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Identifying Sources of Fecal Pollution in the Roanoke River, Roanoke County, Virginia

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ABSTRACT

Antibiotic Resistance Patterns (ARPs) of *Enterococcus* spp. were used as a phenotypic fingerprint to compare and categorize unknown-source isolates in an impaired segment of the Roanoke River, Roanoke County, Virginia. Antibiotic resistance analysis (ARA) of enterococci has been effectively used to differentiate among sources of fecal contamination in many geographic regions in the United States. *Enterococcus* spp. were used as a fecal indicator in a library consisting of 1,562 known-source isolates. Two-way analysis indicated that approximately 95% of the unknown-source isolates collected were of animal origin. A 3-way analysis indicated that 61% of the unknowns were of livestock origin while 34% were of wildlife origin. Of the isolates determined to be of wildlife origin, almost all were from raccoons and geese while enterococci from deer were present at low percentages. For one sample date, 20% of the isolates at one site were of human origin. This bacterial source tracking (BST) data will prove valuable for the development of TMDLs for this impaired waterway.

INTRODUCTION

To date 3,486 km of the 78,000 km of streams and rivers in Virginia are listed as impaired, with only one third being adequately monitored (FORVA). The Roanoke River, used as a source of drinking water and recreation, originates in the mountains of Montgomery County, runs eastward through the highly populated areas of Roanoke County, Salem City, and Roanoke City, continues into North Carolina and empties into Albemarle Sound, North Carolina. In the Roanoke area, land usage is both agricultural, on which horses, cattle and other agriculturally important animals are present, as well as urban from which human indicator bacteria may originate. Large numbers of resident geese and ducks as well as other wildlife are also present.

Of the 803 stream segments in Virginia that are listed as impaired waters, fecal indicator bacteria are the leading cause of the impairment (DEQ). In the next 10 years, Virginia must develop TMDLs (Total Maximum Daily Load) for 600 impaired segments (DEQ). Public watersheds can be restricted from human recreational use if they exceed the Environmental Protection Agency (EPA) standard of 126 *Escherichia coli* or 33 *Enterococcus* colony forming units (cfu) per 100 mL (geometric mean) in fresh water (EPA). Diseases caused by enteric pathogens potentially transmitted through contaminated water include cholera (*Vibrio cholerae*), gasteroenteritis (*Escherichia coli*), giardiasis (*Giardia*), salmonellosis and typhoid fever (*Salmonella* sp.), shigellosis (dysentery, *Shigella* sp.), and viruses, such as hepatitis A and Norwalk group viruses (Parveen et al., 1999; United States Environmental Protection Agency, 2001).

With current water testing procedures, the presence of fecal indicator organisms indicate the presence of fecal material but not the source of the contamination. Transforming a non-point source into a point source is valuable in order to improve water quality, reduce the nutrient load leaving the watershed, and prevent possible transmission of disease (Hagedorn et al, 1999). Several methodologies have been implemented to determine human and non-human sources of contamination. While many methods exist, several have been used extensively and successfully, or show promise. These methods include antibiotic resistance analysis (ARA; Wiggins, 1996; Hagedorn et al, 1999; Wiggins et al., 1999; Bowman et al., 2000; Harwood et al., 2000; Bower, 2001), ribotyping (Parveen et al, 1999; Hartel et al., 2000; Carson et al., 2001), pulsed-field gel electrophoresis (PFGE; Simmons, 2000), and utilization of specific carbon sources (Hagedorn, et al., in review).

In the work presented here, Enterococci were used as a fecal indicator for our library of known-source isolates. While fecal coliforms are the standard indicator in Virginia, ARA using the enterococci has been highly successful (Hagedorn et al., 1999; Wiggins et al., 1999; Bower, 2000; Bowman et al., 2000; Harwood et al., 2000). Enterococci are an appropriate indicator in brackish and salt water primarily because they are more apt to survive in marine environments than fecal coliforms because they can tolerate high (6.5%) salt concentrations (Hagedorn et al, 1999). Enterococci also have a higher survival rate through wastewater treatment processes than fecal coliforms making them an attractive target in fresh water (Harwood et al., 2000). This provides the basis for using antibiotic resistance patterns (ARPs) as a "phenotypic fingerprint" to compare and categorize unknown *Enterococcus* spp. isolates. Although antibiotics are primarily used in humans and livestock, we find antibiotic resistance is widespread and common, even in wildlife such as Canada goose, white-tailed deer, muskrat, and raccoon. We report here that ARA of fecal enterococci from known fecal sources, used in conjunction with discriminate analysis (DA), effectively predicted the sources of isolates taken from four Roanoke river sample sites over three sample dates. We have reinforced the bacterial source tracking method and we report both the extent of fecal contamination in the Roanoke River on these dates and the source of fecal contamination.

