

# **Report as of FY2007 for 2006MN161B: "Development of a DNA Marker Gene System to Determine Sources of Fecal E. coli in Watersheds"**

## **Publications**

- Articles in Refereed Scientific Journals:
  - ◆ Yan, T., M. Hamilton, and M. J. Sadowsky. 2007. High throughput and quantitative procedure for determining sources of Escherichia coli in waterways using host-specific DNA marker genes. *Appl. Environ. Microbiol.* 73:890-896

## **Report Follows**

## **Development of a DNA Marker Gene System to Determine Sources of Fecal *E. coli* in Watersheds**

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### **Project Report**

Many of Minnesota's rivers, lakes, and streams do not meet the Clean Water Acts "swimmable" goal due to elevated numbers of fecal coliform bacteria. Sources of fecal coliform bacteria include runoff from feedlots and manure-amended agricultural land, wildlife, inadequate septic systems, urban runoff, and discharge from sewage systems. Moreover, high levels of fecal bacteria in Minnesota's watersheds threaten the use of these water resources for recreational use and drinking. In this study, we used pooled genomic tester and driver DNAs in suppression subtractive hybridizations to enrich for host source-specific DNA markers for *Escherichia coli* originating from cows, pigs, and humans. For human specific marker DNAs, three separate subtractive hybridizations were done using 5-60 human *E. coli* strains as tester DNAs and 20-50 *E. coli* from other animals as driver DNAs. This generated 576 potential marker genes specific for human *E. coli*. These 576 marker genes were screened by dot-blot Southern hybridization for reactivity to *E. coli* from humans, and 146 reacted with human *E. coli* control DNA. The 146 DNA fragments were also screened by restriction enzyme analysis and the majority were found to be clonal, 44 fragments were unique. The 44 probes were tested for specificity in hybridization reactions with 48 cat, 96 chicken, 96 cow, 96 deer, 96 dog, 81 duck, 135 goose, 42 goat, 78 horse, 210 human, 96 pig, 60 sheep, and 96 turkey *E. coli* isolates. Results of these analyses indicated that 21 cloned DNA fragments showed some hybridization specificity to DNA from *E. coli* isolated from humans, whereas the

remaining 23 probes cross-reacted with *E. coli* from all the animal sources. While our best probes identified greater than 50% of the 210 human *E. coli* strains tested, they also cross hybridized to a significant numbers of non-human strains. Using the same overall strategy, but with different tester and driver strains, we were able to isolate potential DNA probes that detect *E. coli* strains originating from pigs/turkeys, and animals in general. One set of probes developed in this project identified ~ 65% of all of the tested pig *E. coli*. However, they cross-react with ~ 8% of *E. coli* from Turkeys.

Over the past decade, several microbial source tracking (MST) methods have been intensively investigated, leading to the development of a wide variety of potential methods. Most methods to date, however, have suffered from low discriminatory power. In contrast, several genotypic-based methods have been found to be highly efficient in discriminating amongst bacteria originating from different animal hosts. We have developed a genetic marker based detection system (using DNA probes) for host-specific traits that are ecologically meaningful with respect to the microorganism studied. We have been using a multi-strain, genomic comparison approach to identify DNA fragments unique to *E. coli* strains isolated from a particular type of host source. Using this approach we have successfully developed DNA probes specific for *E. coli* strains originating from Canadian geese and ducks.

In our current studies we focused our efforts on the development of marker probes for *E. coli* strains originating from cows, humans, and pigs. The prioritization of these three types of host sources was mainly due to their predominance as contributors to agricultural- and urban-derived fecal contamination in watersheds. To achieve our goals, we used the technique of subtraction suppressive hybridization (SSH) in an attempt to identify DNAs that are specific for *E. coli* originating from humans, cows and pigs.

We used a multi-strain genomic comparison approach for the identification of host-specific DNA markers. The suppression subtractive hybridization (SSH) technique was used to enrich for DNA fragments unique to *E. coli* from each type of host sources. The *E. coli* strains used in SSH and subsequent specificity analyses were obtained from a library of unique isolates previously isolated from the feces of 12 known animal host sources (cats, chickens, cows, deer, dogs, ducks, geese, goats, horses, pigs, sheep, and turkeys), and humans. Suppressive subtractive hybridizations were done using the

CLONTECH PCR-Select™ Bacterial Genome Subtraction kit (BD Biosciences CLONTECH, Mountain View, CA). In initially, three different subtraction hybridizations were done; Human subtraction 1 used 5 human *E. coli* strains as tester DNA , and 5 goose *E. coli* strains as driver DNAs. Following transformed of subtraction products, 192 clones were picked. These were screened by dot-blot hybridization and 11 probes were found to be tester specific (all 11 were confirmed as specific by Southern Hybridization). All 11 probes were tested for specificity by robot arrayed colony hybridization with 48 cat, 96 chicken, 96 cow, 96 deer, 96 dog, 81 duck, 135 goose, 42 goat, 78 horse, 210 human, 96 pig, 60 sheep, and 96 turkey *E. coli* isolates. However, none of the probes reacted with a large number of human strains and cross hybridization with strains from other hosts was pronounced. This suggested that a larger number of tester and driver DNAs were needed.

