

OHHI FY06 Progress Report

Core Name: Pathogen Source Tracking

Project Title: Library Independent Methods Project

Reporting Period: Oct. 2005 – Sept. 2006

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Background and Rationale:

Assays are being developed to: (1) allow rapid, direct detection of pathogenic microbes to better assess risks to human health; and to (2) target host-specific microorganisms to help identify sources of contamination, allowing for effective mitigation and best management plans to be implemented. The focus of the second component, microbial source tracking, is on library-independent detection of host-specific organisms, as this approach is more likely to be practical for routine application than current methods that require construction of large source databases for each watershed of interest (library-dependent methods).

Various platforms are being used to target microbes of public health concern. Proposed assays include: (1) Luminex assays for rapid and simultaneous detection of marine bacterial pathogens and source-specific bacteria, and (2) polymerase chain reaction (PCR) assays for quantitative or real-time detection of indigenous *Vibrio* bacteria and enteric viruses. The genetic targets being identified and validated during this research will also be able to be assimilated into other platforms. For example, this research could contribute toward the goal of real-time monitoring for microbial contaminants as the technology for doing so (buoys, etc. with genetic sensors) is better engineered. Ultimately the assays developed within the microbiology program will allow rapid diagnosis and source tracking of microbial threats to human health along recreational beaches, in estuaries and tidal creeks, and in shellfish.

Objective:

The main objective of the pathogen source tracking program is to develop novel, accurate and reliable techniques to rapidly detect and identify the source of marine microbes that threaten human health. Specific objectives of the library independent methods project include:

- Develop RT-PCR assays for detection of enteric viruses (noroviruses, enteroviruses) from coastal waters and shellfish.
- Adapt the Luminex bead suspension array technology to rapidly and simultaneously detect multiple bacteria targets.

- In communication with other microbiology core members, improve the upstream processing of nucleic acid extraction and amplification methods.
- Collaborate with user groups for assessment of pathogens in coastal waters and shellfish, and to field test novel detection assays.

Accomplishments:

- Developed RT-PCR assays, including competitive internal positive controls, for direct detection of noroviruses, enteroviruses, and coliphage MS2 (viral indicator).
- Developed a multiplex Luminex assay to simultaneously detect *Enterococcus* spp. and *Methanobrevibacter smithii* as indicators of fecal pollution and human-source fecal pollution, respectively.
- Analyzed water and oyster samples from tidal creeks (in the Southeastern US) to determine the densities of fecal indicator organisms and pathogens; this study was conducted in collaboration with the Monitoring and Assessment Core. Preliminary results suggest that headwaters/sediments are reservoirs for fecal bacteria and that bacterial indicators do not adequately model enteric pathogens in these systems. Results from tidal creeks in North Carolina and Georgia demonstrate that these trends are present on a regional basis.
- Analyzed oyster samples from SCECAP/LTD sites along the South Carolina coast for fecal coliforms, enterococci, coliphages, noroviruses, *Vibrio vulnificus*, and *Vibrio parahaemolyticus*. The presence of human pathogens, *i.e.*, *Vibrio vulnificus*, *V. parahaemolyticus*, and enteric viral pathogens in addition to fecal indicator bacteria in these samples suggest that potential human health risks are present for people who consume shellfish from particular sites in the area.

Publications/Presentations:

- Johnston, C., L. Webster, J. Stewart, and J. Gooch. *Application of Luminex Technology for Detection of Marine Microbes of Public Health Significance*. NOAA Oceans and Human Health All-PI Meeting. Charleston, SC Jan. 18-20, 2006.
- Gooch, J.A. and G.I. Scott (2005). *Waterborne Microbial Agents as a Homeland Security Threat*. ThinkTEC Meeting. Dec 1. Charleston, SC.
- Gooch, J.A., B.J. Robinson, B.C. Thompson, R.F. van Dolah, L.H. Coen, G. Riekerk, M.F. Fulton. (2006). *Detection of enteric pathogens in South Carolina oysters*. NOAA Oceans and Human Health Initiative All-PI Meeting. Jan. 18-20. Charleston, SC.

