AMPICILLIN RESISTANCE IN FECAL COLIFORMS OF CANOOCHEE RIVER

Michael L. Gleason[1], Kenneth C. McGill[2] and Lee P. Owen[3]

AUTHORS: [1]Assistant Professor of Biological & Environmental Sciences, [2]Associate Professor of Chemistry, and [3]Biology Undergraduate, Georgia College & State University, CBX81, Milledgeville, GA 31061-0490.
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Abstract. In Spring 2000, the Canoochee River was sampled at eight sites from N32°22.34', W82°7.42' to N32°10.74, W81°49.66' and the samples quantitated by EPA methods for fecal coliform and fecal Streptococci. Fecal coliform levels ranged from 140-540 fecal coliforms/100 ml and coliform/streptococcal ratios ranged from 3.2-10. Fecal coliforms were also tested for resistance to the antibiotic ampicillin (frequency of resistance = 0.11±0.036). Plasmid DNA isolated from selected fecal coliforms conferred resistance to *Escherichia coli* K12-JM101 upon transformation.

INTRODUCTION AND BACKGROUND

The release of untreated sewage because of leaking septic tanks and agricultural activity is a major source of fecal contamination in our Georgian waterways. The result is that our rivers, streams and lakes have become conveyors of transmissible disease (Craun, 1972) and are unsafe for drinking, and in some cases, for recreational use. Public health concerns over water quality and water-usage drive the federal, state, and private-sponsored testing for fecal contamination in our waterways.

Contamination by human and animal fecal waste is commonly monitored by microbiological methods. Among the most commonly employed methods are those that quantify *Escherichia coli*, a fecal coliform (FC), commonly found in intestines of warm-blooded mammals and released in feces (Clesceri et. al, 1998). Fecal streptococcal (FS) are commonly measured in conjunction with *E. coli* to indicate the human or animal origin of the contamination (Feachem, 1974).

The distribution of antibiotic resistant bacteria in surface waters has been the subject of numerous studies and the focus of some concern. Smith's study (1970) represents an early report on the occurrence of river water *E. coli* containing R factors, plasmids encoding antibiotic resistance genes. Other studies have shown a good correlation between metal and antibiotic resistance in river FC (Morozzi et. al, 1986; Lee and Chen, 1991). Another report demonstrates that while native aeroheterotrophs exhibit a uniformly high level of resistance to antibiotics, strains of *E. coli* isolated from upstream rural, low contamination sites had clearly lower resistance than isolates from downstream high-level contamination sites (Boon and Cattanach, 1999). Based on studies like these it has been suggested that antibiotic resistance be considered when establishing bacteriological water quality criteria (Bell et. al, 1983; El-Zanfaly, 1991).

In this paper we report the initial monitoring of fecal contamination at sites along the Canoochee river. Additionally, we have determined the frequency of resistance of isolated *E. coli* strains to ampicillin.

EXPERIMENTAL DESIGN

Our hypothesis was that sewage released from a Claxton chicken processing plant and at its downstream site would have higher levels of FC in river water samples than from upstream sites. Furthermore, because of the common practice of using low-dose antibiotics in animal feed, FC isolates sampled adjacent to the plant and at downstream sites would have higher ampicillin resistance than those upstream. Water sampled upstream and downstream of the plant was tested for FC and FS and ampicillin resistance in FC. Contrary results would cause us to reject or be unable to test our hypotheses.

METHODS

Materials

Growth media and chemicals described were purchased from Sigma Chemical Company, St. Louis,

MO. The Microfil System for filtering and incubation of water samples was purchased from Millipore Corporation, MA. Plates and milk dilution bottles were purchased from Fisher Scientific. Water used to prepare solutions was deionized by a Millipore Ultrapurification System. The laboratory strain of *E. coli* K12 JM101 and plasmid pAMP were obtained from Carolina Biological Supply Company, N.C.

Procedures

Locations of water samples collected from eight bridges (sites) crossing the Canoochee River on March 18, 2000 (from 11:00 am to 2:00 pm) are described in Table I and Figure 1. Site 7 was at the chicken processing plant and Site 8 was about 3 miles downstream. A weighted holder attached to strings contained an autoclaved milk dilution bottle that when submerged in the main channel of the river was opened underwater, filled and then raised. Replicate bottles were collected at each site. Samples were capped after sufficient water was poured off to provide at least 10 ml of air space and stored at ambient river temperature in a cooler. Within 8 hours of collection 10- and 100-ml water samples were filtered onto duplicate 47 mm, pre-sterilized, 0.45 μm pore size, gridded membranes.