In subsequent analyses, 20 human *E. coli* strains were used as tester DNAs and 20 *E. coli* from animals (5 cows, 5 geese, 5 pigs, 1 chicken, 1 dog, 1 cat, 1 horse, and 1 sheep) were used as driver DNAs. Following transformed with the subtraction mixture, we picked 480 clones and screened 75 of these by dot blot Southern hybridization. All 75 clones had strong hybridization signal when probed with pooled DNAs from the tester strains (Figure 1) and only weakly hybridized when probed with driver strains. Sixty-four of these clones were confirmed as tester specific by Southern hybridization and restriction enzyme digestion analysis showed there were 41 unique probes. Of these 15 were tested for specificity by robot arrayed colony hybridization with 48 cat, 96 chicken, 96 cow, 96 deer, 96 dog, 81 duck, 135 goose, 42 goat, 78 horse, 210 human, 96 pig, 60 sheep, and 96 turkey *E. coli* isolates. Nine probes were shown to react predominately with human strains, but only about 10% of the human strains reacted and the same human strains reacted with probes. One probe reacted with 26 of 210 human strains and only 2 chickens, 2 horse, and 1 sheep strain. No further colony hybridizations were attempted because the same human strains were identified with almost all 15 probes.

Consequently, we tried an additional subtraction using 60 human strains as tester DNAs and same 20 animal strains as driver DNAs as discussed above. Following transformation of the subtraction products, we picked 576 clones and screened these by dot blot Southern hybridization. Of these clones, 74 were selected as being tester specific

and 71 of the 74 were confirmed as being tester DNA specific by Southern hybridization. Twelve of these were tested by colony hybridization with same strains as discussed above, and all 12 tested hybridized with greater numbers of human strains than probes from the first or second human subtractions. Some of these 12 clones identified greater than 50% of the 210 human strains. Unfortunately, they also cross hybridized with significantly greater numbers of non-human strains, compared to probes from the first human subtraction (10-30% for several hosts). In one case, nearly 60% of cat strains cross reacted with the tested probes.

We also used the SSH technique in an attempt to isolate marker genes specific for cows. We used DNA from 20 *E. coli* cow strains as tester and 20 DNAs from non-cow strains as driver (*E. coli* from 5 humans, 5 pigs, 5 chickens, 2 horses, 2 sheep, and 1 goose). The cow, human, pig and chicken strains were selected by HFERP dendrogram analysis, and the horse, sheep and goose strains were selected randomly. Subtraction products were cloned and 576 clones were picked for the subtraction library. Of these, 288 clones were tested by dot blot hybridization for specificity for cow *E. coli* – 68 were found to be tester specific. Furthermore, 60 of 68 were also confirmed to be tester specific by southern blot hybridization analysis. Restriction enzyme analysis on the 60 confirmed cow specific clones showed that the clones contained 25 different insert DNAs. Colony hybridizations were done using 14 of the 25 different inserts against an array of *E. coli* from 13 animal hosts and humans. These arrays included *E. coli* strains from the following sources, with the number of strains in parentheses, cat (48), chicken (96), cow (189), deer (96), dog (96), duck (81), goose (135), goat (42), horse (78), human (210), pig (218), sheep (60), and turkey (96). However, none of the insert DNAs tested were specific and results showed that inserts cross hybridized with isolates from many different animal hosts. Inserts hybridized with considerable numbers (>15%) of isolates from source groups not represented or poorly represented in the driver sample. The remaining 11 inserts were not tested, since the isolated probes appeared not be specific for cows.

To potentially increase the specificity of the probes, we chose to modify the subtraction reaction – by both increasing the diversity of strains present in the driver sample and increasing the time of the primary hybridization. To do this, DNAs from 25

cow *E. coli* strains were used as tester and DNAs from 40 non-cow strains used as driver in SSH reactions. The driver sample consisted of 5 strains from each of the following source groups: chickens, goats, geese, horses, humans, pigs, sheep, and turkeys. All strains used in the subtraction were selected by HFERP DNA fingerprint dendrogram analysis. Subtraction products were cloned and 384 clones were initially picked for the subtraction library. We initially analyzed 192 clones by dot blot hybridization to tester and driver DNAs and 35 were found to be tester specific (for cows). Of these, 26 of the 35 were confirmed as tester specific by southern hybridization analysis. Gel electrophoresis analysis of cloned DNA fragments suggested that 10 of the 26 clones contained the same DNA insert. In total, it appears that there are 12 different fragments. It is possible that these inserts have different sequences and nearly the same size. Further analysis through restriction analysis and/or hybridization will be necessary to determine the exact number of different inserts. This is currently ongoing. We also tested 6 cloned insert DNAs by hybridization to a panel of *E. coli* strains from 12 animal hosts and humans. Results of these analyses indicated that 5 of the inserts had a significant percentage of cross-hybridization with non-cow strains, approx 15-25% of all isolates (including cow isolates) hybridize with these inserts. However, 1 insert hybridized only with cow strains, although it only recognized 11 of 189 strains tested. This insert was sequenced and found to be nearly identical to the colicin-N gene of *E. coli*

