Tracking Sources of Fecal Pollution in a South Carolina Watershed by Ribotyping *Escherichia coli*: A Case Study

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Fecal pollution of water systems can exact high risks to human health and can result in significant economic losses due to closures of beaches and shellfish harvesting areas. The ability to effectively track the sources of pollution within a watershed is imperative for proper risk assessment and remedial efforts to be evaluated and enacted. Several microbiological, genotypic, phenotypic, and chemical methods have been proposed as useful tools for tracking the sources of fecal pollution. Here, we describe the effective use of the ribotyping microbial source tracking procedure to determine the source(s) of *Escherichia coli* within a South Carolina watershed. In this study, 92% of animal-derived *E. coli* isolates collected were correctly classified as being of animal origin using our existing ribotype profile database. These results suggest that the temporal and spatial diversity of *E. coli* may not be a significant factor in broad source (human versus animal) classification of *E. coli* by using our ribotyping DNA fingerprinting procedure. In addition, direct matching of ribotype profiles from water samples to those from known animal sources was used as an index of the potential predominant sources of fecal input(s) into this watershed. This method was useful in assessing possible specific animal sources when statistical analysis of a ribotype profile indicated general animal fecal pollution. Prior to investigating potential fecal inputs into this watershed, a significant human source was suspected as the primary input; however, of the 515 *E. coli* isolated from water samples collected during the course of this study, 88% were typed as being of animal fecal origin. Thus, this study was integral in the realization that animals may be a significant source of contamination and that remediation efforts should be redirected to accommodate these findings. This study summarizes one of the few case studies in which ribotyping was successfully utilized to further resolve a “true-life” water quality and management problem.

Keywords: microbial source tracking, ribotyping, *Escherichia coli*

Introduction

Fecal pollution affects the quality and safety of water systems used for drinking, recreation, and in the harvesting of seafood. It can originate from numerous sources, including sewage treatment plant discharges, failing septic systems, agricultural and urban runoff, improper disposal of wastes from boats, and wildlife. Economic losses due to closures of beaches and shellfish harvesting areas can be severe, with estimates reaching billions of dollars annually (NRDC, 2002). Over 11,000 coastal and Great Lakes beaches in the U.S. were closed or had a health advisory in the year 2000, and this number rose to over 12,000 in 2001 (NRDC, 2002). Furthermore, pollution has been suggested as one cause of the degradation of coral reefs (USEPA, 2001). Currently, 10% of the world’s coral reefs are degraded beyond recovery, with 30% in danger of being degraded or destroyed by the year 2050 (USEPA, 2001).

Economic and aesthetic issues notwithstanding, pollution of drinking and recreational waters can pose serious risks to human health. Human fecal pollution has traditionally been considered a greater health risk because it was more likely to harbor human-specific pathogens; however, the emergence of zoonotic pathogens such as *Cryptosporidium*, *Giardia*, and *E. coli* O157:H7 reassert the dangers associated with animal-derived pollution.

The identification of potential sources of fecal pollution is imperative for the enactment of proper remediation efforts. Currently, microorganisms such as total coliforms, fecal coliforms, *Escherichia coli*, and the fecal enterococci are used to indicate the presence of fecal pollution in water. Monitoring for these organisms is useful as it circumvents the need to assay for specific pathogenic organisms, thus reducing the cost of water quality monitoring. Recent research has suggested, however, that most of these organisms fail to predict the presence of specific human pathogens and, in fact, may underestimate the health risks associated with an impaired body of water (Griffin et al., 2001). The ability to characterize water pollution as being of human origin is particularly useful in that it may
indicate the presence of human-specific pathogens such as 
*Shigella* spp., *Salmonella enterica* serovar Typhi, Hepatitis A, 
or human-specific Noroviruses.

Several microbiological, genotypic, phenotypic, and chem-
ical methods have been proposed and utilized to differentiate 
groups of microorganisms, usually indicator organisms, on the 
basis of the host animal or environment from which they were 
derived. This methodology has collectively been termed “Micro-
bial Source Tracking” (MST), and the current state of this tech-
nology has been recently reviewed (Scott et al., 2002; Simpson 
et al., 2002; Sinton et al., 1998).

Our laboratory currently utilizes several MST methods to as-
ssess overall microbiological water quality and to identify potent-
ial point and nonpoint sources of pollution in a given watershed. 
While many MST techniques have been used in feasibility stud-
ies, very few have been utilized in true case studies (Hagedorn 
et al., 1999; Whitlock et al., 2002; Choi et al., 2003). This ar-
ticle summarizes a recent study conducted by our laboratory in 
which the ribotyping (RT) DNA fingerprinting method was ap-
plied to identify sources of *Escherichia coli* in a South Carolina 
watershed. This microbial source tracking study was part of a 
larger effort designed to identify accurately drainage basins that 
potentially discharge high levels of fecal coliforms into the wa-
terway surrounding the City of Isle of Palms, near Charleston, 
South Carolina, so that specific sources could be pinpointed and 
resolved.