- Gooch, J.A., J.R. Stewart, L.F. Webster, B.J. Robinson, B.C. Thompson, and G.I. Scott. (2006). *Microbial source tracking in South Carolina: Lessons learned and future directions*. EPA Mobile Bay Water Quality Monitoring Workshop. Feb 2. Mobile, AL.
- Robinson, B.J. (2006). *Efficiency of Detecting Viruses from Environmental Waters*. Southern Section of AOAC International 20th Annual Meeting. Atlanta, Georgia. April 25, 2006.
- Webster, L.F. (2006). *Detection of E. coli Using the Luminex 100 System*. Southern Section of AOAC International 20th Annual Meeting. Atlanta, Georgia. April 25, 2006.
- Robinson, B.J., B.C. Thompson, L.F. Webster, G.T. DiDonato, J.R. Stewart, and J.A. Gooch. *Microbial Water Quality of Tidal Creek Systems along the South Carolina Coast*. Poster presentation at The American Society for Microbiology General Meeting, Orlando, FL. May 21-25, 2006.
- Gooch, J.A., B.J. Robinson, B.C. Thompson, R.F. VanDolah, L. Coen, G.H.M. Riekerk, A. Hollis and M.H. Fulton. *Determination of Human Enteric Pathogens in Oysters from Coastal South Carolina*. Poster presentation at The American Society for Microbiology General Meeting, Orlando, FL. May 21-25, 2006.
- Webster, L.F., A.K. Leight, B.J. Robinson, S. Friedman, J. Macauley, G. Lauenstein, R.A. Warner, M.H. Fulton. *Post Hurricane Katrina Assessment of Microbial Indicator Levels*. Poster presentation at The American Society for Microbiology General Meeting, Orlando, FL. May 21-25, 2006.
- Gooch, J.A., B.J. Robinson, B.C. Thompson, L.D. Coen, D. Richardson, R.F. vanDolah. (2006). *Joint SCECAP/oyster survey study: Results from summer and winter sampling*. May 30. Brown Bag Seminar, HML. Charleston, SC.
- Gooch, J.A., C. Johnston, B.J. Robinson, J. Gregory, J.R. Stewart, B.C. Thompson, and L.F. Webster. (2006). *Environmental microbiology program at CCEHBR*. S1022 Annual Meeting. June 19. Charleston, SC.

(Additional publications/presentations are listed with the Pathogen Source Tracking Core)

Application/Technology Transfer Relevant to OHH Strategic Goals:

1.0 Scientific Research and Application

- **Luminex assays:** Evaluated Luminex technology for detection of microbes from environmental samples

- Designed and tested oligonucleotide probes and primer sets for environmentally relevant bacteria (*E. coli*, *Enterococcus* spp., *Methanobrevibacter smithii*) for use in Luminex assays.
 - Individually optimized PCR reactions and Luminex assays for each probe and primer set and determined the sensitivity of the assays using bacteria strains from the American Type Culture Collection (ATCC). Detection limits for singleplex Luminex assays ranged between 5 and 50 cells when using genomic DNA extracts from pure cultures. These results are similar to those obtained for real time PCR assays and over a log more sensitive than conventional detection of PCR product on gels.
 - Compared the results from the *Enterococcus* singleplex assay with traditional culturing methods (MPN method) from tidal creek water samples. While both methods were positive for most samples, the Luminex median fluorescent intensity data did not correlate quantitatively with the MPN counts. This result could be explained by inhibition of the PCR reaction, DNA extraction inefficiencies, the presence of dead cells, loose DNA, viable but not culturable (VBNC) cells, or selectivity introduced by culture bias. Controls for these factors must be developed and implemented before the Luminex assays can be considered truly quantitative.
 - A competitive internal positive control (CIPC) was designed for the *Enterococcus* assay to monitor PCR inhibition during amplification. This control will allow us to compensate for PCR inhibition and provide a more accurate estimation of the concentration of the target organism in the sample.
 - Determined that a multiplex assay using a set of group or domain specific PCR primers and multiple species specific probes is not feasible because of the large copy numbers of each target required for detection on the instrument. However, a multiplex PCR using multiple species specific primers can overcome this problem.
 - Developed a multiplex assay to simultaneously detect *Enterococcus* spp. and *Methanobrevibacter smithii* as indicators of fecal pollution and human-source fecal pollution, respectively. Assay will be field tested in FY07 and, pending funding, incorporated in epidemiology studies to test association with health outcomes.
 - Currently optimizing multiplex assays to detect additional targets, as well as investigating methods to increase sensitivity of the assays by improving fluorescent detection and upstream processing of nucleic acids.
- **Enteric virus assays:** Designed and implemented a competitive internal positive control (CIPC) to use with quantitative enterovirus and norovirus (group II) RT-PCR assays. A control of this type is an essential part of ensuring accuracy and quality control for the assays. The advantages of using A CIPC with the newly developed viral assays are listed below:

- Use of the CIPC reduces false negative results, which are often attributed to PCR inhibitors carried over during RNA extraction.
 - Use of the CIPC calibrates reactions to provide accurate estimations of viral concentrations, as well as provide data on intra- and inter-assay variability.
 - Use of the CIPC yields valuable information on the purity of RNA extractions as well as the performance of various quantitative PCR platforms.
- The RT-PCR enterovirus assay specifically identifies pathogens only associated with human fecal contamination, a benefit not found with other conventional indicator organisms.
 - A norovirus quantification standard is currently under development. The standard will allow precise determination of the number (copies) of viruses present in each positive sample.
- **Sample Processing Improvements:** Developed a new, quantitative qRT-PCR assay to detect MS2 coliphage for use in determining virus recovery efficiencies in various concentration and extraction protocols.
 - MS2 coliphage being used in assay development because it is easy to culture, non-pathogenic, and is similar in size and structure of many human enteric viruses.
 - Ease of detection allows for comparison of results from the conventional culture-based EPA method 1602.
 - The MS2 assay will be used in conjunction with the development of new water concentration and viral processing methods to determine filtration and extraction efficiencies.
 - Recent improvements in filtration and RNA extraction have improved sensitivity approximately 20 fold.
- **Tidal Creeks Project:** 32 tidal creek water and 21 tidal oyster samples were analyzed for conventional bacterial indicators, male-specific (F+) and somatic (F-) coliphage, and human Norovirus and Enterovirus from June-August 2006 (summer).
 - Enterococcus densities were higher than fecal coliforms in 18 (86%) of the summer tidal creek oyster samples. Only seven (33%) of these summer oyster samples met the FDA/EPA fecal coliform shellfish guideline (330 fecal coliforms/100g).
 - Enterococcus numbers were higher than fecal coliforms in 18 (56%) of the tidal creek summer water samples. Only 6 (19%) of the tidal creek water samples met the SC DHEC fecal coliform standard (14 fecal coliforms/100 mL) for approved shellfish waters and only 14 (44%) met the SC DHEC water quality standard (200 fecal coliforms/100 mL) for recreational waters.

- Only one (3%) of the tidal creek water samples (Masonboro Creek—10 enterococci/100 mL) met the SC DHEC water quality standard for recreational use (35 enterococci/100 mL).
- Higher concentrations of coliphages were generally observed in the headwaters of creeks. Type I F+RNA coliphages were detected at a variety of sites, indicating presence of animal-source fecal contamination.
- Group II and III coliphages, indicative of human fecal contamination, were detected at one urban site (New Market Creek). Norovirus was detected at Postell Creek, a suburban creek in Georgia, although this assay must be confirmed by sequencing. Enterovirus assays are not complete at this time.

