PROJECT COMPLETION REPORT

98-6 "Development and Application of Multiplex PCR for Screening of Shellfish Pathogens"

Termination Report Period: July 21, 1999 – May 31, 2001

NRAC Total Funding: $142,103 (September 1, 1998 – May 31, 2001)

Principal Investigator: Dr. Richard A. French, University of Connecticut

Participating Investigators

Mazhar Khan	University of Connecticut	Connecticut
Roxanna Smolowitz	Marine Biological Laboratory	Massachusetts
John Volk	Connecticut Department of Agriculture	Connecticut
John Karolus	Connecticut Department of Agriculture	Connecticut
Inke Sunila	Connecticut Department of Agriculture	Connecticut
Lynn Hinckley	University of Connecticut-DTS	Connecticut
Marianne Kalbac	University of Connecticut	Connecticut
Spencer Russell	University of Connecticut	Connecticut
Hillard Bloom	Tallmadge Brothers, Inc.	Connecticut
Robert Smith	Rhode Island Clam Co. Inc.	Rhode Island
Karen Eno	Aeros Cultured Oyster Co., Inc.	New York
Steve Fleetwood	Bivavle Packing Company, Inc.	New Jersey

REASON FOR TERMINATION:

Objectives are completed and the funding period has ended

PROJECT OBJECTIVES:

l. To develop and optimize a multi-species polymerase chain reaction (PCR) (multiplex PCR) for the detection of the eastern oyster *(Crassostrea virginica)* pathogens *Haplosporidium nelsoni* (MSX), *Haplosporidium costale (SSO)* and *Perkinsus marinus* (Dermo).
2. To determine if the multiplex PCR can be used as a specific and quantitative measure of infection by the oyster pathogens *H. nelsoni* (MSX), *H. costale (SSO)* and P. *marinus* (Dermo) .
3. To apply the technology (multiplex PCR) to field samples and or animals at point sources of infection and to evaluate and compare findings with conventional testing procedures.
4. To determine if the specificity and sensitivity of the multiplex PCR method can provide accurate and meaningful data for use as a diagnostic aid in the management of H. *nelsoni* (MSX), *H. costale (SSO)* and P. *marinus* (Derrno) in the eastern oyster.

ANTICIPATED BENEFITS:

Product: A single, rapid multiplex PCR test for *H. nelsoni, H. costale* and P. *marinus* in the eastern oyster *(Crassostrea virginica).* User/Beneficiary: The product will have potential use in commercial and private industry, research, diagnostics, and in certifying and regulatory laboratories. The products are primarily expected to serve the aquaculture industry in the routine screening of oyster seed-stock. In this context, certifying will be possible and the management of disease in the cultivation of oysters can be engineered. Economic benefit: The introduction of multiplex PCR for testing of disease pathogens is expected to increase product availability & quality by increasing disease pathogen monitoring and thus decreasing endemic disease and disease impact.

PROGRESS AND PRINCIPAL ACCOMPLISHMENTS:

OBJECTIVE 1:
- Completed. Results are submitted for publication (see attached manuscript).

OBJECTIVE 2:
- Completes. Results are submitted for publication (see attached manuscript).

- Dr. Soledad Penna conducted the research and defended her masters thesis in May 2000.

OBJECTIVE 3:

- To be completed, September 2001

In 1999, a multiplex polymerase chain reaction (MPCR) was developed in the Department of Pathobiology, University of Connecticut for the simultaneous detection of one member of the Phylum Apicomplexa, *Perkinsus marinus* (Dermo), and two members of the Phylum Ascetospora, *Haplosporidium nelsoni* (MSX) and *Haplosporidium costale (SSO).* After initial testing and review of the initial multiplex PCR of Penna et al., (manuscript attached) improvements were necessary to address quality control and sample variability. To further improve the MPCR and to test its validity, researchers from the Department of Pathobiology have collaborated with Dr. Inke Sunila of the Connecticut Department of Agriculture, Milford Bureau of Aquaculture and Laboratory (Milford, CT.), Dr. R. Smolowitz of the Marine Biological Laboratory (Woodshole, MA.) and numerous other institutions and commercial aquaculture enterprises along the east coast and the Gulf of Mexico. The objective of the project is to validate the new and improved MPCR by comparing its results to conventional diagnostic procedures used today. To complete this objective, a one year research project has been designed to 1) test oysters collected from 10 different Atlantic coast States and 2) test and monitor two commercial oyster production sites within Long Island Sound bimonthly for one full year. In addition we can utilize the samples to further explore molecular diagnostic technology and to develop other specific and sensitive diagnostic tools.

MPCR redevelopment and Field Trials

1. To introduce a 'quality control' gene into the previously developed multiplex polymerase chain reaction (MPCR) for the detection of an 805-bp segment of 28s rRNA gene of *Crassostrea virginica,* as well as the oyster pathogens *Haplosporidium n elson i* (MSX) , *Haplosporidium costale (S SO) ,* and *Perkinsus marin us* (Dermo) in the eastern oyster.
2. To apply the MPCR to field samples and to evaluate and compare findings with conventional testing procedures.

OBJECTIVE 2: - Completed. Results are submitted for publication (see attached manuscript).

The goal of this experiment is to produce a diagnostic test that accurately indicates the presence or absence of each pathogen in any given oyster sample. False positive reactions due to either cross contamination, non-specific crossreactivity and the action of PCR inhibitors can result in partial or complete failure of the PCR to occur (Brightwell, et al 1998). With the increased in reliance on rapid PCR based diagnostic tests for pathogenic micro-organisms it is becoming imperative to incorporate internal standards to the PCR Assay to assure accurate diagnosis and to avoid false positives and negatives (Brightwell, et al 1998). A PCR internal standard is a DNA fragment co-amplified with the target PCR products, which is of different size than the target products (Sachadyn et al 1998). To address this need, an 805-bp specific sequence product from 28s rRNA gene of C. *virginica* has been successfully incorporated into the MPCR to act as an internal, 'quality control' product in our reaction. The positive visualization of an oyster-specific amplicon of greater molecular weight than target amplicons within each sample tested confirms the quality of the DNA isolated for application of the MPCR, and represents DNA amplification under optimized conditions for the primers applied.

RESULTS TO DATE

1. Successfully incorporation of a quality control gene into the MPCR and tested for its Specificity and Sensitivity.
2. Twenty-five samples of thirty oysters each have been processed and tested. Each oyster is subjected to the newly developed MPCR and the conventional diagnostic methods; RayMackin Fluid Thioglycollate Medium assay and Histology.
3. Sampling sites to date:

- Massachusetts
- Rhode Island
- Connecticut
- New York State
- New Jersey
- Maryland
- South Carolina
- Louisiana
- Washington State
- Maine

OBJECTIVE 4:

- To be completed, October 2001

Evaluation of the results and testing procedure will be conducted with industry and state agencies collaborating in the study. Studies have been performned at of the Connecticut Department of Agriculture, Milford Bureau of Aquaculture and

Laboratory (Milford, CT.) by Dr. Inke Sunila, and the Marine Biological Laboratory (Woodshole, MA.) by Dr. R. Smolowitz. The Bureau of Aquaculture reports good results and reproducibility with the MPCR methods and Dr. Sunila is working closely with our group to finalize field trials that have produced excellent results (to be published, see Manuscripts in Preparation). Dr. Smolowitz laboratory (Woodshole, MBL) had difficulties with the assay which are summarized below.
We are moving forward in modifying the test to make it more user-friendly and have been awarded a grant from National Sea Grant Of fice (Development and Application of a Chemiluminescent Quantitative Multiplex PCR for

Summary of Dr. R. Smolowitz (subcontract):