**Materials and Methods**

**Study Area**

The City of Isle of Palms is a barrier island community located 
near Charleston, South Carolina. High concentrations of fecal 
coliform bacteria occur periodically in the waterways surround-
ing the island, and the waters are listed on the Department of 
Health and Environmental Control’s (DHEC) 303(d) list as im-
paired. As a result of poor water quality, the shellfish harvesting 
areas have been closed on numerous occasions. The waterways 
are also heavily used for recreational activities and the impaired 
water quality is an area of significant concern to local citizens 
and community leaders.

**Potential Sources**

There are a number of potential fecal sources contributing to 
the waterway. Three National Pollutant Discharge Elimination 
System (NPDES) outfalls are permitted on the island; how-
ever, effluent monitoring records have indicated that none are 
a significant source of fecal coliforms. Currently, about half of 
residences on the island utilize septic systems for sewage dis-
posal. The remaining residences discharge wastewater into the 
sanitary sewer system. The island is also inhabited by numer-
ous wild animals, including raccoons, squirrels, deer, and many 
bird species. This wild population, combined with numerous 
domesticated animals living on the island, may also potentially 
be a significant source of contamination.

**Collection of Fecal Samples**

Scat samples from birds, cats, deer, dogs, pelicans, raccoons, 
seagulls, and squirrels were collected from specific animals or 
by the identification of feces using known fecal characteristics. 
Samples were placed on ice and shipped to our laboratory in 
Gainesville, Florida, for analysis. Samples were processed and 
assayed for *E. coli* within 24 h of collection. In addition, 3 “un-
known” animal scat samples were collected for analysis. Un-
known fecal samples were those that could not be identified by 
typical fecal characteristics. The breakdown of number of iso-
lates per animal source is depicted in Table 1. Samples were 
diluted in phosphate-buffered saline (PBS) and processed as 
described below for isolation of *E. coli*.

**Isolation of *E. coli* from Fecal Samples**

Fecal samples were streaked onto ChromAgar plates within 
48 h of collection. Plates were incubated at 37°C for 24 h, and 
colonies exhibiting a blue halo were picked and subcultured 
into Luria Broth (LB, Difco) containing 4-methylumbelliferyl-
ß-D-glucuronide (MUG) substrate (Sigma, Inc., St. Louis, MO). 
Tubes that fluoresced under ultraviolet (UV) light were consid-
red to be *E. coli*.

**Collection and Processing of Water Samples**

100 ml water samples were collected, placed on ice, and shipped 
to our laboratory. Samples were analyzed for fecal coliforms by 
most probable number (MPN) in A-1 medium (Difco) according 
to the method outlined in Standard Methods for the Examina-
tion of Water and Wastewater (APHA). A portion of the sample 
was also filtered through membrane filters, which were placed 
ChromAgar plates and incubated and analyzed as described 
above.

**Selection of *E. coli* for Ribotyping**

Between three and five *E. coli* isolates per source (animal or 
water) were selected and analyzed by the ribotyping procedure.

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**Table 1. Sources and number of *E. coli* isolates analyzed in this study**

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of scat samples</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bird</td>
<td>5</td>
<td>18</td>
</tr>
<tr>
<td>Cat</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Deer</td>
<td>5</td>
<td>38</td>
</tr>
<tr>
<td>Dog</td>
<td>5</td>
<td>24</td>
</tr>
<tr>
<td>Pelican</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Raccoon</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td>Seagull</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Squirrel</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>“Unknown”</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>34</td>
<td>160</td>
</tr>
</tbody>
</table>
This number of isolates has previously been shown to be representative of the diversity of isolates within a single fecal or water sample (Parveen et al., 1999; Scott et al., 2003).

Ribotype Profile Database
Over five thousand human and animal-derived *E. coli* were collected previously and were used in the establishment of an original database for isolate classification by ribotype profile and discriminant analysis (Scott et al., 2003; unpublished source isolates). The database contains *E. coli* collected throughout the United States from a variety of human and animal sources. Human source isolates were collected predominantly from septic tanks and human volunteers. Domestic sewage isolates comprise only a small proportion of the human-derived samples as to avoid the inherent cosmopolitan source of fecal inputs into this complex matrix. The approximate breakdown of the database is ∼3800 isolates coming from animals and ∼1500 isolates coming from humans. The discriminatory power of this database was measured by its ability to correctly classify ribotype profiles from animal-derived *E. coli* isolated in this study, which are not included in the existing ribotype database.

