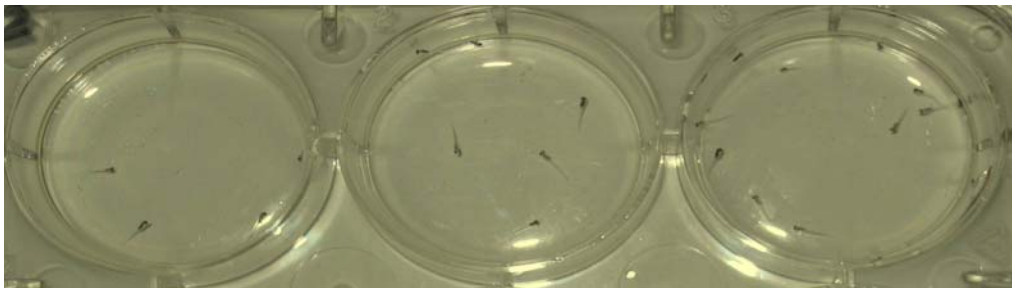

Figure 4. Portion of multi-well plate used in egg disinfection trials. This example shows cod larvae that have recently hatched. Shortly after hatching, survival data is recorded and samples are taken to assess the presence of nodavirus.

Impacts

One of the most significant impacts is our testing of cod fingerlings stocked into sea cages. During a routine health inspection at GBA (Director: George Nardi – participant on current grant) one of the sample pools came up positive for nodavirus. Upon retesting, no positives were registered by GBA's contractor. Samples were also sent out to us (Marine Biological Laboratory) for testing using the Real-time PCR assay and registered negative. Authorities used the results from the Real-time PCR assay and GBA's contract lab in the decision making process to continue to allow transfer of these fish to other sites. GBA has stated that as long as they can afford it, (i.e. through grant support) they will always try to use multiple labs and split samples to ensure accurate results. Without the assay results we provided to managers, a large number of fish might have been destroyed based on the belief that these fish had become infected with nodavirus.

Recommended follow-up activities

As mentioned above, there are two general activities that are recommended to follow-up the current project. These include characterizing etiology of Atlantic cod nodavirus in the environment to have better understanding of factors influencing outbreaks. This could include using the assay developed as part of this research as a tool to evaluate controlled and natural environments. Another recommended activity would be to explore the use of a commercial vaccine. One thing that we did learn from the research is that nodavirus is more widely present than first thought and vertical transmission of the virus is a significant concern. If a vaccine could offer any protection to the virus, this would significantly reduce the risk associated with an outbreak.

Support

	NRAC-USDA Funding	Matching Support (MBL)	Total Support
Year 1	$ 63,017	$ 7,063	$ 70,080
Year 2	$ 61,549	$ 7,063	$ 68,658
TOTAL	$124,612	$14,126	$138,738

Publications, manuscripts, or paper presented

Roberts, and Rick Goetz "*Quantitative real-time molecular methods for disease detection in marine fish"* AQUACULTURE 2004, Honolulu, Hawaii March 1-5 2004

Roberts, Steven and Scott Lindell "*Diagnostic and management techniques to select cod broodstocks and hatchery stocks free from nodavirus*" NACE 2004, Manchester, New Hampshire, December 3, 2004

Lindell, Scott and Steven Roberts "*Egg Disinfection and diagnostic techniques for Atlantic cod nodavirus*" Nodavirus II Workshop, Portsmouth, New Hampshire, December 8, 2004

Steven Roberts "*Diagnostic and management techniques to select cod broodstocks and hatchery stocks free from nodavirus*" NRAC Annual Aquaculture Research, Extension and Business Opportunities Update, Dartmouth, Massachusetts, February 24, 2005

Steven Roberts (2005) New molecular techniques to detect nodaviruses in Atlantic cod hatcheries, Global Aquaculture Advocate, April 2005, 2pp.

PART II – Technical Report

Technical Summary

One of the primary long-term goals of our research was to develop specific and sensitive viral detection techniques. In order to develop a nodavirus detection assay, we have focused on Real-time PCR technology. The specific target of our assay was the gene coding for the coat protein of the virus (RNA2: GenBank Accession #AF445800). A dual-labeled probe was designed, NVcap (FAM- CAT CCC TTG AGA CGC CCG AAC -BHQ1) that is flanked by two primers, NVtmf (TCG CTG GAG TGT ACG TCT CAG T) and NVtmr (GAG TGG TCC GAG GGT TAG GAT). The assay is performed using Invitrogen's Thermoscript One-Step RT-PCR kit with an Opticon Continuous Fluorescence Detection System (MJ Research). Several other comparable instruments (n>10) could also be used to carry out the assay from other manufacturers. For all samples diagnosed as part of this research, Tri-Reagent (MRC Inc.) was used from RNA extraction.

