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Stability of Antibiotic Resistance Patterns in Agricultural Pastures: Lessons from Kentucky, USA

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1. Introduction

Animal and human wastes contain fecal bacteria, including pathogens that can contaminate groundwater, streams, lakes, and reservoirs through runoff and infiltration. Bacterial nonpoint sources of pollution continually impair water quality (Hartel et al., 2002). These pollution sources may come from failed septic systems, large animal operations, land application of wastes, sewage treatment facilities, and wildlife. Fecal pollution of rivers and streams is of great concern due to the direct potential threat to human health, and the increased costs associated with water treatment.

Groundwater contamination from these wastes can be a serious environmental concern in well-drained soils and soils with shallow water tables. Karst topography in Kentucky, for example, constitutes 55% of the land area (KGS, 2002), much of which is in pasture where land application of animal waste is commonly practiced. Of the 4,521 total km (2,810 total mi) of rivers and streams assessed in Kentucky in 2000, 73% were impaired for primary contact (>200 fecal coliforms/100 mL; USEPA, 2000). Similar reports regarding impaired watersheds can be found throughout the United States. Dombek et al. (2000) reported that 47% of assessed river miles in Minnesota were impaired for primary contact due to high levels of fecal coliform bacteria. Graves et al. (2002) reported that approximately 13% of monitored streams and 1% of estuaries in Virginia were impaired, with >60% of the impairments due to fecal contamination. The recurrence of such reports is evidence that tools are needed to identify such pollution sources and facilitate restoration efforts such as implementing total maximum daily loads (TMDLs) or best management practices (BMPs).

The reliance of pollution remediation efforts on TMDLs has been one of the driving forces behind developing techniques to distinguish between human and non-human fecal pollution sources (Johnson et al., 2004). A standard method of assessing water quality impairment based on the potential for pathogenic microbes of intestinal origin is to enumerate commensal bacteria such as coliforms. While total and fecal coliform counts produce an estimate of pollution levels, specific sources of the microbial pollution cannot be determined. Microbial source tracking (MST) techniques offer unique approaches to differentiate nonpoint source pollution. By tracking a pollution source to its origin, resources and management tools may be better allocated to improve water quality. Some

issues that affect the usefulness of these MST techniques include the appropriate database portability, size, and temporal characteristics to yield adequate power of prediction given the diversity of antibiotic resistance patterns in a watershed.

The answers to these issues are as yet undetermined. Several reports agree that MST techniques are most applicable to limited geographical areas such as specific watersheds rather than larger geographic regions (Guan et al., 2002; Johnson et al., 2004; Lu et al., 2005; McClellan et al., 2003). There is also no consensus on whether using *Escherichia coli* (EC) or fecal streptococci (FS) is preferred for use as indicator bacteria. Because of the labor-intensive and time-consuming nature of database building, the characteristics of a useful database are critical in the future applicability of MST methodologies.

There is little research that has concluded with any certainty on temporal variability effects on the ability of a host source database to classify nonpoint sources of pollution. There are research papers that have reported on lack of temporal stability due to among-species variation (Caugant et al., 1981; Gordon, 2001). One assumption made for the use of microbial source tracking is temporal stability or the ability to collect samples from the same source over time with little to no change in the outcome. Gordon (2001) reported on the minimal population differentiation in *E. coli* with only 5% of observed diversity derived from among-species variation, and this was considered inadequate. However, with a representative and adequately sized database, is 5% temporal variation significant?

Gordon (2001) continued to report on the most significant problem using *E. coli* in an MST database in which substantial changes in community occur from host to external environment. Caugant et al. (1981) reported that transient and resident strains of *E. coli* are present in the same host and they pose many questions as to the ramifications this may have to the usefulness of MST techniques. Jenkins et al. (2003) reported on the clonal diversity of *E. coli* among the same Black Angus steers sampled four times through one year. They evaluated ribotypes from two herds and discovered that a high clonal diversity index necessitated a large number of isolates (>900) for a database to be independent of temporal variations; however, they were uncertain whether their 20:1 resident to transient ratio could be overcome. Although Wiggins et al. (2003) studied fecal streptococci, they established that separate geographical databases, i.e., several watersheds, could be merged together to create a large, more representative database. Further, they concluded that the profiles were temporally stable for at least one year using antibiotic resistance analysis.

Phenotypic MST techniques such as antibiotic resistance analysis (ARA) have had moderate levels of success in small and relatively simple aquatic systems to differentiate human and nonhuman sources of pollution, or two-way level of classification (Carson et al., 2001; Guan et al., 2002; Graves et al., 2002; Hagedorn et al., 1999; Hartel et al., 2002; Harwood et al., 2000; Ritchey and Coyne, unpublished data; Ritchey and Coyne, 2009; Wiggins et al., 1999). Correct classification rates of ≥50% including five or more sources are considered useful by resource managers and rates of 60-70% are very useful (Harwood et al., 2000). Guan et al. (2002) conducted a study evaluating profiles using 14 antibiotics with a database consisting of 319 EC isolates collected from nine host sources. The database correctly classified 46% of the domestic, 95% of the wildlife, and 55% of the human sources. When the researchers pooled the nonhuman sources and compared the isolates to human sources, the RCC was 86% for human and 92% for nonhuman isolates. Harwood et al. (2000) constructed a

database of 6144 fecal coliforms FC and 4619 FS isolates from profiles using 9 antibiotics. For the larger database, when the isolates from the animal sources were analyzed separately, the RCC was 54% for the FC human isolates and 61% for the FS isolates. Pooling the animal sources together increased the human RCCs to 69% for the FC isolates and to 76% for the FS isolates. Reducing the number of sources and pooling the animal sources together, greatly increased RCC values (Carson et al., 2001; Guan et al., 2002; Harwood et al., 2000).