We also performed SSH reactions to isolate DNA clones specific for pigs. The DNAs from 21 *E. coli* strains from pigs were chosen as tester and 30 non-pig strains (10 cows, 10 humans, 5 chickens, 1 dog, 1 cat, 1 goat, 1 goose, 1 turkey) were chosen as driver DNAs. All strains used in the subtraction were selected by HFERP DNA fingerprint dendrogram analysis. Subtraction products were cloned and 576 clones were picked for the subtraction library. All 576 clones were tested by dot blot hybridization and 50 were found to be tester (Pig) specific. Of these, 12 of the 50 clones were confirmed as tester specific by Southern blot hybridization to genomic DNAs. Seven of the cloned insert DNAs were tested by colony hybridization. However, none of the inserts were specific to pig isolates, although one insert hybridized predominately with pigs (~40%) and turkeys (~30%). Isolates from other host species cross-hybridized with the probes at <15%, suggesting that this insert may be useful to identify Pig

contamination in waterways not impacted by turkeys. The remaining 11 inserts were not tested, instead we chose to modify the subtraction as described above, by both increasing the diversity of strains present in the driver sample and increasing the time of the primary hybridization . To do this, DNAs from 21 pig strains were chosen as the tester and DNAs from 40 non-pig strains (chickens, cows, goats, geese, horses, humans, sheep, and turkeys) were used as driver DNAs. All strains used in the subtraction were selected by HFERP dendrogram analysis. Subtraction products were cloned and 192 clones were picked for the subtraction library. All 192 clones were screened by dot-blot hybridization to the tester and driver DNAs, and 22 were found to hybridize specifically to the tester (pig) DNAs. Southern blot analysis indicated that 10 of 22 clones were pig specific. Colony hybridizations to all *E. coli* strains in our library indicated that 8 hybridization probes had the ability to detect pig *E. coli*, together they detect ~ 65% of the tested pig *E. coli*. However, they cross-react with ~ 8% of *E. coli* from Turkeys. The basis for the cross-reaction is not known. One of the isolated probes proved to be very interesting, it reacted with Deer, Pigs, Sheep and Goat *E. coli*, but with very few (~1%) of human isolates. This may be useful as a more general animal probe. Moreover, one of the probes reacted with the following percentage of tested isolates as follows: Humans - 1.4%, Horses - 16%, Goats - 2.4%, Sheep - 8.2%, Pigs - 10%, and turkeys 0.08%. Since this probe reacted mostly with horses, this probe may also have use for a general probe for non-human related contamination.

## **Publications, Presentations, or Published Abstracts:**

### Publications

Yan, T., M. Hamilton, and M. J. Sadowsky. 2007. High throughput and quantitative procedure for determining sources of *Escherichia coli* in waterways using host-specific DNA marker genes. *Applied and Environmental Microbiology* 73:890–896.

### Presentations (\* indicates student presentation)

Sadowsky, M.J. 2008. Microbial Source Tracking Methods: Myths and Realities. Oral Presentation. Minnesota Pollution Control Agency, St. Paul, MN.

Sadowsky, M.J. 2007. Development and Use of Marker Genes to Determine Sources and Sinks of Fecal Bacteria and Pathogens in the Environment. Oral presentation. University of Montana Missoula, MT

Sadowsky, M.J. 2007. Library-Dependent Genotypic Methods for MST Studies. Oral presentation. EpiNet, Chicago, IL.

Sadowsky, M.J. 2006. Alternate source and sinks of pathogens in the environment. Oral presentation at the Annual Meetings of the American Society of Agronomy (ASA), Crop Science Society of America (CSSA), and Soil Science Society of America (SSSA), November 12 – 16, Indianapolis, IN.

Sadowsky, M. J. 2006. Development and Use of a High-Throughput Robotic Method to Determine Sources of *E. coli* in the Environment. Oral presentation. University of South Florida, Tampa, FL.

Sadowsky, M. J. 2006. Has Human Activity Outstripped the Environments Ability to Rid Itself of Fecal Bacteria? Albrecht Lecture, Earth Day, University of Missouri, Columbia, MO.

## **Students and Post-doctoral Research Associates supported by this project:**

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**Additional Funding:**

Funding from this current project was used to leverage additional support from the Minnesota Department of Agriculture (MNDAG) for a project entitled: Development of a DNA Marker Gene System for *E. coli* from Cows, Pigs, and Turkeys and Using Small Watersheds to Monitor Bacteria Loadings and Effects of Mitigation Practices. This project was awarded to James Anderson (PI) and Michael Sadowsky (co-PI) for the period March 15, 2007 to June 30, 2009. The MNDAG project is currently supporting the salaries of two graduate students (Charlie Sawdey and Daniel Norat), who continue to work on this project

**Awards and Special Recognition:**

None to date