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Year 1 Report

Microbial Source Tracking

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Preliminary Year 1 Report

August 3, 2007 through May 31, 2008

Validation and Field Testing of Microbial Source Tracking Methodologies in the Gulf of Mexico
Grant/Cooperative Agreement/Interagency Agreement No. _MX-96478707-0

Project Manager: Valerie J. Harwood, Ph.D.

This report covers the project from its inception (08/03/07) through May 2008. A microbial source tracking (MST) workshop was held in Biloxi, MS in November 2006 as a precursor to this project. The attendees included scientists who are experts in the field of MST as well as federal and state regulatory agency officials. The 2006 workshop addressed validation criteria for MST methods and surveyed the attendees for their choice of the MST markers that would be most useful for field studies of beach and shellfish water quality in the near future.

- In general the attendees favored the library-independent MST approach (use of molecular markers rather than libraries to determine the source of fecal pollution).
- Three library-independent MST methods (human *Bacteroides*, *Methanobrevibacter smithii* and human polyomaviruses) are being compared for their usefulness across the Gulf of Mexico for detecting human fecal pollution in environmental waters.
- Three institutions are collaborating in this work: University of South Florida (USF), University of West Florida (UWF) and University of Southern Mississippi (USM).

1. WHAT WORK WAS ACCOMPLISHED FOR THIS REPORTING PERIOD?

SOP Development

Human Bacteroides. Each laboratory (USF, UWF, USM) used a variation of the conventional (+/-) protocol for the human *Bacteroides* marker described by Bernhard and Field (2000) prior to the beginning of this grant. A consensus human *Bacteroides* protocol was developed that employed concentration of bacteria by membrane filtration and a touchdown PCR variant of the original method. The methodology was optimized by the Harwood laboratory and provided to the other two laboratories (UWF and USM).

M. smithii. The same initial processing steps used for the consensus human *Bacteroides* protocol (sample concentration, DNA extraction, and touchdown PCR program) were applied to a consensus *M. smithii* protocol. Time and labor were reduced by keeping assay conditions consistent for human *Bacteroides* and *M. smithii*. The reactions are run in separate tubes (not multiplexed). There is also an option to run the *M. smithii* assay without the touchdown modification, at the annealing temperature of 55.1° C.

Human polyomaviruses (HPyVs). Modifications were made by USF to the protocol previously described by McQuaig et al (2006). The initial step of increasing sample pH to give viral capsids

a negative charge prior to pre-filtration was eliminated due to excessive handling requirements for this step and its failure to contribute to assay sensitivity in most cases. Sample processing now begins dropping the pH to 3.5 to give the viral capsids a net positive charge. This step allows viruses to bind to the 0.45 μm nitrocellulose filter (which has a negative charge) used for concentration of target(s). The modification did not decrease sensitivity of the assay and made it possible to combine sample concentration and DNA extraction for all three markers (human *Bacteroides*, *M. smithii*, and HPyV).

The forward primer previously used for the HPyV PCR assay (Askamit, 1993) was extended by 3 nucleotides. This step was found to increase assay sensitivity by about an order of magnitude (McQuaig et al 2008, submitted).

Each of the three protocols (human *Bacteroides*, *M. smithii*, and HPyV) is now optimized and is ready to be sent to the project collaborators.

Sensitivity and Limit of Detection

The sensitivity of human *Bacteroides*, *M. smithii*, and HPyV was assessed by the ability to amplify the markers from raw sewage. **Each of the human source markers has been very consistently detected in sewage by the USF, USM and UWF laboratories** (with the exception of HPyV for UWF, which has not yet conducted these tests).

In samples collected by USF, all markers displayed 100% positive with sewage samples from 2 wastewater treatment plants (WWTP), 2 lift stations, and a septic tank pump-out truck. Sewage samples were serially diluted to determine the limit of detection of each assay with respect to actual pollutants.

Human Bacteroides.

- Positive results were consistently obtained from a 10^{-5} dilution of sewage in buffered water, which is equivalent to ~ 70 CFU enterococci. The experiment was repeated in river water and coastal water, where detection levels were found to be the same as in buffered water.
- Results of co-PIs at USM and UWF were similar for human *Bacteroides* in buffered water. Both USM and UWF obtained positive results from a 10^{-4} dilution of sewage in coastal waters. UWF obtained positive results in a 10^{-5} dilution of sewage in fresh water. USM noted inhibition of the human *Bacteroides* assay when sewage was diluted in water from a duck pond. Efforts are ongoing to resolve the inhibition issue (see Problems and Solutions).

Methanobrevibacter smithii.