Filters were aseptically placed into petri dishes (Millipore) containing either mFC broth suspended in a sterile glass filter or KF agar. Selective bacteriological growth of FC and FS on the filters was obtained with mFC incubated at 44.5°C and KF at 35°C, respectively (Clesceri et. al, 1998). The number of blue colonies on mFC media (FC) and red colonies on KF media (FS) were counted after incubation at 24 and 48 hours, respectively.

Further bacteriological and molecular methods are as described by Sambrook et. al (2000). From sites

Table 1. Identity of Collection Sites

Site Number	Sampling Sites
1	I-16 Crossing
2	23/121 Crossing
3	129 Crossing
4	199 Crossing
5	169 Crossing
6	Hendrix Rd. Crossing
7	US Highway 25 Crossing
8	Daisy-Nevils Rd. Crossing

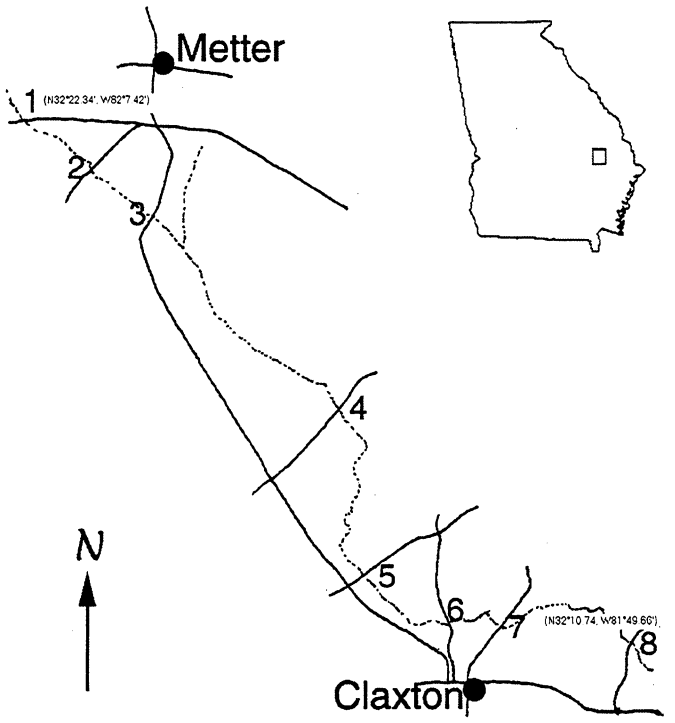

Figure 1. Map of collection area.
Dotted (light) line indicates Canoochee River and solid (heavy) lines roads. Crossings (collection sites) described in Table I are indicated as numbers (1-8) in figure. Inset within Georgia map in upper-right corner indicates location of study area detailed in this figure.

6, 7 and 8, thirty-two isolates of FC on mFC plates were individually picked and streaked for isolation on LB plates and incubated for 24 hours at 37°C. Colony morphology of FC on LB plates and microscopic analysis at 1000X with Grams staining, when compared with *E. coli* controls, was consistent with the isolation of *E. coli* from the Canoochee River. FC isolates were then transferred to LB plates containing 100 μg/ml of ampicillin and grown for another 24 hours at 37°C. Resistance to ampicillin was counted if a well-formed streak of growth was present on the LB with ampicillin plate. Ampicillin-sensitive *E. coli* K12 JM101 and ampicillin-resistant *E. coli* K12 JM101 [pAMP] were used as controls. Statistical analysis of mean by standard deviation and comparisons by student's t-test were employed.

Six ampicillin resistant isolates (sites 6 and 7) were restreaked for subcloning on LB with 100 μg/ml ampicillin plates. Plasmid midiprep DNA was prepared from isolates by inoculating 50 ml cultures of LB broth containing 100 μg/ml ampicillin and growing to mid-log with shaking at 37°C. At mid-log, cultures either

had 100 μg/ml of chloramphenicol added or not, and were then grown for another 4 to 6 hours. Midiprep DNA was either purified by detergent-alkaline midiprep followed by diatomaceous affinity purification or Rnase/phenol-chloroform extraction. Plasmid DNA was resuspended in 10 mM Tris, 1 mM EDTA, pH 8.0. Plasmid DNA was analyzed by standard 0.8% agarose minigel electrophoresis with ethidium bromide staining and UV-transillumination. Transformation of non-resistant *E. coli* strain K12-JM101 with plasmid DNA isolated from FC isolates used the standard $CaCl_2$-based transformation method.