DNA Extraction
*E. coli* isolates were grown overnight in Luria-Bertani Broth (LB), and DNA was extracted using a Masterpure DNA purification kit (Epicentre, Madison, WI) according to manufacturer’s instructions.

Determination of DNA Concentration
DNA concentration was determined using a TKO 100 fluorometer according to manufacturer’s instructions.

Restriction Enzyme Digestion
Approximately 1 μg of DNA was digested with *HindIII* restriction enzyme (Roche Molecular Biochemicals, Indianapolis, IN) according to manufacturer’s instructions. Digested DNA was separated on a 1.0% agarose gel at 30 V for 16 h in 1X Tris-Borate-EDTA (TBE) buffer, stained with ethidium bromide and viewed under UV light.

Southern Blot Analysis
After electrophoresis of restriction-digested DNA, agarose gels containing restricted DNA were depurinated in 0.2 M HCl for 10 min, denatured in 0.5 M NaOH/1.5 M NaCl for 35 min, and neutralized in 0.5 M Tris-HCl (pH 7.2)/1.5 M NaCl (0.1 mM) disodium EDTA for 45 min. DNA was blotted from gels onto nylon membranes (Roche Molecular Biochemicals, Indianapolis, IN) using a vacuum blotting system (VacuGene XL) and fixed with shortwave UV light for 5 min.

Probe Preparation
*E. coli* 16S and 23S rRNA (Roche Molecular Biochemicals, Indianapolis, IN) was reverse transcribed into cDNA with avian reverse transcriptase and labeled with digoxigenin (DIG)-dUTP according to the manufacturer’s instructions (Roche Molecular Diagnostics, Mannheim, Germany).

Hybridization and Detection
Membranes were prehybridized at 65°C for 30 min in 20 mM Na2HPO4 and 7% SDS (pH 7.2) and then hybridized in the same solution containing the digoxigenin-labeled probe at 65°C for 16 h. After hybridization, membranes were washed twice for 60 min each time with 20 mM Na2HPO4 and 5% SDS (pH 7.2) at 65°C followed by 2 washes for 30 min with 20 mM Na2HPO4 and 1% SDS (pH 7.2) at 65°C. Membranes were then reacted with alkaline phosphatase-conjugated anti-DIG antibody and visualized by using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate for colorimetric detection according to the manufacturer’s instructions (Roche Molecular Diagnostics, Indianapolis, IN).

Statistical Analysis of RT Profiles as Predictors of Source
RT banding profiles were visualized and DNA patterns were translated into binary code where the presence or absence of bands at a specific length was recorded as a 1 or 0, respectively. Binary codes were examined using statistical discrimination methodology as implemented in SAS software (SAS Institute, Inc., Cary, NC). Discriminant analysis results were summarized, and the performances of the discriminating functions were defined by the average rate of correct classification (ARCC) and the percentage of correctly and misclassified isolates in a classification table created using 10-fold cross-validation. Initial analysis examined the ability of RT profiles to predict the source of animal *E. coli* isolated in this study from birds, cats, deer, dogs, pelicans, raccoons, seagulls, and squirrels as being animal derived when compared to our pre-existing ribotype database. The second analysis compared RT profiles generated from isolates obtained from water samples to the 515 DNA fingerprints generated from the aforementioned isolates. Direct matching of RT banding patterns was considered a presumptive indication that fecal pollution from the representative animal source was present in the water column.

Results
Classification of RT Profiles from Known Animal Sources by Discriminant Analysis (DA)
In order to test the robustness of our existing ribotype fingerprint database, 34 individual scat samples from several animal sources suspected of contributing fecal pollution to the watershed were collected and 160 distinct DNA fingerprints from *E. coli* were generated by the ribotyping procedure. The resulting fingerprints were analyzed by discriminant analysis and classified as either
animal or human derived. The results of this test are of particular interest because the isolates collected in this study are not included in our existing database and therefore represent an external validation of the far-reaching (temporal and spatial) capacity of the ribotyping procedure for 2-way (animal versus human) discrimination (Scott et al., 2003). The results of this analysis are depicted in Table 2. Ribotype profiles that could not be classified or gave poor probability of classification were identified as indeterminate. As can be seen in the Table 2, the average rate of correct classification of animal isolates was 92%.

Analysis of Water Samples and Prediction of Source of E. coli by DA

Five hundred fifteen DNA fingerprints were generated from E. coli isolated from water samples submitted to our laboratory. Of these, 454 (88%) were classified as animal derived, 59 (11%) were classified as human derived, and 2 (<1%) were classified as indeterminate by discriminant analysis. These results are typical of a watershed with multiple fecal inputs; however, these data strongly indicate a predominant animal source of fecal pollution into this watershed.