Generally, the primary concern of water resource managers and public health officials is discriminating human and nonhuman sources of contamination followed by secondary information to determine the source of the animal contamination (Harwood et al., 2000). Ritchey and Coyne (unpublished data) reported rates of correct classification of 66% for human and 67% for nonhuman sources at the two-way level of classification. However, caution must be exercised when testing the portability or spatial variability of the database by applying it on a large geographic-scale and in more complex systems. Many researchers (Guan et al., 2002; Johnson et al., 2004; Lu et al., 2005; McLellan et al., 2003) have concluded that because of the importance for an MST database to represent an area, yet exhibit limited temporal variability despite the genetic diversity of bacteria, MST techniques may be more useful when applied to limited geographical areas such as specific watersheds. Work presented by Harwood et al. (2000) with a database of 6144 isolates produced acceptable RCCs. Those RCCs were lower than the RCCs obtained by Wiggins (1996) when using limited geographic areas, but similar to Wiggins et al. (1999) when the geographical area of their study was increased.

Regardless if one is evaluating an aquatic or terrestrial system, the same unresolved issues remain that affect the ability of MST techniques to ascertain the source of contamination from fecal sources to an adequate level of predictability. These issues predominantly lie in the intrinsic resistance and stability, which can vary considerably based on antibiotic profiles chosen, source and type of fecal bacteria, portability, temporal characteristics, and soil and/or water conditions. Based on past and current research, complex environmental systems still require considerable research to adequately evaluate the application of MST.

2. Terrestrial systems and a Kentucky study

Numerous studies show that pollutant concentrations from manure-amended agriculture lands often exceed water quality standards (Howell et al., 1995; Reddy et al., 1981). Bacteria survival also influences bacterial contamination from manure-amended agriculture lands through runoff and infiltration. Some important factors influencing bacteria survival in soil are soil type, moisture, temperature, sunlight, pH, antibiotics, competitive organisms, available nutrients, organic matter, and clay content (Ellis and McCalla, 1978). Few studies have evaluated sod management practices on fecal bacteria survival, but Entry et al. (2000) showed that vegetation type in riparian filter strips had no effect on fecal coliform survival in soils.

Various studies have investigated the correlation behind surface derived fecal sources and elevated fecal levels in groundwater, via infiltration, and surface water, via overland flow or erosion. Several studies have reported that moisture levels play a primary role in determining bacterial growth and duration, while temperature was a secondary factor (Berry and Miller, 2005; Collins, 2004; Sinton et al., 2007; Stoddard et al., 1998; Unc and Goss,

2003). Soil physicochemical characteristics including soil type, structure, depth to water tables, and bedrock, i.e. karstic, should be considered when investigating non point sources of contamination. Stoddard et al. (1998) studied leachate of manure treated and untreated shallow karst tilled and no-tilled soils from central Kentucky (the Bluegrass region). Neither timing of manure application nor tillage method significantly affected leachate concentration of fecal coliforms. Movement of fresh fecal bacteria, within 60 days of application, moved below the root zone upon sufficient rainfall events.

Based on past and current research, complex environmental systems still require considerable research to adequately evaluate the application of MST techniques. Mowing is a common sod management practice that could affect fecal bacteria survival and antibiotic resistance patterns in poultry manure-amended pasture lands because it subjects fecal bacteria to environmental stress. In the Kentucky study Ritchey and Coyne (unpublished data), fecal bacteria survival was examined in frequently mowed, poultry manure-amended sod on Maury silt loam soil for 70 days. Simultaneously evaluated was the efficacy of antibiotic resistance analysis across time for this known animal waste source.

3. Study conditions

The soil consisted of Maury silt loam (fine, mixed, mesic, Typic Paleudalf) in undisturbed sod-covered plots with mixed grass vegetation dominated by fescue. There were 14 experimental units measuring 2.4 m wide by 6 m long, spaced 2.5 m apart. The treatments were undisturbed (n=3), disturbed every week (n=4), and disturbed biweekly (n=4). Disturbance was simulated by using a push mower. Each treatment had one unmanured plot as a control. All treatments received poultry manure at a rate of 20 kg per plot (14 Mg ha^{-1}) in a completely randomized design. Sampling was conducted on day 0, 7, 14, 21, 28, 49, and 70. Because source was known, monitoring the environmental conditions to determine how these sources reacted with the respective condition changes on pasture areas was possible. In this way, behavior of these fecal coliforms with measurable environmental conditions could better be predicted.

Two soil cores, 5 cm deep, were extracted from random locations in each plot, before mowing, at each interval during the experiment. The soil cores were bagged separately and stored at 4 ^{0}C until analysis within 24 h of collection. Each core was separated into vegetative cover and soil for analysis. Fecal coliforms and fecal streptococci from vegetation and soil were enumerated separately. Composite samples of vegetative cover along with the surface residue were prepared from the two cores in each plot and 3 g of composite sample was added to 90 ml of 2 mM phosphate buffer (pH 7.2). The surface 5 cm of soil from each core in each plot was thoroughly mixed and 10 g of field moist soil was added to 90 ml of phosphate buffer. The buffer and samples were agitated on a reciprocating shaker at approximately 160 rpm for 30 min to extract the bacteria. Oven dry weights of vegetative and soil samples were determined and all fecal bacteria concentrations were expressed on a dry weight basis. Samples were analyzed for fecal coliforms and fecal streptococci within 24 h using a spiral plater (Autoplate ® 4000 spiral plater, Spiral Biotech, Inc., Bethesda, MD). Fecal coliforms were incubated on mFC agar (Difco™ mFC Agar, Detroit, MI) at 44.5 °C for 22 h, and fecal streptococci were incubated on KFS agar (Difco ™ KFS Agar, Detroit, MI) at 35 °C for 48 h.

After EC and FS colonies were counted, at least five isolates from each plot were selected at random. To verify the presence of EC and FS, the isolates were grown on EC-MUG broth and mEnterococcus agar, respectively. The positively identified isolates were spiral plated in duplicate onto Mueller Hinton agar. Immediately after plating the *E. coli* isolates, antibiotic diffusion discs (BBL™ Sensi-Disc, Sparks, MD) were placed onto the agar surface using an 8-place dispenser (BBL® Sensi-Disc 8-place dispenser, Cockeysville, MD). Seven antibiotics were evaluated at the following concentrations: ampicillin (10 µg), cephalothin (30 µg), erythromycin (15 µg), rifampin (5 µg), streptomycin (10 µg), tetracycline (30 µg), and trimethoprim (5 µg). The cultures were incubated at 35 °C for 24 h and zones of inhibition for each antibiotic disc were measured at the end of the incubation period. The same procedure was followed for the fecal streptococci isolates except the positively identified isolates were grown in Tryptic Soy Broth (Difco ™ Tryptic Soy Broth, Detroit, MI) at 35 °C for 48 h and then spiral plated onto Mueller Hinton agar that was incubated at 35 °C for 48 h.

