



WATER RESOURCES RESEARCH GRANT PROPOSAL

Title: Ribotype Source Library of *Escherichia coli* Isolates from Georgia

Focus categories: NPP, WQL, AG

Keywords: agriculture, animal waste, bacteria, biotechnology, microbiology, ribotyping, watershed management, water quality

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Principal investigator:

Peter G. Hartel
Dept. of Crop & Soil Sciences
University of Georgia
Athens, GA 30602-7272
Georgia Water Resources Institute

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Statement of critical regional water problems

The proposed research will isolate a fecal coliform, *Escherichia coli*, from the feces of a wide variety of warm-blooded animals, including humans, from at least two locations in the State of Georgia and will catalog the ribosomal DNA fingerprint (ribotype) of each isolate. This genotypic (DNA-based) method, called ribotyping, shows considerable promise in being able to associate specific *E. coli* ribotypes to specific animal hosts. This proposal establishes a ribotype source library against which any isolate of *E. coli* from a water source in the State of Georgia can be compared to identify its host origin. The ramifications of this for the State are important and far-reaching, particularly for waters in the State that are out of compliance with respect to fecal coliform numbers.

With regards to the Georgia Water Resources Research Program Priorities for FY2000, the proposed research responds directly to three priorities. This research responds directly to the priority "pollution source and transport assessment" under surface water quality protection. Because of ribotyping's discriminatory power, it should be possible not only to identify the pollution source of many *E. coli* isolates from any Georgia water source, but also, in some cases, how far the isolate has moved. This research also responds directly to the research priorities for "pollutant source control" under environmental protection and "state-wide assessment and long-range planning " under water

management. With this research in hand, water resource managers will be able direct their efforts at controlling sources of *E. coli* (as fecal coliforms) where reasonable control is possible (e.g., human sewage) and not at sources over which they had limited control (e.g., wildlife). The information gained in this research would also allow water resource managers to reassess surface waters that were out of compliance with respect to fecal coliforms if wildlife were the greatest contributors of these bacteria.

Statement of the results, benefits, and/or information

The main benefit of ribotyping, being able to identify the host origin of unknown *E. coli* isolates (as fecal coliforms) from state water sources, is not possible if a source library from a wide variety of warm-blooded animals does not exist. The more extensive the ribotype source library, the greater the likelihood is of obtaining matches. Beginning last November, I began to assemble a library of ribotypes of *E. coli* from Georgia. At this point, I have obtained several hundred *E. coli* ribotypes from beef cattle, swine, poultry, and Canada goose in Georgia. The results are promising. The intent of this proposal is to ribotype a minimum of 1,000 *E. coli* isolates from a variety of other warm-blooded animals at two locations in Georgia in order to establish a ribotype source library for the State of Georgia.

The benefit of the proposed research is that if water resource managers are able direct their efforts at controlling sources of *E. coli* (as fecal coliforms) where reasonable control is possible (e.g., human sewage) and not at sources over which they have limited or no control (e.g., wildlife), then this constructive allocation of State resources would maximize the possibility of improving the State's water quality. With respect to the proposed research, the results will be published in a refereed scientific journal, and the ribotype source library will be placed on the world-wide web so that it is publicly accessible.

Nature, scope, and objectives of the research

A. Nature and scope

Fecal coliforms consist of several bacterial genera from the family Enterobacteriaceae that can grow on a selective medium at 44.5 °C for 24 hours. Fecal coliforms normally inhabit the intestinal tract of warm-blooded animals and their presence in soil or water is a good indicator that the soil or water was contaminated by bacterial pathogens. For example, when numbers of fecal coliforms exceed 2,000 per 100 mL of water, the likelihood of bacterial pathogens in the water is 98.1% (Geldreich, 1970). Fecal coliform counts are typically used to monitor Georgia's recreational waters.

