

Escherichia coli and Coliphage Analyses to Differentiate Pollution Sources in Surface Waters of South Carolina

J.R. Stewart¹, B.C. Thompson¹, L.F. Webster¹, D.E. Chestnut², D.A. Graves², G.I. Scott¹

¹National Ocean Service, Center for Coastal Environmental Health and Biomolecular Research. Charleston, SC 29412

²South Carolina Department of Health and Environmental Control, Bureau of Water. Columbia, SC 29201

ABSTRACT

Human fecal contamination of environmental waters poses a serious threat to public health. Despite existing sanitation efforts, contaminated waters continue to cause illness through drinking water supplies, recreational water uses, and shellfish harvesting. This project attempts to distinguish indicator microorganisms which originate in the gastrointestinal tracks of humans from those with wild animal or domestic animal origins. Selected sites with high coliform counts in the Saluda-Edisto watershed of South Carolina were sampled from which fecal coliforms, male-specific coliphages and somatic coliphages were quantified. Reference wastewater effluent and chicken farm litter were also tested. Three methods were compared for their ability to distinguish pollution sources: multiple antibiotic resistance (MAR) assays of *E. coli* isolates, ribotyping of *E. coli* isolates, and genetic typing of F⁺RNA coliphage isolates. Seventeen percent (n=42) of surface water and 22% (n=102) of all tested *E. coli* isolates displayed multiple antibiotic resistance. Nine DNA banding patterns were repeated through 60% (n=102) of ribotyped *E. coli* isolates. Of the 49 surface water F⁺RNA coliphage isolates tested, 47 typed as group I, 1 typed as group II and 1 typed as group III. Human-source contamination was identified in three of five surface water sites, suggesting a risk of enteric pathogen contamination. Human-source contamination appears unlikely for the other two surface water sites examined. Additional research is required to determine which of the three tested methods is most effective for contamination source identification.

INTRODUCTION

Multiple pollution sources are inherent in urbanized watersheds, making it difficult for resource managers to identify and correct sources of contamination. Identification of human fecal pollution in environmental waters could implicate pollution sources (i.e. sewage outfalls or septic tanks) and allow policy changes intended to prevent the spread of pathogenic microorganisms. Microbiological techniques for pollution source tracking are being developed to address these issues. MAR testing and ribotyping of *E. coli*, and F⁺RNA coliphage typing are among the most promising methods proposed. Parveen and colleagues (1997 and 1999) have successfully distinguished point and nonpoint pollution in Florida using MAR and ribotyping methods. Furuse (1983) and Griffin (2000) have successfully identified human and animal source pollution using F⁺RNA coliphage typing. The rationale behind MAR is that bacteria from human sources are more resistant to antibiotics than bacteria from wild animal sources because humans more commonly undergo antibiotic therapy. Bacteria originating from domestic animals have different MAR patterns than those from humans because humans are treated with different antibiotics than livestock. Ribotyping involves ribosomal RNA analysis of *E. coli* to differentiate between clonal groups of the bacteria. F⁺RNA coliphage isolates identify sources of contamination by typing isolates into one of four subgroups using genetic hybridization or serological methods. Groups I and IV are generally indicative of animal feces whereas groups II and III are more sewage- specific, although geographical variations in coliphage distributions have been observed. This project employed source tracking of microorganisms from state water quality stations in South Carolina with high coliform counts. *E. coli* and coliphages were analyzed from five surface water stations in the Saluda-Edisto watershed. Reference samples (two sewage treatment plants and three chicken farms) were analyzed from the same watershed. The sites were then compared to distinguish between point source human origin, point source nonhuman origin, and nonpoint source pollution.