Statistical analysis for fecal coliform and fecal streptococci concentrations in sod and soil was performed separately using the PROC MIXED procedure in SAS ® (SAS version 8.2, SAS Institute Inc., Cary, NC) for the analysis of variance and means separation among the treatments were determined by difference in least square means. A linear regression model using a first order decay model (log CFU g^{-1} = K Days + Constant; K= mortality rate) was used to estimate the fecal coliform and fecal streptococci mortality rates in sod and soil. Statistical analysis for *E. coli* and fecal streptococci antibiotic resistance patterns was performed using the PROC GLM procedure in SAS. Repeated measures were used for analysis of variance to detect differences in treatments, collection dates, and interaction of treatments by collection dates. The LSD procedure was used to detect significant pairwise differences. Principle components analysis was performed using the PROC PRINCOMP procedure in SAS. Discriminant analysis was used to evaluate correct rates of classification and was performed using the PROC DISCRIM procedure in SAS.

4. Results and discussion

4.1 Population and survival

The background fecal coliform and fecal streptococci concentrations in the plots were not significantly different from one another prior to manure application. The fecal coliform concentrations in background ranged from non detectable (<1) to 100 CFU g^{-1} in sod and non detectable to 50 CFU g^{-1} in soil. The fecal streptococci concentrations in the background ranged from 1,000 to 16,000 CFU g^{-1} in sod and 100 to 800 CFU g^{-1} in soil. The poultry manure contained approximately 7.9 x 10^8 CFU g^{-1} fecal coliforms, and 2.5 x 10^9 CFU g^{-1} fecal streptococci.

Manure application significantly increased the fecal coliform and fecal streptococci concentrations in sod and soil compared to the respective unmanured controls and remained so for the duration of the experiment. The fecal coliform and fecal streptococci concentrations exceeded 10^6 CFU g^{-1} sod and 10^5 CFU g^{-1} soil seven days after manure application (Tables 1 and 2). Fecal coliform and fecal streptococci concentrations in unmanured control plots also increased after manure application, but this was most likely due to cross contamination resulting from mowing and sample collection. Mowing frequency neither increased nor decreased the fecal coliform and fecal streptococci concentration in either sod or soil in the

Treatment	Day 7	Day 14	Day 21	Day 28	Day 49
Sod					
Never Mowed	6.9a* †	7.9a*	6.4a*	6.0a*	5.1a*
Never Mowed control	4.1	4.0	4.1	4.0	BD
Mowed Biweekly	6.8a*	7.2ab*	6.3a*	6.4a*	4.6a*
Mowed Biweekly control	4.4	3.9	4.2	3.6	1.9
Mowed Weekly	6.6a*	6.9b*	5.7a*	6.1a*	4.5a*
Mowed Weekly control	5.1	4.9	4.4	4.1	3.2
Soil					
Never Mowed	5.5a*	4.1a*	4.4a*	4.0b*	2.6a*
Never Mowed control	2.9	2.4	ND	1.2	BD
Mowed Biweekly	5.1a*	5.1a*	5.1a*	3.8b*	2.6a*
Mowed Biweekly control	3.1	2.4	1.9	BD	BD
Mowed Weekly	5.1a*	4.5a*	4.3a*	4.3a*	3.0a
Mowed Weekly control	3.6	3.3	3.1	3.3	3.6

† Values at each interval and sample type sharing the same letter are not significantly different (p ≥ 0.05). None of the controls were significantly different from one another. * Significantly different (p ≤ 0.05) from unmanured controls. ND- Not determined. BD – Below detection levels.

Table 1. Average fecal coliform concentration (log CFU g^{-1}) from sod/soil in mowed and poultry manure-amended sod plots.

Treatment	Day 7	Day 14	Day 21	Day 28	Day 49	Day 70
Sod						
Never Mowed	6.5a*†	6.2a*	6.3a*	6.3a*	6.4a*	4.9a
Never Mowed control	4.8	4.7	4.3	5.1	3.8	4.6
Mowed Biweekly	6.3a*	6.1a*	6.2a*	5.9a*	3.9a*	5.4a
Mowed Biweekly control	4.0	4.8	4.3	4.3	5.1	BD
Mowed Weekly	6.6a*	6.3a*	6.3a*	6.6a*	5.9a*	4.9a
Mowed Weekly control	4.6	4.9	3.9	4.4	4.4	BD
Soil						
Never Mowed	5.0a*	5.0a*	5.0ab*	4.5a*	4.0a*	3.3a
Never Mowed control	4.1	3.8	3.3	3.1	3.1	3.2
Mowed Biweekly	5.1a*	5.3a*	5.1a*	4.5a*	3.7a	3.6a
Mowed Biweekly control	3.9	2.8	3.7	2.7	3.0	3.7
Mowed weekly	5.2a*	5.2a*	4.6b	4.5a*	4.0a*	3.3a
Mowed weekly control	4.0	4.0	3.9	3.6	3.1	3.5

† Values at each interval and sample type sharing the same letter are not significantly different (p ≥ 0.05). None of the controls were significantly different from one another. * Significantly different (p ≤ 0.05) from unmanured controls. ND- Not determined. BD - Below detection levels.

Table 2. Average fecal streptococci concentration (log CFU g^{-1}) from sod/soil in mowed and poultry manure-amended sod plots.

manure-amended plots. These results were consistent with previous rain simulation studies on bacterial survival and infiltration in frequently mowed sod plots (Gandhapudi, 2004) in which mowing did not significantly affect the fecal bacteria concentrations recovered in lysimeters pans.

Because mowing had no effect on bacteria survival, different treatments were used in the study as replicates to study fecal coliform and fecal streptococci survival in sod and soil. The fecal coliform concentration in sod increased from 7 to 14 days after manure application, suggesting net growth, and thereafter decreased slowly for the rest of the study period. In contrast, the fecal coliform concentrations in soil slowly but continuously declined after manure application, without any evidence of net growth during the first 14 days. The fecal coliforms in sod and soil had only an approximate 25-fold decrease in 49 days. However, fecal coliform concentration in sod and soil declined below the detection limits (1000 CFU g^{-1} in sod and 100 CFU g^{-1} in soil) 70 days after manure application. The difference in detection limits was due to the difference in initial dilution.