One of the most vexing problems in isolating fecal coliforms from water samples is not knowing the host origin of these bacteria. In the past, the only way to identify the host origin of a bacterium was to observe the bacterium's various phenotypic markers (i.e., characteristics expressed by the bacterium, like multiple antibiotic resistance). The main problems with using phenotypic markers are their lack of reproducibility and lack of

discriminatory power (ability to distinguish two closely related strains). However, in recent years, it has become possible to identify the host origin of a bacterium based on its DNA. This alternative method, called genotyping, not only has increased reproducibility, but also has increased discriminatory power. The most common of these genotypic methods include chromosomal DNA restriction analysis, plasmid typing, pulsed field gel electrophoresis, various polymerase chain reaction (PCR) methods, and ribotyping (Farber, 1996).

Each genotypic method has its advantages and disadvantages with respect to strains that can be typed, reproducibility, discriminatory power, ease of interpretation, and ease of performance. In this proposal, the genotypic method I selected is ribotyping. Ribotyping is based on ribosomal RNA (rRNA). Ribosomal RNA is present in all bacteria, and is composed of three species, 5S, 16S, and 23S. The DNA in the bacterium that codes for these three species of rRNA is usually present in 2 to 11 copies and is highly conserved (or similar; Grimont and Grimont, 1986). In ribotyping, the DNA is isolated from the bacterium and cut with a special enzyme that only recognizes certain DNA sequences (i.e., a restriction enzyme). The DNA is electrophoresed in a gel and the DNA transferred to a nylon membrane (this is called Southern blotting). The membrane is probed with a chemiluminescent copy of the 5S, 16S, and 23S portions of the DNA and, when properly treated, the membrane gives a pattern that can be scanned with an imager. As a method for distinguishing a subspecies of a bacterium, ribotyping is considered to have excellent reproducibility, good discriminatory power, excellent ease of interpretation, and good ease of performance (Farber, 1996).

In this proposal, the fecal coliform I selected for ribotyping is *Escherichia coli*. This bacterium was selected for five reasons. First, as a fecal coliform, *E. coli* is accepted by the American Public Health Association as a good indicator of pathogenic bacteria (Clesceri et al., 1998). Second, most environmental ribotyping has been done with this bacterium (see Related Research). As a result, the methodology for ribotyping this bacterium is established. Third, there is good scientific evidence that specific strains of *E. coli* are associated with different host species (Faith et al., 1996). Fourth, *E. coli* does not exist as a stable population in the environment unless the source of contamination is persistent (Savageau, 1983). Fifth, *E. coli* is easy to isolate and easy to manipulate genetically.

With an extensive library of *E. coli* ribotypes from Georgia, one should be able to isolate *E. coli* from any water source (as well as from other sources, like soil) in Georgia and identify the host origin of that *E. coli* isolate. To develop this library, a large number of *E. coli* isolates must be obtained from warm-blooded animals and ribotyped. An isolate of *E. coli* from a water source can then be ribotyped and compared to the source library to identify its host.

At present, I have ribotyped over 200 *E. coli* isolates from Georgia. These include *E. coli* isolates from beef cattle, poultry (broilers), sheep, and swine. In addition, I have obtained 70 *E. coli* isolates from the study of Buchan et al. (1997) to add to my collection. These isolates from beef cattle (23 isolates), Canada goose (19 isolates), and poultry (28

isolates). I have recently obtained the necessary software to analyze the gels of all these *E. coli* isolates. Although the comparisons are not yet complete, a simple visual inspection of one of the gels shows clear differences among the ribotypes of the three different animals (Fig. 1, next page). Currently, besides my own library of several hundred *E. coli* isolates, no other library containing *E. coli* ribotypes from Georgia exists anywhere in the world.