- **Joint SCECAP/Oyster Survey Study:** Collaborative project between the South Carolina Department of Natural Resources and NOAA sampled 27 and 32 tidal creek oyster beds during winter (Jan-Feb., 2006) and summer (Aug-Oct., 2006), respectively. These beds consisted of SCECAP and DNR long term disease (LTD) sites along the South Carolina coast. These samples were analyzed for FC, enterococci, male-specific (F+) and somatic (F-) coliphages, human Norovirus and Enterovirus, *Vibrio vulnificus* and *V. parahaemolyticus*. This project was jointly funded by NOAA and SC DNR.

- Fecal coliforms were higher than enterococci in 20/27 (74%) of the SCECAP/LTD oyster samples in winter, possibly because the FC are able to survive lower temperatures better.
- Enterococcus bacteria were higher than fecal coliforms in 31/32 (97%) of summer SCECAP/LTD oyster samples, possibly because the enterococci population outgrew the fecal coliforms at the higher temperatures.
- According to the FDA/EPA shellfish guideline (330 fecal coliforms/100g), 22/27 (81%) of the SCECAP/LTD winter oyster samples met the standard. The 5 winter oyster samples that exceeded the FC guideline were:
 - Murrell's Inlet LTD—480 FC/100g
 - RO-056102—Calibogue Sound--480 FC/100g
 - RT-052095—Adams Ck--1000 FC/100g
 - RT-052118—Church Ck--1000 FC/100g
 - RO-056112—SC Aquarium--5600 FC/100g

Only 13/32 (41%) of the SCECAP/LTD summer oyster samples met the FDA/EPA standard. The 19 summer oyster samples not meeting the FDA/EPA FC guideline were:

- SC Aquarium—60000 FC/100g
- RT-06024—Seven Reaches Creek—480 FC/100g
- RT-06008—Conch Creek—480 FC/100g
- Toler's Cove Ltd—480 FC/100g
- Inlet Creek LTD—480 FC/100g
- RT-06037—Euhaw Ck (broad River)--480 FC/100g
- RO-06326—Lucy Point Creek—480 FC/100g
- North Inlet LTD—1000 FC/100g

- Murrell's Inlet LTD—1000 FC/100g
 - RT-06033—Old Man Creek—480 FC/100g
 - RO-06325—Upper Broad River--480 FC/100g
 - RT-06028—Clauson Creek—480 FC/100g
 - RT-06032—Little Papas Creek—480 FC/100g
 - Warsaw Flats LTD—480 FC/100g
 - RT-06035—Unnamed Ck to Eddings Point Ck—1600 FC/100g
 - RO-06320—Cooper River (Patriot's Point)—460 FC/100g
 - Price Creek LTD—480 FC/100g
 - RO-06315—Leadenwah Creek—480 FC/100g
 - RT-06027—Unnamed Ck to Ocella Ck—480 FC/100g
- Both somatic and male-specific coliphage concentrations were higher in the winter months. Group I male-specific RNA coliphages, indicative of animal fecal contamination, were detected at a number of sites during the winter and summer samplings. Detecting animal fecal contamination is important to help focus remediation efforts and because of the possibility of zoonotic disease transmission.
 - Neither Norovirus nor Enterovirus was detected in any of the winter samples. Summer sample Norovirus and Enterovirus analysis is in progress.
 - A majority of the winter oyster samples contained *V. vulnificus*, with a maximum density of 2,600 CFU/g. *V. parahaemolyticus* counts were well below the FDA standard of 10,000 CFU/g shellfish meat. The maximum winter oyster density was 900 CFU/g. Summer oyster sample analyses are in progress.
 - The presence of pathogens and indicators in oysters suggests the potential for human health risks associated with shellfish consumption from the positive sites.
- **Confirmation of *Vibrio vulnificus* isolates:** A published real-time quantitative polymerase chain reaction (qPCR) assay for *Vibrio vulnificus* is being used to confirm isolates previously isolated using biochemical and hybridization methods.
 - Approximately 90 environmental isolates and 10 positive controls have been tested to confirm the presence of *V. vulnificus*.
 - Isolates that were negative for the qPCR assay and isolates that were biochemically atypical but positive for the PCR assay will be speciated.
 - This assay may allow for greater confirmation of *Vibrio vulnificus* isolates from environmental samples.