FIELD TRIALS USING MULTIPLEX PCR AS A METHOD OF BIVALVE DISEASE DIAGNOSIS AT THE MBL Roxanna Smolowitz, DVM Joe Cavanaugh, Technician

The objective of this study was to explore and test the use of the multiplex PCR diagnostic method, developed by Dr. Richard French and his associates at U. Conn., in another bivalve diagnostic laboratory. Funding, methods and appropriate reagents were provided in Feb. 2001 from NRAC/U. Conn. and testing occurred over a 5 month period from that date. Tissues used for testing in this method originated from oysters collected from two locations on Martha's Vineyard, MA (SSO and Dermo positive stock) and one location in Dennis, MA (Dermo and MSX positive stock). In addition to examination using multiplex PCR, animals were examined histologically and/ or with thioglycollate culture for these three diseases. Results of the PCR testing were compared to the more traditional testing methods.
Conclusions and Recommendations for Future Testing of Multiplex PCR: Because of the problems in the establishment and use of the Multiplex PCR method in our laboratory, we were not able to do any real comparison between traditional and multiplex PCR diagnostic tests for sensitivity and reproducibility. However, based on our short and limited experience with this test, we do have recommendations for use in its further development, especially if it is to be used as a standard testing method in various diagnostic laboratories.
1. Quantify the DNA concentrations of several individual oysters taken from different areas to determine the average variance between individuals and areas. (Pl Comments: This procedure is routinely performed in the laboratory of the Pl and is requiredfor standardization of laboratory results)
2. Sample oysters at the appropriate time of year when the various pathogens are known to be present to increase the number of expected positive individuals tested. (Pl Comments: The testing procedure is being tested over the course of two full years and includes more than 12 sites {see objective 3, above2. The goal is to cover periods of low and high disease prevalence and regions of variable disease epizootiology)
3. Do further work to establish the best location from which to consistently biopsy tissue sections so that identical locations on oysters can be used to standardize the multiplex PCR method. (PI Comments: The PI 's laboratory recognizes tissue sampling as a limitation and recommends sampling of gill, digestive gland and anus, and is in the process of assessing data relative to conventional testing methods)
4. Test reproducibility of multiplex PCR by running samples with single primer pairs to determine if there are any artifacts of the multiplex PCR. The appearance of spurious bands in some of the multiplexed individuals and those individuals with only dermo primers are cause for concern. There could be some competitive exclusion for binding of the DNA polymerase or a bias towards optimal amplification with some primer pairs over others when all primers are multiplexed together. It appears this should be a standard part of the controls needed for conducting this test. (PI Comments. This work has been conducted and as it was the ground work required to develop and optimize the MPCR methodology. A semi-quantitative MPCR method is in development)
5. Offer training sessions for laboratories interested in using the fully developed test. (PI Comments. The MPCR testing methodology is being modif ed to make the test more user friendly, faster, and a more applicable diagnostic tool to industry and researh. we have expanded the use of the MPCR by utilizing the amplif ed products in a semiquantitative, sensitive, reverse dot blot test.)

IMPACTS:

We have interesting results in preliminary field trials, which indicate a higher prevalence of SSO in the LIS then previously recorded by conventional histology. We are working with VIMS (Nancy Stokes and CT Bureau of Aquaculture (Inke Sunila) in interpreting and following up on our findings with regards to SSO prevalence and the possibility of new

Haplosproidium strains and/or species. The field trials are expected to be enlightening with regards to the prevalence of Dermo, SSO, and MSX in LIS and the region relative to histology. That is, the multiplex PCR has proven to be much more sensitive (~2X) than conventional methods.

RECOMMENDED FOLLOW-UP ACTIVITITES:

To develop and optimize a reverse dot-blot assay for the semi-quantification measure of infection by the oyster pathogens MSX, SSO, and Dermo, in the eastern oyster. To make the multiplex PCR a more user friendly, faster, and more applicable diagnostic tool to industry and research, we plan expand the use of the multiplex PCR by utilizing the amplified products in a semi-quantitative, sensitive, reverse dot blot test.