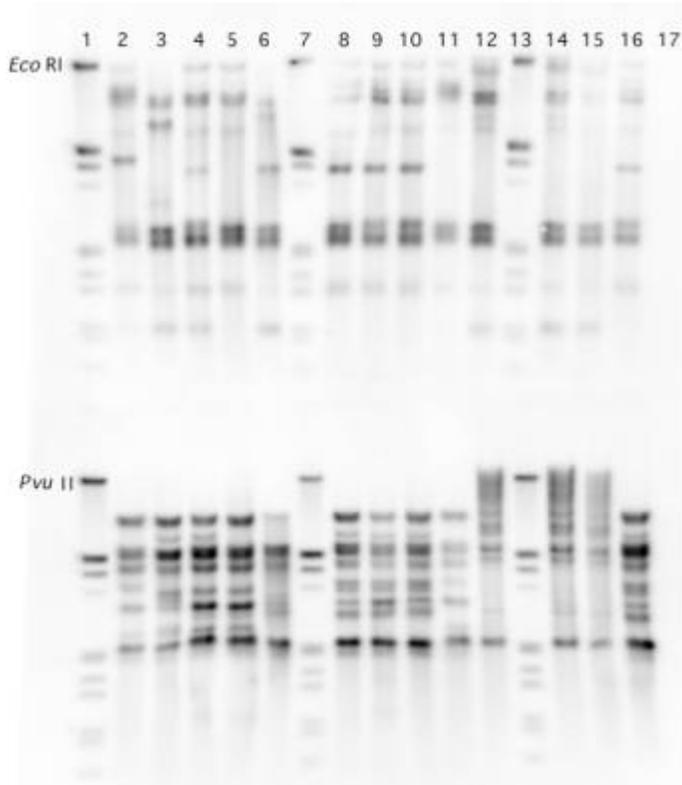


Fig. 1. Lanes 1, 7, and 13 are λ -DNA cleaved with *Eco*RI and *Hind*III (molecular weight marker and known DNA control; Lanes 2, 3, 4, and 5 are beef cattle isolates; Lanes 6, 8, 9, 10, and 11 are poultry isolates; Lanes 12, 14, 15 and 16 are Canada goose isolates; Lane 17 is the no DNA control. The lanes for no DIG-labeled DNA control, and DNA from type culture *E. coli* ATCC #11775 are not shown. The DNA was cut with two restriction enzymes, *Eco*RI (top) and *Pvu*II (bottom) and run as a double gel. The ribotype patterns of *E. coli* isolated from beef cattle, poultry and Canada goose are clearly different.

Objective: To construct a ribotype source library of *E. coli* isolates from a wide variety of warm-blooded animals in Georgia in order to determine the host origin of *E. coli* isolates from Georgia waters.

Methods, procedures, and facilities

A. Obtaining fecal samples for the source library

Fresh fecal samples will be obtained as aseptically as possible from a variety of warm-blooded animals at the two Georgia locations, Athens and Tifton. The principal agricultural animals to be tested will be cattle, poultry, swine, horses, and sheep. The wild animals to be tested will be waterfowl and deer. Other sources will be humans and dogs. With the exception of humans, fresh fecal samples will be collected with an ethanol flame-sterilized spatula and will be transferred to sterile Whirl-Pak bags. Collection information will consist of the animal species, person collecting, and the date and place collected. The bags will be kept on ice and will be sent by overnight mail to the University of Georgia for *E. coli* isolation. Because of the restrictions on the use of humans, human isolates of *E. coli* will be collected from septic tanks. These septic tanks will be from families without outside sources of *E. coli* (e.g., dogs, cats). Septic tanks will be sampled with sterile dilution bottles and will be treated in the same manner as the fecal samples. It is important to note that poultry litter or swine lagoons will not be sampled as these sources may contain *E. coli* from other sources (e.g., broiler litter may contain rodent feces).

B. Obtaining isolates of E. coli from the fecal samples

Escherichia coli will be isolated from the fecal or septic tank samples by serial dilution. Fecal samples will be diluted from 10^{-1} and 10^{-8} . A 10-g or 10-mL sample will be placed in sterile 0.1% Bacto-Peptone (Difco Laboratories, Detroit, MI) contained in a 160-mL dilution bottle. After 1 minute of shaking, a 10-mL sample will be transferred to another 160-mL bottle containing 90 mL of the same peptone diluent and so forth. Samples of each dilution will be filtered through 0.45- μ m filters (Nalgene, Rochester, NY) and the filters will be placed on mTEC agar plates (Difco). The plates will be wrapped in quadruple Ziploc bags and will be incubated submerged in a water bath at $44.5 \pm 0.2^\circ\text{C}$ for 24 h. All dilutions will be done in duplicate. Yellow colonies growing on mTEC agar (Difco) after 24 h incubation will be considered as presumptive *E. coli*.