The RNA extraction protocol includes the following basic steps;

1. Homogenization: 1 ml TRI REAGENT + 50-100 mg tissue
2. Phase separation: homogenate 0.2 ml chloroform.
3. RNA precipitation: aqueous phase + 0.5 ml isopropanol.
4. RNA wash: 1 ml 75% ethanol.
5 RNA solubilization: water.

Detailed instructions on RNA extraction can be found in the manufacturer's protocol (http://www.mrcgene.com/tri.htm). When water samples were tested, water was first filtered using a 0.22 um PES filter. The filter was then treated in a similar manner as tissue. A schematic outlining the steps required to complete the molecular assay is shown on the following page. We did explore the possibility of developing a reagent kit, however given the rapid improvement in commercial reagents and decrease in reagents costs this approach is not practical.

The initial cDNA synthesis and two-step PCR cycling program (40 cycles) is performed consecutively in the same reaction well by incubating samples first at 50 ^{o}C for 30 min, followed by PCR. For PCR, an initial 5-min 94 ^{o}C incubation is performed followed be 40 cycles of denaturation (94 ^{o}C for 15s) and annealing/extension (60 ^{o}C for 1 min). Fluorescent detection is performed after each annealing/extension step. To determine the lower detection levels of the molecular assay and dynamic range, nodavirus RNA was generarted *in vitro*. In other words, synthetic RNA was generated that can be directly correlated with nodavirus quantity. Specifically, cod nodavirus RNA was transcribed *in vitro* using a T7 polymerase and the RiboMAX Large Scale Production System (Promega).

1: AY847947. Reports Atlantic cod nerv...[gi:57115615]

Features Sequence

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LOCUS       AY847947               670 bp    RNA     linear   VRL 10-JAN-2005
DEFINITION  Atlantic cod nervous necrosis virus strain DB5y5_85 putative RNA
            dependent RNA polymerase gene, partial cds.
ACCESSION   AY847947
VERSION     AY847947.1  GI:57115615
KEYWORDS    .
SOURCE      Atlantic cod nervous necrosis virus
  ORGANISM  Atlantic cod nervous necrosis virus
            Viruses; ssRNA positive-strand viruses, no DNA stage; Nodaviridae;
            Betanodavirus.
REFERENCE   1  (bases 1 to 670)
  AUTHORS   Roberts,S.B.
  TITLE     Molecular detection of Atlantic cod nodavirus
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 670)
  AUTHORS   Roberts,S.B.
  TITLE     Direct Submission
  JOURNAL   Submitted (06-DEC-2004) Marine Biological Laboratory, 7 MBL Street,
            Woods Hole, MA 02543, USA
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Figure 5. NCBI Genbank entry for nodavirus RNA polymerase gene.

In order to explore additional targets for molecular diagnostics the other portion of the Atlantic cod nodavirus was isolated. This was completed using a degenerative PCR based approach. This nucleotide was information was immediately made available to the public through the National Center for Biotechnology Information so other researchers could use it to study the organism (Figure 5). Our molecular assays showed that target this nucleotide sequence was not superior to targeting the capsid protein.

Another objective of our research was to screen other species from regional hatcheries. We have evaluated samples for several research and commercial based production facilities (Figure 2). To accommodate the request to test species other than cod, we developed an assay that is similar to the dual labeled probe assay for cod described above, however instead of the cleavage of a dual-labeled probe emitting fluorescence, SYBR green I dye is included in the reaction that in turn, fluoresces when it binds to the final product (double-stranded DNA). For this assay we used Stratagene's Brilliant SYBR Green Master Mix kit. The primers that proved to be the most successful for this assay were:

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Funiv  TCT TCC AGC GAT ACG CTG TTG A
Runiv  TCA GTG TTG TTG CCG RCA CAC A
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Numerous egg disinfection trials were conducted as part of this research. As summarized in the previous section three agents were used; iodine, ozone, and RNase A. Trials were carried out in triplicate using 5cm plastic Petri dishes (Figure 4). Iodine disinfection was carried out at 100ppm for 2, 10, and 20 minutes. Ozone treatments were carried out at 2 ppm for 1, 2, and for minutes. The levels for the first two treatments were based on levels commonly used by the industry. To our knowledge, RNase A had never been used as an egg disinfection agent two doses were selected based on the amount of RNA reported to be degraded by one unit of enzyme. The two doses included 0.1 ppm and 1.0 ppm, both for a duration of 1 minute. Neither of these treatments appeared to significantly effect survival. The table below shows the specific survival rates for iodine and ozone treatments.

Treatment	**Exposure Time**	**Survival (7 d post hatch)**
Iodine (100ppm)	2 min	93%
Iodine (100ppm)	10 min	92%
Iodine (100ppm)	20 min	81%
None (Iodine control)	na	90%
Ozone (2ppm)	1 min	96%
Ozone (2ppm)	2 min	90%
Ozone (2ppm)	4 min	98%
None (Ozone control)	na	88%