OBJECTIVES

- Identify *Escherichia coli* isolates from study site coliforms using Analytical Profile Indexing (API)
- Analyze confirmed *E. coli* isolates (up to 10/site for surface water and 15/site for reference samples) by multiple antibiotic resistance (MAR) testing and ribotyping
- Detect and enumerate coliphages using Single Agar Layer (SAL) and Enrichment Presence/Absence methods
- Type all confirmed F⁺RNA coliphage isolates into one of four prototypical subgroups
- Compare MAR, ribotyping and F⁺RNA coliphage typing for their ability to identify fecal pollution sources

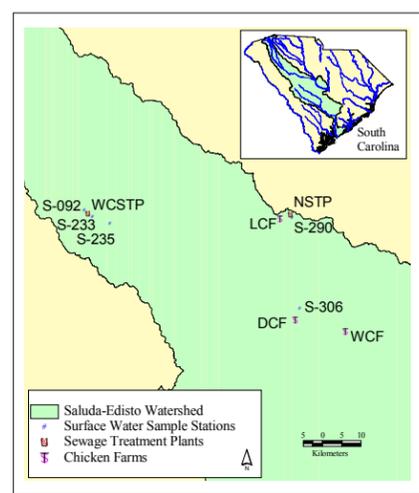
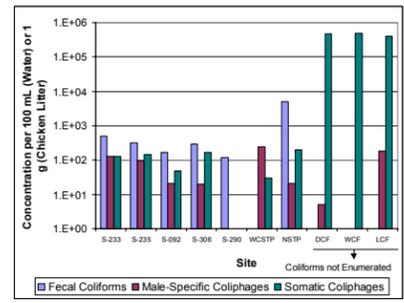


Figure 1. Surface water and potential pollution source sites sampled in the Saluda-Edisto watershed.

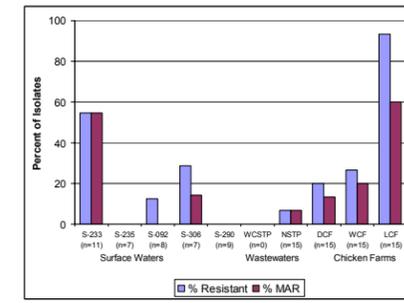
RESULTS

Figure 3. Concentration of indicator microorganisms



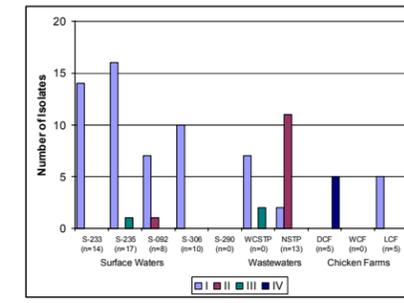
- Coliforms outnumbered coliphages in all but the Wilson Creek Effluent sample, where coliforms were not detected.
- No correlation was found between the concentration of fecal coliforms, male-specific coliphages and somatic coliphages (correlation coefficients between -0.1 and -0.3).

Figure 4. Percent of *E. coli* isolates exhibiting antibiotic resistance



- 19% of surface water *E. coli* isolates were antibiotic resistant and 17% exhibited multiple antibiotic resistance.
- 37% of the isolates from known sources were antibiotic resistant and 25% exhibited multiple antibiotic resistance.

Figure 5. F⁺RNA coliphage subgroups



- Surface water samples contained predominantly group I F⁺RNA coliphage. A group II isolate was identified from S-092 and a group III was identified from S-235.
- One wastewater sample contained predominantly group II isolates and a lower level of group I. The other contained predominantly group I with a lower level of group III.
- Groups I and IV were identified from chicken litter.

SITE INDEX	
S-233, S-235, S-092, S-306, S-290	= Surface Water Sample Stations
WCSTP	= Wilson Creek Sewage Treatment Plant Effluent
NSTP	= Newberry Sewage Treatment Plant Effluent
DCF	= Donavic Chicken Farm, Chicken Coop Litter
WCF	= Whittle Chicken Farm, Chicken Coop Litter
LCF	= Livingston Chicken Farm, Chicken Coop Litter

METHODS

Analytical Profile Index (API). API is a bacteria identification system comprised of a series of 21 biochemical tests arranged on a strip. The strips were inoculated and incubated according to package instructions with the API 20E test kit, and results were decoded using the API database (bio Merieux Vitek; Hazelwood, MO).