Presumptive isolates of *E. coli* will be transferred to tryptic soy agar (TSA; Difco) and will be incubated overnight at 35°C . This step will be repeated to ensure pure isolates. After two streakings, each isolate will be struck a third time on TSA as well as on urea agar (Difco) and Simmons citrate agar (Difco). To minimize agar use, the urea agar and Simmons citrate agar will be as 1.5-mL slants contained in 24-well cell culture cluster plates (Costar, Cambridge, MA). In addition to the *E. coli* isolates, type cultures from the American Type Culture Collection (ATCC, Manassas, VA) will be used as appropriate controls. These organisms represent almost all bacteria that can be found on mTEC agar plates after appropriate dilution. The type cultures include *Escherichia coli* #11775 (urease⁻, citrate⁻), *Klebsiella pneumoniae* subspecies *pneumoniae* #13883 (urease⁺, citrate⁺), *Citrobacter freundii* #8090 (urease⁺, citrate⁺), and *Enterobacter aerogenes* #13048 (urease⁻, citrate⁺). After overnight incubation at 35°C , fecal isolates that can grow on TSA but are urease⁻ and citrate⁻ will be subjected to an oxidase test. If the isolate is also oxidase⁻ (cytochrome oxidase P450 negative), then the isolate will be considered as presumptive *E. coli* and will be frozen. All other isolates will be autoclaved and discarded. To freeze each *E. coli* isolate, a loopful of each isolate will be transferred from the third streaking of the TSA plate to two labeled cryovials, each containing a 3.5:1

mixture of saline/phosphate buffer (8.5 g of NaCl, 0.65 g of K₂HPO₄, and 0.35 g of KH₂PO₄ per liter of distilled water) and cryoprotectant (50 mL of sterile glycerol and 50 mL of dimethylsulfoxide). The two cryovials, one representing the working stock and the other the reserve stock, will be kept in different -80°C freezers.

A total of 1000 *E. coli* isolates will be obtained, with 500 isolates being obtained at Athens and 500 isolates at Tifton. The minimum number of isolates from an animal species will be 50. The number of isolates from humans and other warm-blooded animals at each location will be (number of isolates): human (100), cattle (100), deer (50), dogs (50), poultry (50), sheep (50), swine (50), and waterfowl (50). To maximize ribotype diversity, each human or animal will contribute no more than 5 isolates. For example, 10 different poultry facilities will be visited in order to obtain the necessary 50 poultry *E. coli* isolates.

C. Obtaining ribotypes of *E. coli*

Presumptive isolates of *E. coli* will be struck from the -80°C freezer onto TSA and incubated overnight at 35°C. A single clone will be inoculated into 10 mL of Luria-Bertani broth (Maniatis et al., 1982) and incubated on a rotary shaker at 100 rpm overnight at 35°C. The DNA from a turbid, 1-mL sample will be obtained with a commercial kit (DNeasy tissue kit, Qiagen Inc., Valencia, CA). The DNA will be quantified with a UV spectrophotometer at 260 nm (DNA) and 280 nm (protein). Samples with an acceptable 260:280 ratio (i.e., >1.75) will be used for ribotyping.

A digoxigenin (DIG)-labeled probe will be prepared. To do this, 6.3 µL of distilled water, 1.4 µL of 16S and 23S RNA (= 5.4 µg of RNA) and 3.3 µL of 5S RNA (= 2.6 µg of RNA), 2 µL of hexanucleotide mix, 2 µL of dNTP, 4 µL of reverse transcriptase buffer, and 1 µL of reverse transcriptase (all ingredients except the distilled water are from Boehringer-Mannheim, Indianapolis, IN) will be added to a microfuge tube to yield a total of 20 µL of DIG label. The label will be incubated overnight at 37 °C. The labeling will be stopped by adding 2 µL of 200 mM EDTA (pH 7.5) to the microfuge tube and the DIG label then quantified against a kit standard (Boehringer-Mannheim). This procedure will yield a reverse transcribed, DIG-labeled probe of *E. coli* 5S, 16S, and 23S rRNA.