MATERIALS AND METHODS

Bacterial Library

Fresh fecal material, ranging from a swab to several grams, from known sources (horse, human, raccoon, sheep, chicken, cow, white-tailed deer, Canada goose, and muskrat), was diluted in sterile distilled water. Samples from humans are presumed to be a mixture of isolates from several individuals from a portable toilet or pump out truck. Multiple manure samples were collected and mixed, while goose isolates were from single individuals. Several horses were swabbed to obtain fecal material. One hundred L fecal suspension was pipetted onto each mEnterococcus agar (Difco) plate and spread with a sterile glass hockey stick. Plates were inverted and incubated at 37C for 48h. Burgundy and pink colonies were picked off with sterile toothpicks and placed into 200μL enterococcosel broth (BBL) in sterile 96-well plates. Black wells (positive for *Enterococcus* spp.) were noted. A 48 prong replicaplater was used to transfer isolates onto Trypticase Soy Agar with lecithin and polysorbate 80 (BBL) Antibiotic Plates (Table 1). Antibiotic plates were inverted and incubated for 48h at 37C, and

TABLE 1. Antibiotics and Final Plate Concentration Used for Antibiotic Resistance Analysis.

Antibiotic	Plate Concentration (g/mL)
Chlortetracycline	60,80,100
Oxytetracycline	20,40,60,80,100
Streptomycin	40,60,80,100
Cephalothin	10,15,30,50
Erythromycin	10,15,30,50
Tetracycline	10,15,30,50,100
Neomycin	40,60,80
Vancomycin	2.5
Amoxicillin	2.5

were then evaluated for growth (1) or no-growth (0). Results were entered into the SAS Institute statistical program JMP IN® version 4.2. Using discriminant analysis, the library was evaluated for correct classification prior to evaluation of unknown source isolates (Table 2).

Bacterial Unknown Source Isolates

Water samples were obtained using standard methods (Greenberg, 1992) from four Roanoke River sample sites spanning approximately 34 kilometers in Roanoke County, Virginia (Roanoke Wayside, 37°14.90N 80°10.48W; Green Hill Park, 37°16.55N 80°06.84W; Route 11, 37°16.10N 80°02.29W; 14th Street, 37°15.88N 79°45.94W, obtained via hand-held Magellan GPS 4000). Roanoke Wayside is a small public park adjacent to VA Route 460 upstream from Salem, VA, with a main channel depth at the time of the study of approximately 53cm. Green Hill Park is a large public park just outside Salem City with a main channel site depth of approximately 85cm. The Route 11 site is a shallow site within Salem City with a main channel depth of 26cm. The 14th Street site is just upstream from the Roanoke City water treatment facility with a main channel depth of approximately 42cm. Water depths are approximate based on several sample dates from the main channel and could vary seasonally.

Water was filtered on the same day through sterile 0.45μm filters (Gelman), placed onto mEnterococcus agar in 9 X 50mm Petri dishes (Gelman), and incubated for 48h at 37C. The procedure was then identical as for the creation of the library. Once antibiotic growth versus no-growth data was obtained, the isolates were run as unknowns in the library against the known antibiotic resistance patterns (Table 3).

RESULTS AND DISCUSSION

Antibiotic Resistance Patterns (ARPs) were created for 1,562 enterococci for the development of the library (Table 2). Most published *Enterococcus* libraries range in size from 830 to over 4000 isolates (Harwood et al, 2000; Wiggins et al, 1999; Bower 2000; Graves, 2000; Bowman et al., 2000). The average rate of correct classification (ARCC) of the 2-way analysis of the library was 88% while the ARCC for the 3-way analysis for the library was 81% (Table 2). Three-way wildlife, 4-way livestock and 8-way analyses were also performed (Table 2).