Direct Matching of RT Profiles of Water Samples and Submitted Scat Samples

In an effort to pinpoint specific fecal inputs, RT profiles generated from E. coli isolated from water samples were compared to those generated from submitted scat samples. The results were classified as no match, unique match, or multiple matches. It has been previously determined that significant overlap exists between RT profiles generated from E. coli isolated from different animals (Scott et al., 2003). However, unique matches identified in a relatively large, diverse study such as this one may indicate the identification of a source-specific ribotype profile and could therefore be used as an index of fecal pollution from that animal source. Of the 454 animal isolates analyzed, 51 RT profiles were directly matched from a specific animal source. Of these, 22 (43%) were classified as coming from deer feces and 9 (18%) directly matched those generated from dog feces. The remaining isolates were spread among the other animal sources, with the exception of squirrel, in which no direct matches were observed. Eighty RT profiles matched patterns from multiple animal sources, thus indicating the degree of overlap among and between animals. The remaining 323 patterns were unique and did not match any known animal sources, thus indicating the diversity of possible RT profiles within a single watershed (Table 3).

Discussion

The results of this study further solidify the utility of the ribotyping procedure as a method to identify microbial indicators of fecal pollution as being either human or animal derived. While this method has received mixed reviews for its ability to further discriminate isolates as coming from a specific animal source, recent research has indicated that a binary classification scheme is still a viable application of this technique (Scott et al., 2003). With the exception of the aforementioned study, previous literature regarding the ribotyping procedure has not tested the robustness of the known-source database by challenging with multiple isolate profiles not contained within the database (Carson et al., 2001, 2003; Parveen et al., 1999). Our ability to correctly classify 92% of the animal isolates collected in this study again suggests that temporal and spatial diversity may not be a significant factor in broad source classification of E. coli. In addition, direct matching of RT profiles from water samples to those from known sources was used as an index of the potential predominant sources of fecal input(s). Because of the high diversity of profiles examined in this watershed, it is important to note that direct matching analysis was only used as a probability index and not as rigorous scientific proof.

Nonetheless, prior to investigating potential inputs into this watershed, a significant human source was suspected as the

<table>
<thead>
<tr>
<th>Source (no. of isolates)</th>
<th>No. (%) classified as animal</th>
<th>No. (%) classified as indeterminate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Birds (18)</td>
<td>17 (94)</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Cat (20)</td>
<td>20 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Deer (38)</td>
<td>38 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Dog (24)</td>
<td>22 (92)</td>
<td>2 (8)</td>
</tr>
<tr>
<td>Pelican (3)</td>
<td>3 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Raccoon (30)</td>
<td>25 (83)</td>
<td>5 (17)</td>
</tr>
<tr>
<td>Seagull (6)</td>
<td>6 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Squirrel (12)</td>
<td>8 (67)</td>
<td>4 (33)</td>
</tr>
<tr>
<td>“Unknown” (9)</td>
<td>8 (89)</td>
<td>1 (11)</td>
</tr>
<tr>
<td>ARCC*</td>
<td>92%</td>
<td></td>
</tr>
</tbody>
</table>

*ARCC, average rate of correct classification.

Table 2. Classification of ribotype profiles from E. coli isolated from wildlife by discriminant analysis

Table 3. Results from direct comparison of RT profiles generated from water isolates to those generated from animal sources

<table>
<thead>
<tr>
<th>(No.) isolates classified by DA as</th>
<th>Bird</th>
<th>Cat</th>
<th>Deer</th>
<th>Dog</th>
<th>Pelican</th>
<th>Raccoon</th>
<th>Seagull</th>
<th>Squirrel</th>
<th>Multiple matches</th>
<th>Unique profiles (no match)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal (454)</td>
<td>2</td>
<td>1</td>
<td>22</td>
<td>9</td>
<td>5</td>
<td>7</td>
<td>5</td>
<td>0</td>
<td>80</td>
<td>323</td>
</tr>
<tr>
<td>Human (59)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>58</td>
</tr>
<tr>
<td>Indeterminate (2)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>
primary input. This study was integral in the realization that animals may be a significant source of contamination and that remediation efforts should perhaps be redirected to accommodate these findings. This study also summarizes one of the few case studies in which ribotyping was utilized to further resolve a true-life water quality and management problem. With the Environmental Protection Agency (EPA)-mandated total maximum daily loads (TMDLs) being calculated for water bodies throughout the United States, it is important that microbial source-tracking techniques be applied and evaluated for their ability to aid in solving problems from a practical standpoint. It is likely that a combination of strategies will be used to definitively pinpoint sources of fecal pollution in future investigations. This study suggests that the ribotyping method is worthy of being at least one of those techniques.

References