The fecal streptococci concentration in sod and soil declined very slowly after manure application for the duration of experiment (70 days). There was only an approximate 15-fold decrease in fecal streptococci concentration observed in 70 days and the fecal streptococci concentration exceeded 4×10^4 CFU g^{-1} in sod and 2.0×10^3 CFU g^{-1} in soil even 70 days after manure application. However, fecal streptococci concentrations at 70 days were not different from unmanured control plots.

4.1.1 Mortality rates

A first order die-off model was used to describe fecal coliform and fecal streptococci mortality in this study because the first order die-off model has been widely and successfully used to describe fecal bacteria mortality in bacteria survival studies (Edwards and Daniel, 1992; Reddy et al., 1981; Stoddard et al., 1998). A 35-day model (between Day 14 and Day 49), excluding the periods with net growth, was used to describe the fecal coliform mortality in sod and a 49-day model (between Day 7 and Day 49) was used to describe the fecal coliform mortality in soil. The linear regression model (log CFU g^{-1} = k Days + constant) describing mortality rates indicated that there was no difference between mortality rates in sod and soil, and that the average fecal coliform mortality rate (k) was 0.06 log cells day^{-1} (R^2= 0.57 in sod; R^2 = 0.53 in soil). Redistribution of fecal bacteria in manure during mowing can presumably have facilitated growth, but attempts were not made to calculate growth rates for individual treatments. Lack of significant differences in net mortality rates was likely due to confounding effects of growth and mortality.

A 70-day linear regression model (log CFU g^{-1} = k Days + constant) was used to describe fecal streptococci mortality in sod and soil. The average fecal streptococci mortality rates in this model were 0.02 log cells day^{-1} (R^2= 0.53) in sod and 0.03 log cells day^{-1} (R^2 = 0.69) in soil. We assume that very low fecal streptococci mortality rates in sod and soil might have been influenced by the background fecal streptococci populations. The fecal streptococci mortality rate in the control plots did not correlate with the fecal streptococci mortality rates in the manure-amended population. In unmanured controls, the fecal streptococci concentrations did not change significantly throughout the study.

Studies that used poultry litter as a soil amendment have reported mortality rates ranging from 0.06–0.29 day^{-1} for fecal coliforms and 0.06-0.357 day^{-1} for fecal streptococci (Crane et al., 1980; Zhai et al., 1995). Our mortality rates were comparable to the mortality rates reported elsewhere, although the simple first order die-off model used in this study to describe the mortality of fecal coliform and fecal streptococci in sod and soil showed a poor R^2, indicating that the model was not a good fit for the data.

Moisture, temperature, and nutrient availability are important factors that influence fecal coliform and fecal streptococci mortality in soil. Entry et al. (2000), for example, reported that decreasing soil moisture and increasing soil temperature substantially increased the mortality of total coliforms and fecal coliforms in soil. Our results suggest that fecal coliform and fecal streptococci mortality rates were also influenced by soil moisture and temperature. During the study period almost every sampling period was preceded by a rain event that substantially increased the gravimetric moisture content in the soil to 29-40% (Fig. 1). Weekly rainfall exceeded 5-year averages in 6 of 10 sample periods (Fig. 2). The average 70-day temperature during the study was 17–27 °C in sod and 22–25 °C in soil.
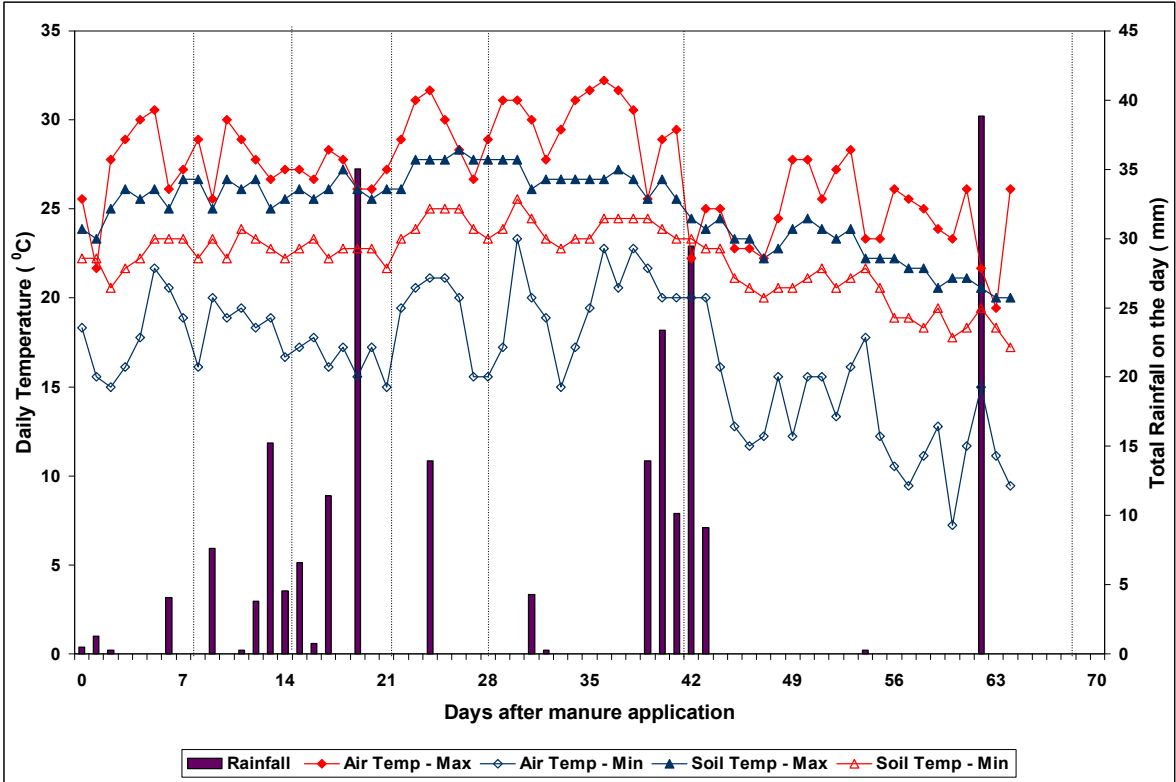

Fig. 1. Daily maximum and minimum air and soil temperatures, and total precipitation during the study period (July 2003 – October 2003) (weather data from Maine Chance Research Farm, Lexington KY). Dotted lines in the graph indicate the sampling periods during the study.