- A positive result was consistently obtained from a 10^{-3} dilution of sewage in buffered water. A positive result was obtained from a 10^{-3} dilution of sewage in river water, and a positive result of a 10^{-4} and 10^{-3} dilution of sewage in coastal water. River and coastal water experiments will be repeated to determine the consistency of these results.
- Results of co-PIs at USM and UWF did not differ substantially from USF's results. USM

was able to detect the target at a 10^{-4} dilution of sewage in PBS and in coastal water. Inhibition of the PCR occurred in samples diluted in duck pond water. UWF was able to detect the target at a dilution of 10^{-5} in PBS and coastal water. Fresh water tests will be conducted soon.

HPyVs.

- Positive results were obtained from sewage dilutions ranging from 10^{-2} to 10^{-3} . In order to increase assay sensitivity we have explored the 1) use of hollow fiber filtration (HFF) to concentrate large sample volumes and 2) modification of the forward primer.

We have detected human polyomavirus (pure strain from culture) and HPyVs in sewage using HFF. Both a 10-L volume of buffered water seeded with 5 ml of sewage (1:2000 dilution) and a 10-L volume of buffered water seeded with 2.5×10^4 viral particles resulted in a positive PCR result.

The same experiment was repeated using river water; however, the PCR assay was inhibited. The template, which was extracted from the HFF concentrate (river water seeded with HPyVs), was diluted in an attempt to reduce inhibition. The 1:10, 1:100 and 1:1000 dilutions were positive, indicating that inhibition was overcome. HFF holds promise for concentration of large samples into small volumes, and therefore detection of dilute targets, but the inhibition effect must be carefully accounted for.

- USM has performed the HPyV PCR assay successfully, although they are still carrying out experiments to determine the limit of detection. They have also carried out hollow fiber filtration (HFF) experiments, and have found that the concentrated template from coastal waters spiked with HPyV must be diluted 1:1000 for detection of the target.
- UWF is in the first stages of performing the HPyV assay.

Specificity

All fecal samples are first tested with primers for total *Bacteroides* or bacterial 16S rDNA to ensure there is no inhibition (false-negative).

Human Bacteroides

- Horse (n=8), sparrow (n=3), duck (n=4), cow (n=1) and dog (n=7) fecal samples have been processed, resulting in one positive cross-reaction with feces from one dog. More samples are being processed including cow, seagull and crane.

M. smithii

- Horse (n=8), sparrow (n=3), duck (n=1), cat (n=4), cow (n=5), and deer (n=1) fecal samples have been processed resulting in no cross-reactivity.

HPyV

- Dog (n=29), cow (n=6), duck (n=1), cat (n=5), sparrow (n=3), and deer (n=1) fecal samples have been processed resulting in no cross-reactivity.

The USF lab sent DNA extracted from dog feces to Chris Singalliano's NOAA laboratory (Miami). Protocols for all three human marker assays were sent to Dr. Singalliano and to Dr.

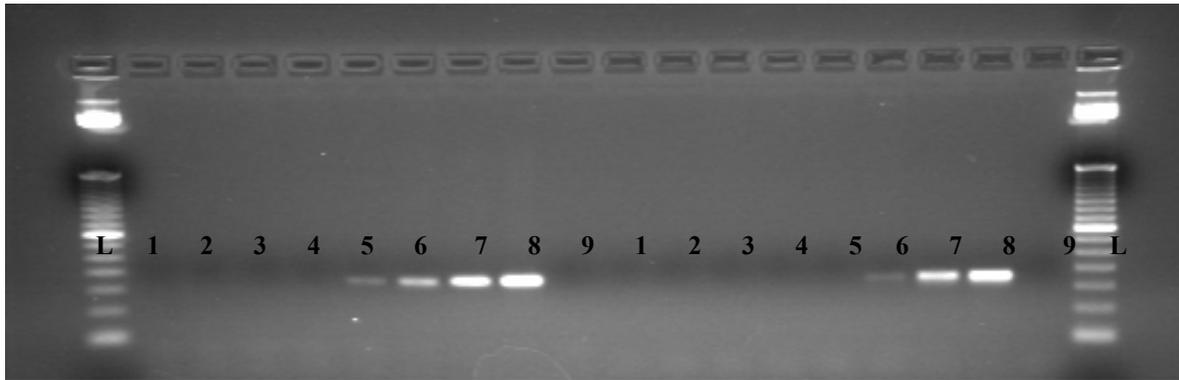
Kelly Goodwin (NOAA). Their dog-specific QPCR assay found 90% of the samples (n=29 total) to be positive for dog marker. More samples including nontarget DNA for the dog assay were sent 5/14/08.

A Quality Assurance Project Plan (QAPP) for environmental sampling was prepared and submitted to Danny Wiegand, the Program Manager.