Multiple Antibiotic Resistance (MAR). Confirmed *E. coli* isolates were tested for antibiotic resistance using a method adapted from Parveen et al (1997). Isolates were grown in duplicate on Mueller-Hinton plates with each antibiotic and on control plates without an antibiotic. Digital images of the plates were stored electronically, and colony sizes were compared using Sigma Scan Pro software (SPSS Inc., Chicago, IL). Isolates were scored as either resistant or sensitive to each antibiotic based on whether there was a < 15% reduction or ≥15% reduction in colony diameter, respectively.

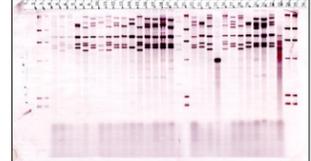
$$\text{MAR index for an isolate (\%)} = \frac{\text{number of antibiotics to which the isolate was resistant}}{\text{number of antibiotics tested}} * 100$$

$$\text{MAR index for a site (\%)} = \frac{\text{number of antibiotics to which all isolates from a site were resistant}}{(\text{number of antibiotics tested})(\text{number of isolates for the site})} * 100$$

Ribotyping. *E. coli* DNA from study samples was isolated, quantified fluorometrically, diluted to a common concentration, digested with *Hind*III, and separated electrophoretically along with a λ bacteriophage molecular weight marker. The DNA was dephosphorylated, denatured and neutralized, then transferred to a nylon membrane by the Southern Blot Method. The DNA was crosslinked to the membrane by uv radiation exposure. The membranes were then hybridized with a cDNA probe which had been digoxigenin-labeled while being reverse transcribed from *E. coli* 16S and 23S rRNA. Finally, the membranes were incubated with an anti-digoxigenin-AP conjugate, washed with the Genius Buffer System, and exposed to NBT/BCIP color substrate solution (Boehringer-Mannheim Corp.) to visualize the bands. Band sizes were measured and banding patterns were assigned a letter code. The codes were then compared for each sample and reference site.

Coliphage Typing. Male-specific (*E. coli* FAMP host) and somatic (*E. coli* CN13 host) coliphages were detected by the single agar layer and enrichment presence/absence methods. RNase testing was used to distinguish F⁺RNA and F⁻DNA isolates. Confirmed F⁺RNA isolates were then typed into one of four subgroups by hybridization with digoxigenin-labeled nonradioactive oligonucleotides (Hsu, 1995). Identification of type II or III isolates were considered indicative of human-source fecal pollution.

Figure 2. Ribotype banding patterns of *E. coli* from study sites



Resistance Patterns	Surface Waters					Wastewaters		Chicken Farms		
	S-233 (n=11)	S-235 (n=7)	S-092 (n=8)	S-306 (n=7)	S-290 (n=9)	WCSTP (n=0)	NSTP (n=15)	DCF (n=15)	WCF (n=15)	LCF (n=15)
A, C, K, Ne, O, P, T				1						
A, C, O, P, T	3									
A, C, O, P, Su, T	1						1			
A, P, Su										
C, O, St, T								1		1
C, O, T	2								2	
O, St, T								1		
O, T									1	
P			1							
St				1						5
St, Su										8
Su								1		
T										1
Sensitive to All (%)	5 (45%)	7 (100%)	7 (88%)	5 (71%)	9 (100%)	NA	14 (93%)	12 (80%)	11 (73%)	1 (7%)
MAR Index	25	0	1	11	0	NA	4	5	6	16

A = ampicillin, C = chlortetracycline, K = kanamycin, Na = nalidixic acid, Ne = neomycin, O = oxytetracycline, P = penicillin G, S = streptomycin, Su = sulfathiazole, and T = tetracycline

- Seven surface water isolates had resistance patterns which included chlortetracycline, oxytetracycline and tetracycline
- The majority of isolates from LCF were resistant to streptomycin and sulfathiazole