TABLE 2. Rates of Correct Classification (RCC) of Known-Source Library Isolates by Discriminant Analysis

Source	Number of Isolates[A]	Number Correctly Classified	Rate of Correct Classification (%)
2-way			
Animal	1,215	1,154	95
Human	347	277	80
Total	1,562	1,431	88
3-way			
Human	347	264	76
Livestock	804	698	87
Wildlife	411	333	81
Total	1,562	1,295	81
4-way (livestock)			
Chicken	44	21	48
Cow	440	365	83
Horse	235	155	66
Sheep	85	76	89
Total	804	617	72
3-way (wildlife)			
Deer	141	121	86
Goose	174	166	96
Raccoon	94	89	95
Total	409	376	92
8-way			
Horse	235	195	83
Human	347	234	68
Raccoon	94	77	82
Sheep	85	74	87
Chicken	44	36	82
Cow	440	123	29
Deer	141	112	79
Goose	174	127	73
Total	1,560	978	73

[A]Animal, number of samples collected, and average number of isolates per sample: Horse, 6, 39; Human, 7, 50; Raccoon, 4, 24; Sheep, 2, 43; Chicken, 1, 44; Cow, 6, 62; Deer, 6, 24; Goose, 4, 44; Muskrat, 1, 2. Muskrat isolates excluded from 3-way wildlife library and 8-way library analyses.

An average of 41 unknown-source *Enterococcus* strains were examined per Roanoke River site per sample date. Two-way analysis indicated that approximately 95% of the unknowns collected were of animal origin (Table 3). Our 3-way analysis indicated that approximately 61% of the unknowns were of livestock origin while approximately 34% were of wildlife origin.

TABLE 3. Two-way, Three-way, and Eight-way Classifications of Unknown-Source *Enterococcus* Isolates by Discriminant Analysis

Sample Site/ Date	CFU/ 100mL	Number of Isolates	2-Way Classification%		3-Way Classification %			8-Way[A] Classification%							
			Animal	Human	Human	Livestock	Wildlife	Horse	Human	Raccoon	Sheep	Chicken	Cow	Deer	Goose
SEPTEMBER															
Roanoke Wayside	364	44	98	2	2	43	55	43	2	43	0	0	0	0	11
Green Hill Park	149	48	98	2	2	63	35	60	0	35	2	0	0	0	2
Route 11	256	47	100	0	0	60	40	58	0	34	0	0	1	0	6
OCTOBER															
Green Hill Park	47	47	100	0	0	47	53	43	0	53	0	0	0	0	4
14th Street	65	38	100	0	0	79	21	76	0	8	0	0	3	5	8
JANUARY															
Roanoke Wayside	34	37	92	8[B]	8[B]	60	32	57	3	11	16	0	0	5	8
Green Hill Park	26	44	80	20[B]	20[B]	66	14	70	18	5	0	0	0	2	5
Route 11	24	35	94	6[B]	6[B]	69	26	69	3	8	3	0	3	3	9
14th Street	27	27	96	4	4	63	33	56	4	19	4	0	4	4	11

[A]An 8-way classification is given as an indication of specific animal source only and should not to be interpreted as accurate as the 2-way and 3-way classifications. Muskrat isolates excluded from 8-way classification.

[B]Percent isolates classified as of human origin that fell above the misclassification rate of animal (2-way), or livestock and wildlife (3-way)

TABLE 4. Analysis of Wildlife Unknowns by 3-Way Wildlife Library

Sample Site/ Date	Number Wildlife Isolates[A]	% Total Sample	Classification %		
			Deer	Goose	Raccoon
SEPTEMBER					
Roanoke Wayside	24	55	8	21	71
Green Hill Park	17	35	0	6	94
Route 11	19	40	5	26	68
OCTOBER					
Green Hill Park	25	53	0	8	92
14th Street	8	21	25	25	50
JANUARY					
Roanoke Wayside	12	32	17	33	50
Green Hill Park	8	20	13	38	50
Route 11	8	23	0	25	75
14th Street	9	33	11	33	56

[A]Based on a 3-way analysis of all unknowns. Muskrat isolates excluded from analysis.

We obtained unknowns of human origin on several sample dates, but only in January did the % classification rise above the error rate for the database. Error is assessed as the average percentage of isolates misclassified as human. For example, in the 3-way analysis in which the sets of data were categorized as human, livestock, and wildlife (Table 2), 30 livestock isolates were misclassified as human (3.7%) and 22 of the wildlife isolates were misclassified as human (5.4%) yielding an average incorrect rate of classification (IRCC) of 4.5%. Therefore, any unknown set yielding a number of isolates from human sources above 4.5% would be considered significant. In the January sample at Green Hill Park, 20% of the isolates were of human origin, and 6 to 8% human isolates were obtained at Route 11 and Roanoke Wayside respectively (Table 3).