PAPERS PRESENTED:

• Papers Presented:
Soledad Penna, J. Volk, J. Karolus, I. Sunila, and R. A. French. Development and application of multiplex PCR for screening of shellfish pathogens. The 18th Milford Aquaculture Seminar, February 23 25, 1998.

R. A. French. Multiplex PCR: A Rapid Diagnostic Method for Screening of Disease Pathogens of Animals and Public Health Significance, Universitv of Illinois, Department of Pathobiologv. ADril 22. 1998

Soledad Penna and R. A. French. Development and application of multiplex PCR for screening of shellfish pathogens. New England Association of Parasitologists (NEAP IV), May 9, 1998.

Soledad Penna and R. A. French. Development and application of a multiplex PCR for screening of shellfish pathogens. Universtiv of Connecticut Graduate Research Forum. November 20. 1998.

Richard A. French, Salvatore Frasca, Jr., Sylvain De Guise, and Herbert J. Van Kruiningen. Aquatic Animal Health and UCONN Aquaculture Program: New Faculty and Opportunities. The 19th Milford Aquaculture Seminar, February 22-24 1999.

Soledad Penna and Richard A. French, John Volk, John Karolus, and Inke Sunila, Roxanna Smolowitz. Diagnostic Screening of Oyster Pathogens: Preliminary Field Trials of Multiplex PCR. 92st Annual Meeting of the National Shellfisheries Association, Halifax, Nova Scotia, April 18-22, 1999.

Spencer Russell, Soledad Penna, Richard French. Comparative evaluation of the multiplex PCR with conventional detection methods for Haplosporidium nelsoni (MSX), Haplosporidium costale (SSO), and Perknsus marinus (Dermo) in the eastern oyster (Crassostrea virginica). The 20th Milford Aquaculture Seminar, February 28 - March 1, 2000.

Spencer Russell, Soledad Penna, Richard French. Comparative evaluation of the multiplex PCR with conventional detection methods for *Haplosporidium nelsoni* (MSX), *Haplosporidium costale (SSO),* and *Perknsus marinus* (Dermo) in the eastern oyster *(Crassostrea virginica).* 93rd Annual Meeting of the National Shellfisheries Association, Seattle, Washington, March 19-23, 2000.

Spencer Russell, Soledad Penna, Richard French. Diagnostic Techniques for the Detection of Dermo, MSX, SSO in the Eastern Oyster, *Crassotrea virginica.* Long Island Sound Research Conference, University of Connecticut, Stamford Campus, Stamford, CT, November 17-18, 2000.

S Russell, S Penna, R. A. French. Diagnostic Techniques for the Detection of Dermo, MSX and SSO in The Eastern Oyster, *Crassostrea virginica.* Proceedings, 2000 Long Island Sound Conference, The Long Island Sound Foundation, Inc., November 2000.

• Prospective Conference Presentations
National Shellfish Association Annual aeneral meetinz. Mvstic Seasort Connecticut March 2002.

MANUSCRIPTS:

Maria-Soledad Penna, and Richard A. French. Development of a multiplex PCR for the detection of *Haplosporidium nelsoni, Haplosporidium costale,* and *Perkinsus marinus* in the eastern oyster *(Crassostrea virginica,* Gmelin, 1971) (submitted 03/01, Molecular and Cellular Probes)

MANUSCRIPTS IN PREPARATION:

1. "Multiplex PCR field trials for the diagnosis of oyster pathogens MSX, SSO and Dermo: An Atlantic coast perspective."
Published in " Diseases of Aquatic Organisms" or "Journal of Shellfish Research""

2. "A Multiplex PCR and reverse dot-blot for the specific non-radioactive detection of MSX, SSO and Dermo in the Eastern oyster, *Crassostrea virginica."* Published in "Diseases of Aquatic Organisms" of " Molecular and Cellular Probe

The Full Report with all the data, graphs and tables is available at the NRAC office upon request.