To perform a restriction digest of the genomic DNA, a 2-µg sample of DNA will be added to each of two microfuge tubes and each brought to a 16-µL volume with distilled water. A 2-µL sample of *EcoRI* or *PvuII* will be added to a 2-µL sample of the appropriate restriction enzyme buffer to give a final microfuge volume of 20 µL (sufficient for loading one well in the gel). The mixture will be incubated at 37 °C.

After overnight incubation, 4 µL of loading dye will be added to each tube of restricted DNA (total volume, 24 µL). A 14 µL-portion of DNA will be added to each well of a 0.7% agarose gel. Additional wells will be set aside for DNA ladders of λ-DNA cleaved with *EcoRI* and *HindIII* (molecular weight marker; Boehringer-Mannheim), no DNA control, no DIG-labeled DNA control, and DNA from type culture *E. coli* ATCC #11775.

The gel will be submerged in 1X Tris acetate EDTA buffer before electrophoresing at 55 volts for approximately 3 hours.

The gel will be placed on a Nytran nylon membrane contained in a vacuum blotting assembly (VacuGene, Pharmacia Biotech, Uppsala, Sweden). The gel will be sequentially washed with denaturing solution (10 min), neutralizing buffer (10 min), and transfer buffer (40 min). After transfer (this is a Southern blot), the gel will be discarded and the membrane washed briefly (5 min) in 2X transfer buffer before the DNA on the membrane is fixed with shortwave UV light (Hofer UV Crosslinker, Amersham Pharmacia Biotech, Piscataway, NJ).

The membrane will be hybridized with preheated DIG-labeled probe overnight at 42°C. The membrane will be washed in a series of stringency washes before equilibrating in washing buffer for 1 min. The membrane will be incubated in blocking solution for 60 minutes at room temperature. The blocking solution will be discarded and a new batch of blocking solution containing anti-DIG-alkaline phosphatase will be added. After 30 minutes incubation at room temperature, the solution will be discarded and detection buffer will be added for 2 min. The membrane will be removed from detection buffer and will be treated with a chemiluminescent substrate (CPSD, Boehringer-Mannheim). The chemiluminescence will be quantified with an Alpha Innotech FluorChem 8000 imager (Alpha Innotech, San Leandro, CA) and the image saved as a TIFF file. The banding patterns contained in the image will be quantified with gel analysis software (GelCompar II, Applied Maths, Inc., Kortrijk, Belgium). Relationships among the isolates will be examined by cluster analysis, and cluster dendrograms will be plotted with the same gel analysis software.

Related research

Introduction

There are only four teams in the world determining the host origin of *E. coli* isolates in environmental water samples by ribotyping. All four teams are in the United States. The principal investigators of the four team include (university): Mansour Samadpour (University of Washington), George Simmons (Virginia Tech) , Mark Tamplin (University of Florida), and myself (University of Georgia). Because the research is so new, published literature is scarce. The literature has been divided according to these research teams.

Samadpour (University of Washington)

This team has contributed to four nonrefereed reports on ribotyping environmental *E. coli* isolates. In the first report, Samadpour and Chechowitz (1995) matched 421 of 589 *E. coli* isolates (71%) from Little Soos Creek (Washington State) to cow, deer, dog, duck, fish, horse, humans, llama (!), swine, and poultry. The primary contributors of *E. coli* to the creek were cows and dogs.