2.0 **Public Information and Outreach**

- Helped train Orange County, CA Sanitation District (OCSD) personnel in *E. coli* and Enterococcus qPCR techniques and procedures. Training was performed in part by Jason Gregory (NOAA) at a workshop sponsored by the Southern California Coastal Water Research Project (SCCWRP) and was conducted to allow OCSD employees to beta test rapid methods for use by local water quality labs.
- As demonstrated by the presentations cited above, this project is disseminating timely information on tools for detecting and tracking pathogens to a variety of targeted audiences and end-users.

3.0 Capacity Building

- Improvements have been made on space-related issues pertaining to molecular protocols. Previous sample nucleic acids were extracted and amplified in the same room as field samples, creating a risk for sample contamination and assay failure. Newly acquired space at the Hollings Marine Laboratory (BSL”3” labs) allows separate facilities for nucleic acids extraction and amplification, greatly decreasing chances of contamination and increasing reliability of molecular methods, such as quantitative polymerase chain reaction (qPCR).
- Evaluated qPCR machines in hopes of increasing the sample volume and speed of processing tidal creek and oyster samples for enteric viruses. The current equipment only analyzes 16 samples simultaneously and is not as sensitive as two 96 well format instruments the lab has been able to try on a short-term basis. An evaluation of current 96 well format instruments showed that samples could be processed faster and with greater sensitivity and we are now hoping to acquire a new instrument.

Project Abstract:

The HML OHH pathogen and source tracking project is developing tools to allow rapid pathogen detection from coastal waters and shellfish, and to allow sources of pollution to be identified. Recent accomplishments include microbial assessments of tidal creek systems and shellfish beds along the South Carolina coast. The tidal creek project has revealed a gradient of microbial concentrations in tidal creeks, with higher concentrations typically observed in the headwaters of the creeks. The shellfish project has demonstrated that shellfish from some sites harbored selected pathogens, and that pathogen abundances are not necessarily correlated with those of standard bacterial indicators. These field studies are also demonstrating the applicability of methods developed to date. Additional methods for pathogen detection, including Luminex xMap™ technology that allows for rapid, simultaneous detection of numerous microbial species, are in early stages of development. The capacity to apply Luminex methodology for bacterial pathogens and real-time PCR methods for viral pathogens will provide NOAA the biotechnology and scientific information to integrate its resource assessments and research activities with those of public health institutions including NIH, NIEHS,

NSF, CDC, State Health Departments and academic research centers. Together, these new tools can be used for coastal ecosystem assessments and protection of public health.

Unresolved Issues:

- It is currently problematic to isolate pathogens from large volumes of water and other samples without also concentrating compounds that inhibit subsequent molecular analyses. Recovery efficiencies tend to be low (20-90%), both for concentrating protocols as well as the nucleic acid extraction and purification steps that typically follow. The low recoveries decrease overall detection sensitivity and increase chances that organisms of public health concern could go undetected when they are present in low concentrations.
- Funding is needed to participate in an epidemiology study starting in summer 2007. The study is organized by the Southern California Coastal Water Research Project (SCCWRP) in collaboration with Dr. Jack Colford, an epidemiologist at the University of California, Berkeley. We have an opportunity to apply our novel detection technology to beach waters that will be collected simultaneously with health data. This is a rare opportunity to link water quality data and health data.

Budget Summary:

	OHH funds	Matching (base funds)	Total
Year 1	\$131,000	\$85,000	\$216,000
Year 2	\$154,000	\$158,000	\$312,000
Year 3	\$200,000	\$178,000	\$378,000
Total	\$485,000	\$421,000	\$906,000