2. PROBLEMS AND SOLUTIONS.

- USF, UWF, and USM all agreed to eliminate re-amplification from PCR assays due to high risk of contamination and production of spurious bands. This step had been previously specified in some versions of the human *Bacteroides* and HPyV assay.
- Because PCR detects minute amounts of DNA, contamination is a constant threat. Method blanks (processing DNA-free PBS through the ENTIRE sample preparation protocol, including filtration) are essential to detection of contamination if it is occurring. When processing water samples, method blanks are always used throughout the entire assay. The contamination threat also occurs when solid fecal samples are processed; however the issue is somewhat different because no filtration occurs. Because of the high concentration of DNA extracted from feces, one must be very careful to avoid cross-contamination among samples. To ensure that positive results with fecal samples are not due to contamination, the number of samples processed for DNA extraction at one time is limited to 13, plus a reagent blank (reagents from DNA extraction kit and no feces). The reagent blank is processed with the samples and if it yields a positive for PCR, the samples are thrown out.
- A tradeoff exists between the volume of water sample processed and filtering time. Filtration times were compared in order to determine if certain steps could be eliminated to decrease the sample preparation time. Using a 0.45 micron filter instead of a 0.2 micron filter for sample concentration of 500 ml of pond water decreased filtering time by 90%. However, in certain water types, particularly eutrophic tributaries, the full 500 ml volume cannot be filtered through a 0.45 μm filter. Two options are given in the protocol: (1) pre-filter the sample or (2) filter for 15 min., record the volume filtered, and discard the remaining sample.
- We have shown that the limit of detection for the *M. smithii* and HPyV assay from sewage samples is substantially higher (~2 orders of magnitude) than that of human *Bacteroides*. In other words, one can detect a much more dilute sewage sample (10^{-5}) with the human *Bacteroides* assay compared to the other two (10^{-3}). Presumably, there are fewer target organisms per unit volume for these assays than for human *Bacteroides*. A modified forward primer has been developed for the HPyV assay and both hollow fiber filtration and amendments to PCR are under investigation to decrease the limit of detection of these assays. Figure 1 shows the increase in sensitivity gained from substituting the new SM2 primer for the previously used P5 primer for the HPyV assay.

Figure 1. Sewage spike in coastal water comparing the two primer sets for HPyV



50bp ladder – amplicons are between the 150 and 200bp ladder marks

First set of 8 lanes are with the modified forward primer

1. Method blank
2. Reagent blank
3. 5µl sewage/500ml coastal water
4. 50µl /500ml
5. 500µl /500ml
6. 5ml/500ml
7. Spike 10 µl of BKV into 500ml of coastal water
8. PCR positive 1µl template of BKV
9. PCR negative

- Extensive concentration of environmental waters leads to inhibition of the PCR reaction, particularly in the case of hollow fiber filtration (HFF). Dilution of the template has alleviated this problem, but it is also counter-productive in terms of concentration factor. There is obviously a balance between sample concentration and PCR efficiency that must be achieved. The USM laboratory is focusing on testing various PCR enhancers to increase the robustness of the assays to inhibition.
- The USM lab also noted positive results for human *Bacteroides* in a coastal water body that receives effluent from a wastewater treatment plant. The team hypothesized that the marker may be detected in disinfected effluent due to the survival of DNA through the wastewater treatment process. Further testing of wastewater treatment plant effluents was therefore initiated.

USF tested for all three human markers on effluent from a wastewater treatment plant that had been disinfected with chlorine and subsequently dechlorinated with sodium thiosulphate. Three separate samples (collected on different days) were processed and found to be negative for HPyV and *M. smithii*. Only one sample tested positive for human *Bacteroides*, but it should be noted that all samples contained fecal coliforms (≤ 4 CFU/100 ml).

USM tested human *Bacteroides* and *M. smithii* on lagoon effluent and found both markers present.

3.WHAT WORK IS PROJECTED THROUGH THE END OF YEAR 1?

- Complete validation (replication of results) and extend specificity testing to more individual samples.
- Send SOPs and control DNA to collaborators. Collaborators have been contacted and are still eager to participate
- Complete literature review

4.Is the project work on schedule?

a. Yes

5.Does the project funding rate support the work program?

To date, funds are adequate. There may be a need to expedite some quantitative PCR work; this will be discussed with the Program Manager.

6.What has been spent to date? What is your plan for spending the remaining funds?

Cumulative expenses through May 2008 are in the amount of \$177,146 (including encumbered funds). This is a preliminary report, and we expect to spend the remainder of year 1 funds by the beginning of year 2 (August 2008) on supplies and salaries. Remaining funds will be spent as outlined in the project budget.

7.Have you submitted a quarterly voucher for reimbursement? A copy should be attached to the quarterly report.

Not applicable -- payment handled through ASAP.gov; however, see above for cumulative expense through April 2008.

8.Is there a change in project manager?

No change

9.If you have a multi-year project or will not complete the project as projected, have you submitted your request for a funding amendment?

This report is a precursor to the request for the next year of the project.

11. Projected goals for Year 2

See proposal, but in brief, projected goals for Year 2 are unchanged and focus on:

- Validation of methods at field sites (Year 2)
- Validation of methods by collaborators

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