4.2 Antibiotic resistance patterns

Antibiotic resistance patterns of *E. coli* (EC) and fecal streptococci (FS) changed with time. The *E. coli* in sod and soil generally lost resistance followed by a return to initial patterns of

resistance; whereas, the fecal streptococci in sod and soil generally had periods of increased resistance followed by a return to initial patterns. A summary of the significant differences of means by antibiotic and date from sod and soil for EC are shown in Table 3. A summary of the significant differences of means by antibiotic and date from sod and soil for FS are shown in Table 4.
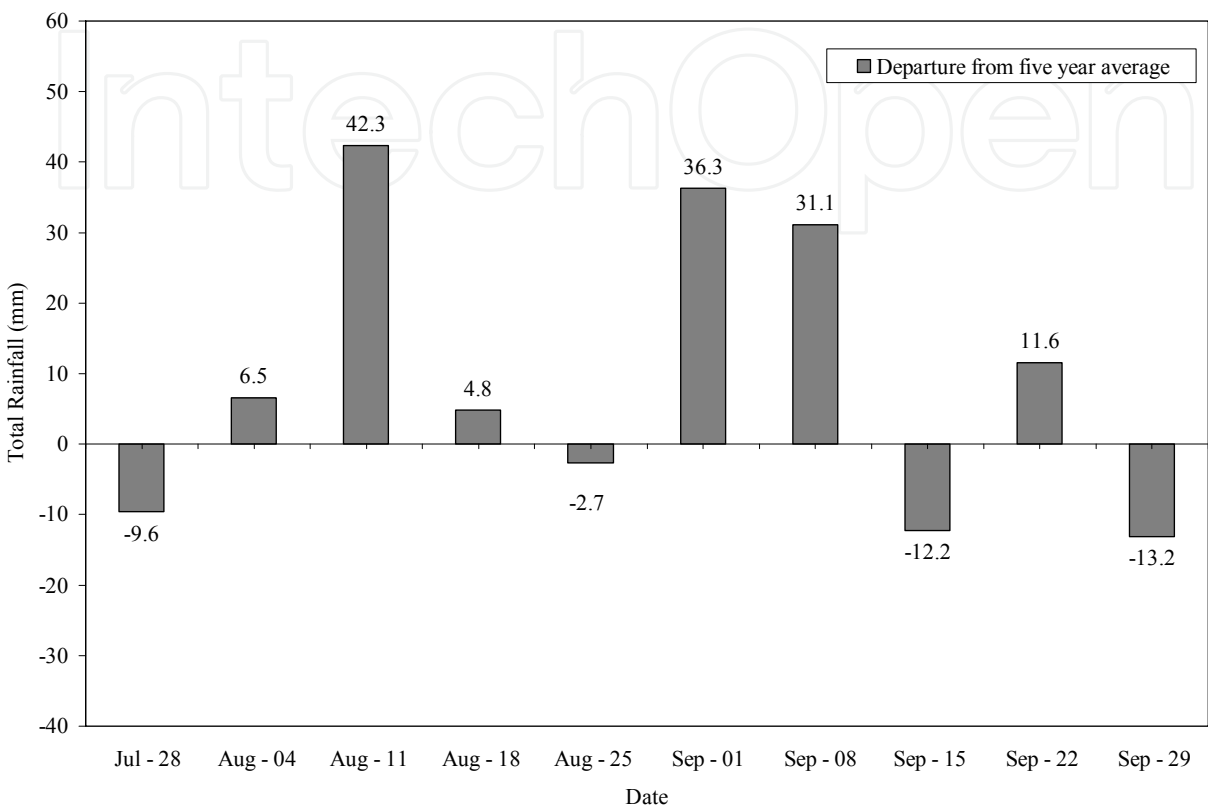

Fig. 2. Weekly rainfall departure during the study period (July – September 2003) from the past five year average (1998-2002). (Data obtained from Spindletop Research Farm Weather Station, Lexington KY).

Escherichia coli (EC) Isolates – Sod. There were five sampling dates for the sod EC isolates. The samples were collected at days 0, 7, 14, 28, and 49 after poultry application. The choice of antibiotic(s) for the study played a large role in the detection of bacterial changes with time. This was evident based on different resistance patterns among the seven antibiotics, thus providing more unique 'signatures' for each isolate. Statistical analysis of the sod data indicated that there was no significant treatment or sampling date main effect or interaction for ampicillin and cephalothin. The remaining antibiotics (i.e., erythromycin, rifampin, streptomycin, tetracycline, and trimethoprim) all produced significantly different antibiotic resistance patterns (ARPs) with sampling date. There were no significantly different date by treatment interactions or treatment main effects.

As the area surrounding the antibiotic or zone of inhibition increases, the resistance of the bacteria to the antibiotic decreases, and vice versa. The resistance of EC to erythromycin, rifampin, streptomycin, tetracycline, and trimethoprim significantly decreased with time. Generally, the bacteria showed initial changes in resistance at day 14 for all of the antibiotics that had significant date effects.

Antibiotic	Day 0	Day 7	Day 14	Day 21	Day 28	Day 49
Sod						
Ampicillin	17a†	16a	17a	ns‡	18a	18a
Cephalothin	18a	15a	17a	ns	17a	18a
Erythromycin	9a	8a	13b	ns	16c	17c
Rifampin	9a	8a	10a	ns	13b	13b
Streptomycin	14a	15a	17b	ns	19c	18bc
Tetracycline	21a	20a	23b	ns	24b	24b
Trimethoprim	18a	23b	26b	ns	29c	28c
Soil						
Ampicillin	18a	19a	ns	18a	19a	17a
Cephalothin	17a	16a	ns	17a	19a	17a
Erythromycin	11a	8a	ns	10a	18b	17b
Rifampin	9a	8a	ns	9a	13b	12c
Streptomycin	16a	17a	ns	17a	20b	18c
Tetracycline	20a	22ac	ns	19a	25b	24bc
Trimethoprim	23a	25ab	ns	26b	28c	28c

† Values at each interval and sample type sharing the same letter are not significantly different ($p \geq 0.05$). ‡ ns = not sampled

Table 3. Date and mean values of antibiotic inhibition zones (mm) for *E. coli* isolates from sod and soil.