The work presented here shows the usefulness of ARA in the source tracking of fecal contamination in the Roanoke River. While the results of this study are encouraging, and is essential in the development of TMDLs for this impaired waterway, it again emphasizes the need for multi-year studies to determine the seasonality of sources over time. Virginia is required to develop TMDLs for 600 impaired waters over the next 10 years. Based on current ARA methodology, a 2-way (animal and human) and 3-way analysis (human, livestock, wildlife), or small sub-libraries such as "wildlife" appear to be the most accurate analyses for any known-source database (Hagedorn, personal communication).

Our library had good average rate of correct classification (ARCC), ranging from 72-92% which is good compared to published percentages (54-91%, Wiggins et al., 1999; 73% Human and 89% cattle, Bower, 2000; 87-94%, Graves, 2000; 34-88%, Harwood et al., 2000). The 3-way wildlife library (92% ARCC) and 8-way classifications (73% ARCC) prove to be of interest because of their specificity. One must use caution, however. Unknown isolates indicated as non-human may most effectively be

analyzed in a specific non-human sub-library such as our 3-way wildlife library (deer, goose, raccoon, Table 2) with an ARCC of 92%. In the analysis of unknowns determined to be from wildlife, based on the 3-way wildlife library, most isolates were determined to be from raccoon or goose (Table 4). We feel this approach is better suited to determination of the origin of specific isolate than does an 8-way library (ARCC of 73%) which includes all animals including humans. The creation of sub-libraries (exclusion of human-source isolates for example) controls the number of possible sources and therefore potentially reduces misclassification. This is indicated by the ARCC for each: 92% and 73%, 3-way wildlife and 8-way respectively. Even the 8-way database however indicated that most of the wildlife isolates were from raccoon and goose, and had a respectable ARCC. Over the three sample dates, the source of fecal contamination was generally from the same sources.

An average of 61% of the unknown-source isolates were classified as livestock, with an average of 59% of these over the three sample dates classified as from horse based on the 8-way analysis (Table 3). We know based on general observation that both horses and cattle are present along the river. While the rate of correct classification (RCC) for horse in the 8-way library was 83%, it was only 29% for cow. Cow was misclassified as horse for 32% of the known-cow isolates. We feel therefore that many of the unknowns classified as horse may actually have been from cattle. In the 8-way analysis, only 2% of horse isolates were misclassified as cow, with horse isolates most often misclassified as goose (8%). We feel that with most of the cattle isolates originating in Montgomery County, the regional difference may have contributed to this error rate. While many of the samples originated in both Montgomery (upstream from Salem) and Roanoke Counties as well as Salem City, these specific populations of bacteria may have differed significantly from those actually entering the river. If cattle are suspected contributors of fecal pollution, further specific isolation and comparison would be needed. We feel future studies should also include known-source isolates from urban animals such as cats and dogs, as well as resident ducks. Pigeons may also be an important contributor of fecal material in urban settings such as Salem, as the birds are known to frequent bridges as well as power lines suspended over water.

Using ARPs with discriminant analysis may prove to be a more efficient methodology for bacterial source tracking when compared to molecular methods. Although molecular methods may provide more precise identification of specific types of sources, they have high per-isolate costs, very complex and time-consuming procedures, and are not as feasible in assaying large numbers of samples in a reasonable time frame. We feel that while ARA may not be an appropriate method to discriminate to the level of specific animal using a single multi-animal library, current levels of classification are quite useful. We speculate however that adding a molecular component to ARA may aid in the precision of the analysis. With ARA one may quickly assess general sources of contamination while a molecular methodology may indicate more precisely the specific source if needed. We feel studies are needed comparing multiple methodologies, such as ARA, Ribotyping, PFGE, and Biolog, simultaneously to determine the limitations of each technique.

To date, molecular methods offer similar ARCCs as ARA (Matched 71% of isolates, Samadpour and Chechowitz, 1995; 83% 2-way discriminant analysis, Perveen et al., 1999; 78-100% band matching, Dombeck et al., 2000; 51% band matching, Simmons et al., 2000; 48-96%, Carson et al., 2001). We also recommend samples be

taken from our sample sites during summer months with more frequent sampling, possibly at high and low water events. We reason that greater contamination by human isolates will be evident during higher water events (personal communication Bill Tanger, FORVA) which may have been a contributing factor for the higher human-source *Enterococcus* numbers in January.

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