In the second report, Farag and Goldstein (1998) isolated fecal coliforms in Grand Teton National Park (Wyoming). The *E. coli* isolates were sent to the M. Samadpour for ribotyping. The isolates from Cascade Creek in the park matched source ribotyping patterns of humans, birds, deer, dogs, and elk. The total number of no matches is not given. This study was followed by a more extensive third report the next year in the same park but other locations (Tippets, 1999). Of 104 *E. coli* isolates, ribotyping matched 14 as human, 13 as deer or elk, 11 as avian or goose or duck, 6 as marmot, 6 as rodent, 5 as bird, 5 as bear, 3 as beaver, 3 as dog, 2 as raccoon, 1 as cattle, and 35 as no match. Therefore, ribotyping was able to identify 69 of the 104 isolates (66%).

A fourth report by Berghoff (1998) involved the Glen Canyon National Recreational Area, Utah. Again, the *E. coli* isolates were sent to the M. Samadpour for ribotyping. Ribotyping was able to identify 47 of 248 *E. coli* isolates (19%). Of the isolates where the host was identified, 24 were from cattle, 15 from humans, 4 from birds, 2 from dogs, and one each from cat and elk or deer.

Simmons (Virginia Tech)

This team has contributed to two ribotyping reports. In the first report, Simmons et al. (1995) attempted to determine the source of fecal coliforms that were forcing closure of oyster beds in the Chesapeake Bay. Fecal samples were collected from raccoon, waterfowl, otter, muskrat, deer, and humans in the area. The sources of the fecal coliforms in the oyster beds were matched raccoons and deer. When these animals were removed by either hunting or trapping, numbers of *E. coli* declined by up to two orders of magnitude, permitting the oyster beds to reopen.

In the second report, Simmons and Herbein (1998) initiated studies to determine the source of fecal coliform contamination at a beach in San Diego, CA. Of 83 *E. coli* isolates, 72 (87%) matched *E. coli* isolates from harbor seals with a similarity of 80% or better.

Tamplin (University of Florida)

This team has contributed one refereed journal article on ribotyping. Parveen et al. (1999) ribotyped a total of 238 *E. coli* isolates from human and nonhuman sources. The isolates were collected from the Apalachicola National Estuarine Research Reserve (Florida), associated sewage treatment plants, and directly from animals. The human isolates had 41 different ribotype profiles; the nonhuman isolates had 64 different profiles. When the ribotype profiles were clustered, discriminant analysis showed that 100% of the human profiles and 97% of the nonhuman profiles were correctly classified.

Hartel (University of Georgia)

I have one refereed journal article on ribotyping *E. coli*. In this study, I used a Riboprinter (Qualicon, Inc., Newark, DE) in an attempt to automate the ribotyping of the *E. coli* isolates from two small streams in Georgia (Hartel et al., 1999). Although the RiboPrinter

uses only the 16S portion of rRNA as a probe and one restriction enzyme at a time (and therefore yields fewer bands), it automates ribotyping such that at many as 32 isolates can be processed in a day. The RiboPrinter was able to discriminate among ribotypes of *E. coli* from a pasture stream, a wooded stream, and cow manure, but the discrimination was insufficient within a site. For this reason, I went back to the more discriminating (but more time-consuming) method in this proposal involving 5S, 16S, and 23S portions of *E. coli* rRNA and two restriction enzymes, *EcoRI* and *PvuII*. These results have yet to be published.

Summary

Ribotyping bacteria from environmental sources is in infancy. As the above research shows (e.g., Samadpour and Chechowitz, 1995), when a good source library of *E. coli* exists, ribotyping is good at identifying the host origin of the isolates. However, when only a limited source library exists (e.g., Berghoff, 1998), ribotyping can identify the host origin of only a few of the isolates. This reason for the current proposal to develop a strong ribotype source library.

Also implicit in the development of strong ribotype source library is the idea that some geographic separation of ribotypes must occur. Otherwise, the large database developed by Samadpour, which reflects *E. coli* isolates from the Pacific Northwest, should be able to identify most isolates from the Glen Canyon National Recreational Area in Utah. This was not the case—only 19% of the isolates were identified. Therefore, it is even more important to develop a database specifically for Georgia.

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