E. coli (EC) Isolates – Soil. There were five sampling dates for the soil EC isolates. The samples were collected at days 0, 7, 21, 28, and 49 after poultry application. Similar to the sod data, there was no significant treatment or date main effect or interactions with ampicillin and cephalothin. The sampling dates were significantly different from each other using erythromycin, rifampin, streptomycin, tetracycline, and trimethoprim. The EC from the soil also had decreased resistance to all of the antibiotics with time. However, the initial changes in resistance occurred at day 21 which was approximately one week later than the sod. This would suggest that migration of fecal bacteria from the sod to the soil may have occurred with time.

The treatment by sampling date interactions were significantly different for erythromycin and tetracycline. The resistance of EC decreased with time in the 'Mowed Every Week' and 'Mowed Biweekly' treatments for erythromycin, including the control plots. In the mowed treatments, the significant decrease in antibiotic resistance occurred between day 0 and day 21. However, the 'Never Mowed' treatments receiving poultry manure had increased resistance of bacteria at day 21 followed by a decrease to initial levels of resistance. The resistance of EC to trimethoprim decreased by day 28 and remained at that suppressed level until the last sampling date on day 49 for both the 'Never Mowed' and 'Mowed Every Week' treatments. Similar to the 'Never Mowed' treatment for erythromycin, the 'Mowed Biweekly' treatment for trimethoprim resulted in an increased level of resistance by day 28 and returned to initial levels for the remaining sampling dates. There were no significant changes with time in the control treatment.

Antibiotic	Day 0	Day 7	Day 14	Day21	Day 28	Day 49	Day 70
Sod							
Ampicillin	22a†	23a	22a	ns‡	24a	21a	23a
Cephalothin	15b	10a	9a	ns	17b	14b	18b
Erythromycin	17a	11b	9b	ns	19a	20a	20a
Rifampin	16a	14ab	10b	ns	17a	18a	16a
Streptomycin	6a	7a	6a	ns	9b	10b	7a
Tetracycline	21b	11a	11a	ns	16ab	21b	23b
Trimethoprim	26a	25a	23a	ns	27a	21a	25a
Soil							
Ampicillin	23ab	25a	24a	22ab	23ab	20b	16c
Cephalothin	16ab	9c	10c	16ab	19a	13bc	12bc
Erythromycin	18a	10b	12b	18a	17a	16a	16a
Rifampin	21a	12cd	10d	18ab	15bcd	14bcd	16abc
Streptomycin	8abc	6c	6c	9ab	10a	7bc	7bc
Tetracycline	21a	9c	13bc	17ab	16ab	20a	19a
Trimethoprim	25ab	29a	22b	24ab	20bc	23b	16c

† Values at each interval and sample type sharing the same letter are not significantly different (p ≥ 0.05). ‡ ns = not sampled

Table 4. Date and mean values of antibiotic inhibition zones (mm) for fecal streptococci isolates from sod and soil.

Fecal Streptococci (FS) Isolates – Sod. There were six collection dates for the sod FS isolates. The samples were collected at days 0, 7, 14, 28, 49, and 70 after poultry application. Trimethoprim produced no statistically significant main effects or interactions. Cephalothin, erythromycin, rifampin, streptomycin, and tetracycline produced significant date main effects. There were no significant treatment main effects for any antibiotic used in this study. The statistical analysis indicated that ampicillin and streptomycin produced significant treatment by date interactions. All of the antibiotics that had significant date effects except for streptomycin went through a phase of increased resistance at approximately day 7 with a subsequent phase at day 28 that was generally not significantly different from day 0. Streptomycin showed an opposite trend, whereby the bacterial response was a slight decrease in resistance at day 28 followed by an increase back to initial patterns of resistance at day 70. This may be an artifact of significant treatment by date interactions for streptomycin.

The most significant differences for treatment by date interactions of ampicillin occurred in the 'Mowed Biweekly' treatment when compared to the other treatments. There was a two phase cycle that was marked by increased resistance of FS to ampicillin followed by a return to initial (day 0) patterns of resistance patterns. There were two of these cycles that were documented over the 70-day sampling period. The first cycle began at day 7 and ended at day 28. The second cycle began at day 49 and ended at day 70. The 'Mowed Weekly' treatment for treatment by date interactions of streptomycin contained ARPs that indicated decreased periods of resistance occurring at day 49 and returning to day 0 patterns at day

70. The treatment by date interactions for the control treatments of ampicillin and streptomycin were also statistically significant. There were decreases in resistance to both antibiotics at day 28.

Fecal Streptococcus (FS) Isolates – Soil. There were seven collection dates for the soil FS isolates. The samples were collected at days 0, 7, 14, 21, 28, 49, and 70 days after poultry application. All seven of the antibiotics used in this study produced significant date main effects, but there were no significant treatment effects or treatment by date interactions. The results for FS in soil were the most ambiguous for this study. The results for FS in sod and EC in sod and soil had definitive resistance patterns that changed similarly with time across antibiotics within each organism and medium. However, upon evaluation of FS in soil, the data suggested an increased resistance at day 7 through day 14. This was more pronounced with cephalothin, erythromycin, rifampin, streptomycin, and tetracycline. Ampicillin and trimethoprim produced ARPs that showed progressively increasing resistance of FS throughout the sampling dates. These results, as those for EC, may be explained by a gradual migration of the organisms from sod to soil.

4.2.1 Rates of correct classification based on antibiotic resistance

The rate of correct classification or RCC for sod and soil by date was analyzed for EC and FS. The six total distinct dates used for EC were divided into two date groups, early and late, and the initial background (day 0) was excluded. The early date included 7, 14, and 21 days after poultry application. The late date included 28 and 49 days after application. The seven total distinct dates for FS were also divided into two groups, early and late, with exclusion of the initial background. The early date for FS included 7, 14, and 21 days after poultry application. The late date included 28, 49, and 70 days after application.