Site	Site MAR	RT Banding Patterns (# of Isolates)	Coliphage Types (# of Isolates)	Pollution Source Conclusion (No. of Methods Supporting)
S-233	25	A (2), E (1), G (2), unique (7)	I (14)	Possibly Human (3/3)
S-235	0	A (1), F (2), unique (5)	I (16), III (1)	Possibly Human (1/3)
S-092	1	A (1), F (3), unique (4)	I (7), II (1)	Possibly Human (1/3)
S-306	11	unique (7)	I (10)	Indeterminant, but Nonpoint
S-290	0	A (3), H (2), unique (4)	NA	Unlikely Human (2/3)
WCSTP	NA	NA	I (4), III (1)	Human
NSTP	4	B (4), D (4), E (2), unique (5)	I (2), II (11)	Human
DCF	5	A (5), B (5), C (2), E (1), unique (1)	IV (5)	Nonhuman
WCF	6	A (2), I (6), unique (6)	NA	Nonhuman
LCF	16	A (13), unique (2)	I (5)	Nonhuman

- Human-source fecal contamination may be present at three tested surface water sites

SUMMARY AND CONCLUSIONS

- Coliphages survived better than coliforms through sewage treatment processes at the Wilson Creek Plant. These alternative indicators appear more appropriate for detection of enteric viral pollution than coliforms.
- Resistance patterns for *E. coli* isolates from site S-233 and wastewater included ampicillin and penicillin resistance, and the isolates from these samples had RT banding pattern E in common. These results suggest an impact of human-source fecal pollution.
- The presence of groups II and III F⁺RNA coliphages in samples from sites S-092 and S-235 (respectively) suggests an impact by human-source fecal pollution in the vicinity. These waters may pose a public health risk.
- Each *E. coli* isolate from S-306 displayed a unique banding pattern. One of the isolates displayed the highest MAR index (70) while five of the isolates were sensitive to all tested antibiotics (MAR isolate index = 0). Therefore, nonpoint source pollution is likely the cause of high coliform levels at site S-306.
- No F⁺RNA coliphages could be detected from site S-290, and *E. coli* isolated from the site showed no resistance to tested antibiotics. Therefore, site S-290 may be impacted by nonhuman fecal contamination.
- All three tested methods show promise for their ability to identify pollution sources, but more research is necessary to conclude which is most reliable.

REFERENCES

Furuse K, T Sakurai, Y Inokuchi, H Inoko, A Ando and I Watanabe (1983). Distribution of RNA coliphages in Senegal, Ghana and Madagascar. *Microbiol. Immunol.* 27:347-358.

Griffin DW, R Stokes, JB Rose, JH Paul 3rd (2000). Bacterial Indicator Occurrence and the Use of an F⁽⁺⁾ Specific RNA Coliphage Assay to Identify Fecal Sources in Homosassa Springs, Florida. *Microb Ecol.* 39:5664.

Hsu FC, Y-S C Shieh, J van Duin, MJ Beekwilder and MD Sobsey (1995). Genotyping male-specific RNA coliphages by hybridization with oligonucleotide probes. *Appl. Environ. Micro.* 61: 3960-3966.

Parveen S, L Murphee, I Edmiston, CW Kaspar, KM Portier and M Tamplin (1997). Association of multiple-antibiotic-resistance profiles with point and non-point sources of *Escherichia coli* in Apalachicola Bay. *Appl. Environ. Microbiol.* 63:2607-2612.

Parveen S, KM Portier, K Robinson, I Edmiston and ML Tamplin (1999). Discriminant analysis of ribotype profiles of *Escherichia coli* for differentiating human and nonhuman sources of fecal pollution. *Appl. Environ. Microbiol.* 65:3142-3147.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Mark Sobsey from the University of North Carolina-Chapel Hill for providing coliphage and host stocks for this research. Special thanks to Dr. Jan Gooch (NOAA) for her assistance in the laboratory, and to James Daugomah (NOAA) and Jeanie Eidson (DHEC) for their GIS assistance. Bill McDermot (DHEC) is gratefully acknowledged for sample collections, as is Dr. Salina Parveen (University of Florida) for sharing her wisdom about the MAR and ribotyping techniques.

This project was funded in part by the US EPA under a section 319 grant through the SC department of Health and Environmental Control.