Table 2. Fecal Coliform and Fecal Streptococci Counts by Site

Site	FC/100 ml	FS/100 ml
1	160	43
2	140	41
3	210	33
4	320	32
5	330	47
6	540	170
7	230	51
8	210	4

CONCLUSIONS

Sampling along the eight Canoochee river sites began at site 1, a rural area just downstream of Metter, GA, and well upstream of Claxton, GA, and concluded at site 8 three miles downstream of a chicken processing plant (Table I and Figure 1). The processing plant is just downstream of the city limits of Claxton and adjacent to site 7. As expected river water sampled at sites (1-2) had the lowest FC counts observed. Surprisingly though, the levels of FC were highest (site 6) just upstream of the chicken processing plant and within the city limits of Claxton. Ratios of FC/FS ranged from 3.2 to 10. Unfortunately, since a sample collection over time could not be performed, the significance of this finding is not certain as the survival of FC and FS vary considerably (Feachem, 1974). In cases when timed sampling verifies that high FC/FS ratios are valid (>4.0), the fecal contamination may be deemed to be of human origin (Feachem, 1974). Other factors significantly limit interpretations of FC/FS ratios, one being that a FS counts less than 100 FS/100 ml should not be used in ratio analysis (Clesceri, 1998). Only at one site did FS levels surpass 100 FS/100 ml (site 6, 170 FS/100ml), and in this case the FC/FS ratio was 3.2. The high levels of FC immediately upstream of the city limits of Claxton (sites 3-4) and within it (sites 5-6) may reflect the release of untreated human sewage into the river from upstream source(s), i.e., downstream of site 2 and upstream of site 6. The increasing FC load moving downstream from sites 3 to 6 may reflect the spread or growth of a FC plume (Clesceri et. al, 1998). Levels of FC measured at site 8 clearly indicate the sewage released from the chicken processing plant was not a major factor contributing to its FC load. The entry of treated water from the City of Claxton, down stream of site 6, but upstream of site 7 may account for the lower than expected values at sites 7 and 8, but why this would be so is not clear. Based on these observations the first part of our hypothesis that the levels of FC present at sites 7 and 8 would be higher and of chicken origin are rejected. Whether this reflects satisfactory treatment of sewage from the plant or an undetected periodic release of untreated or poorly treated sewage is unknown.

Ampicillin resistance was determined for sites 6, 7 and 8 as described in the Methods. Respectively, the frequencies of ampicillin resistance were 0.09, 0.16, and 0.09 (mean 0.11±0.036) for these sites. The difference in resistance frequency measured for site 7, compared to sites 6 and 8 was not significant in pair-wise testing ($p>0.05$). To confirm that the resistance was due to plasmid-borne resistance and not chromosomal genes, FC isolates were treated as if they contained low-copy number plasmids and prepared for midiprep isolation of plasmid DNA. While plasmid DNA was not readily visible in all minigel analyses of midipreps, transformation of a non-resistant laboratory strain of *E. coli* (K12 JM101) yielded transformants that could grow in the presence of ampicillin (results not shown.) Furthermore, upon cold-storage wild-type isolates and transformants frequently lost resistance, a property common to low-copy number plasmids. It seems likely then that the ampicillin resistance of the wild-type FC isolates was plasmid-borne. Studies are underway to determine the molecular nature of these plasmids. Unfortunately, since there was no apparent contribution of chicken FC to the FC load of the river water sampled at sites 7 and 8, it was not possible to address our second hypothesis that low-level use of antibiotics in feed may increase the number of antibiotic resistant FC present in released chicken sewage.

RECOMMENDATIONS

The high levels of FC measured in our study may warrant the attention of state authorities as recreational use of the river (i.e. fishing) was evident near site 6 (540 /100ml). Special attention might focus on discovery of a fecal contamination source upstream of Claxton, GA.

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