The RCC for EC using resubstitution analysis showed that the database correctly classified 92% of the sod isolates and 70% of the soil isolates for the early collection dates. The database correctly classified 70% of the sod isolates and 85% of the soil isolates for the late collection dates. These results coincided with the analysis of the significant differences of means by antibiotic and date from sod and soil as discussed previously. That is, the data support the idea that migration of fecal bacteria from the sod to the soil may occur with time. The correct classification of bacteria is highest for the early dates of sod while the majority of the bacteria resides in the surface or sod portion of the profile. Over time, the bacteria migrate to the lower area or the soil portion of the profile. For these dates, the RCC becomes lower for sod and higher for soil.

The RCC for FS using resubstitution analysis showed that the database correctly classified 85% of the sod isolates and 59% of the soil isolates for the early collection dates. The database correctly classified 69% of the sod isolates and 58% of the soil isolates for the late collection dates. The results for FS are more ambiguous than those reported for EC. While the RCC for sod also decrease over time, the RCC for soil remain relatively unchanged. It is worth noting that host source origin is typically classified. In this case, the medium, i.e., sod and soil, is effectively being classified with moderate success. Rates of correct classification of 60% or higher are considered useful by resource managers (Harwood et al., 2000), which makes the rates reported here of significant value.

Principle Component Analysis (PCA) was a useful tool when applied to the EC database. The variables used to compute the PCA were the seven antibiotics used for the profiles. The cumulative percent of variability accounted for by the first two axes was 72% which described most of the variability among the seven antibiotics. Axis one, which accounted for 55% of the variability, appeared to be associated with the antibiotics erythromycin, rifampin, and streptomycin. The second axis appeared to have large loadings for ampicillin and cephalothin. The graph of the PCA output for EC is shown in Figure 3 where period 1 = 0 days (background), period 2 = 7, 14, and 21 days, and period 3 = 28 and 49 days after poultry litter application. The dates were combined into early (background, period 1 = 0 days), intermediate (one to three weeks after application, period 2 = 7, 14, and 21 days), and late (anything after three weeks, period 3 = 28 and 49 days). Graphing the two axes based on period reveals grouping in the data. Period 1 data are not structured and agrees with previous findings for these are background data collected prior to poultry application. The data for period 2 resolve as two groups suggesting that a transitional period occurs between one and three weeks after poultry application as bacterial populations migrate from sod to soil. The data for period 3 are grouped together with no further changes up to 7 weeks after poultry application. The groups change relative to axis 1 which suggests that date primarily affects erythromycin, rifampin, and streptomycin antibiotic resistance patterns.
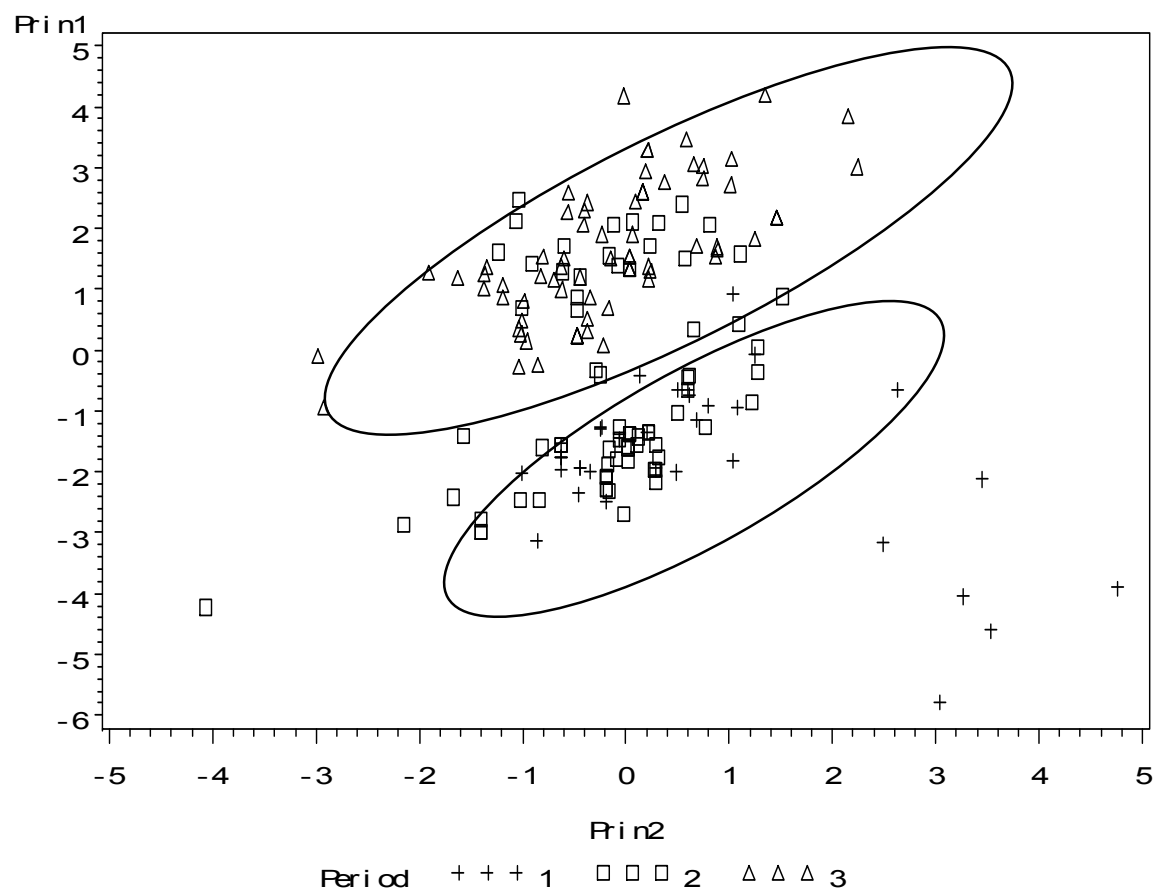

Fig. 3. Principle components analysis for *Escherichia coli* by date. Sod and soil isolates were combined in this analysis.

The graph of the PCA output for FS is shown in Figure 4. The PCA for the FS dataset showed that the cumulative percent of variability accounted for by the first two axes is 64%, which described most of the variability among the seven antibiotics. Axis one, which accounted for 43% of the variability, appeared to be associated with the antibiotics cephalothin, erythromycin, and rifampin. Axis two had large loadings for ampicillin and trimethoprim. The dates are the same as those described for EC with an addition of 70 day data added to period 3. There appeared to be no structure to the data presented in the plot, and no distinct grouping patterns.
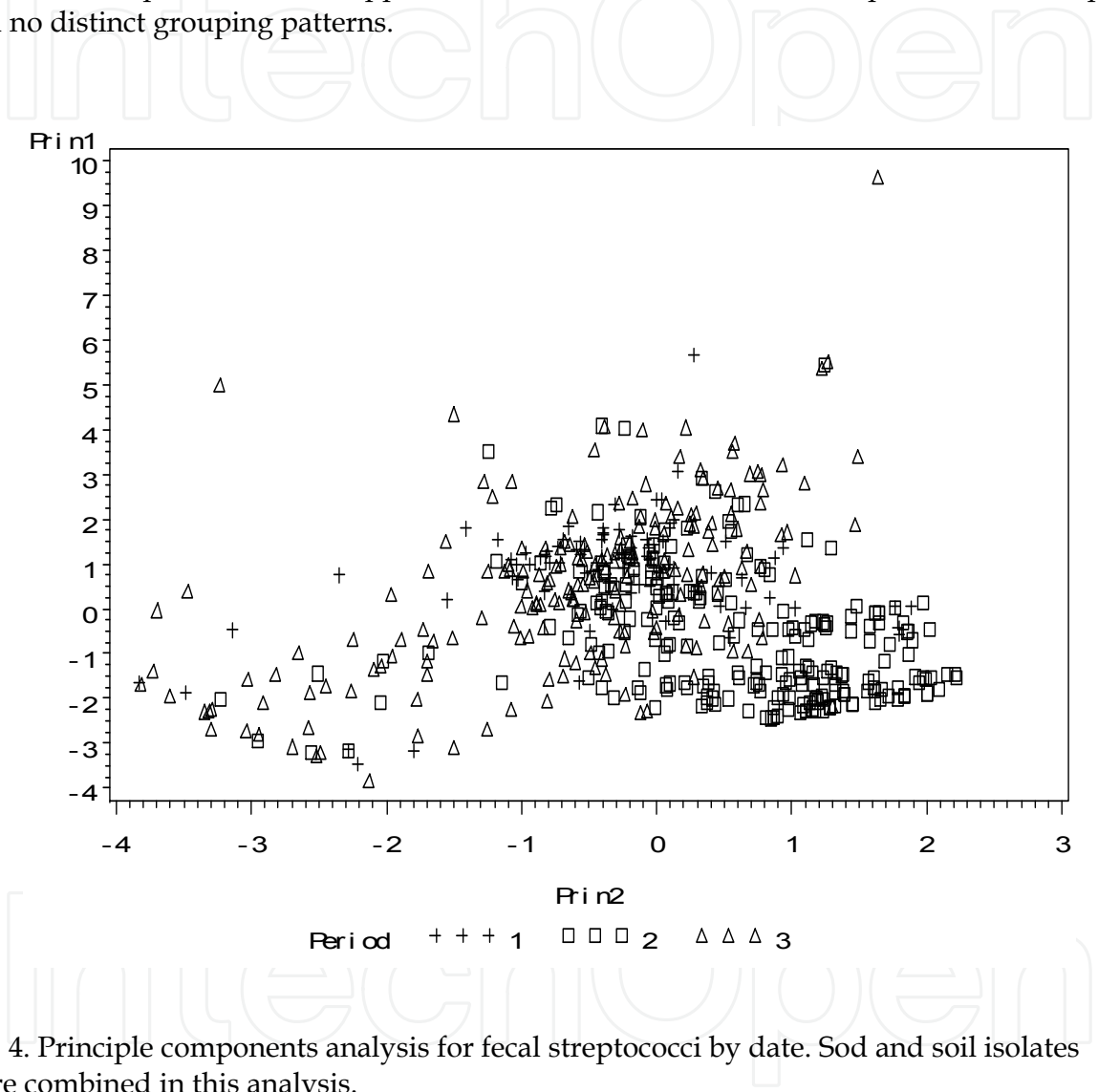
Fig. 4. Principle components analysis for fecal streptococci by date. Sod and soil isolates were combined in this analysis.

5. Conclusions

This study showed that disturbance (e.g. mowing) had little or no effect on EC and FS mortality in sod or soil in our study environment. It was suspected that the selection of mowing height, preservation of residue, and consistently wet weather combined to minimize treatment effects. The relatively prolonged survival of the fecal bacteria promoted the potential for runoff during the study, as well as potential for phenotypic variability as revealed by the MST profiles. The fecal bacteria appeared to persist in the environment for

extended periods. Mowing frequency did not appear to affect the resistance profiles of *E. coli* and fecal streptococci for seven antibiotics. However, characterization of the same fecal bacterial population by means of MST was not consistent for that same time period; thereby suggesting that MST by this method was a time-dependent technique. Sampling time after our initial poultry manure application did appear to significantly affect the profiles recovered. Ampicillin and cephalothin were considered good indicators of antibiotic resistance over time for *E. coli* in sod or soil as there were no significant differences between sampling dates.

The selection of antibiotic to identify changes in microbial populations over time appears to play an important role in the effective use of MST. Based on the results from this study (Ritchey and Coyne, unpublished), ampicillin and cephalothin may be good choices to determine sources of EC in soil or sod and trimethoprim may provide useful information when studying FS in sod because there were no significant differences with time which indicates temporal stability when using these antibiotics.

6. Acknowledgement

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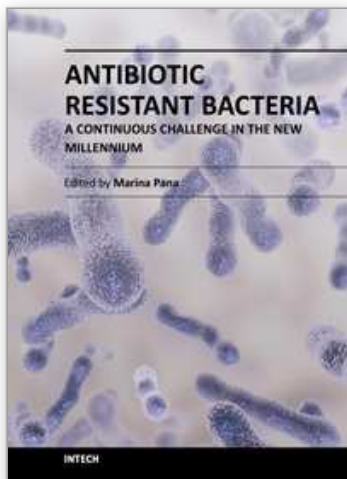
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Edited by Dr. Marina Pana

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