ABSTRACT

LIWIMBI, LLOYD CHIPILIRO SIYANI. Antibiotic Resistance and Water Quality: Land Application of Swine Lagoon Effluent as a Potential Source of Antibiotic Resistant Genes in Surface Water. (Under the direction of Dr. Alexandria Graves).

The use of antibiotics in animals is suspected to be a major route for transfer of antibiotic resistance genes (ARGs) to human pathogens, although different antibiotics are used in animals than in humans. North Carolina has the second largest swine production industry in the USA which is concentrated in a small geographical area in the southeastern area of the state. This may increase the risk of antibiotic resistant bacteria reaching nearby surface waters. The goals of this study were 1) to identify and quantify ARGs in *Escherichia coli* isolates from swine feces, lagoon effluent, cattle, wildlife and ground and surface waters 2) to develop a data base for antibiotic resistance patterns for *E. coli* isolated from these sources 3) to evaluate the possible use of this database in tracking movement of fecal bacteria from confined swine operations to a nearby stream 4) to determine the relationship between antibiotic resistance genes found in *E. coli* strains from manure and water with the actual phenotypic expression of the resistance to their corresponding antibiotics. *Escherichia coli* isolates were recovered from manure and water samples by basic microbiological culture and IDEXX Colilert methods, respectively. Antibiotic resistance genes were identified from the isolates using the polymerase chain reaction (PCR) method. On average, *E.coli* counts in the surface water were 272.1 CFU/100 mL, 10 fold higher than that in ground water (21.1 CFU/100 mL). A total of 1,208 *E. coli* isolates from swine feces, lagoon effluent, cattle, deer, dog, bird and nearby ground and surface waters (n=238, 234, 192, 48, 48, 48, 200, and 200, respectively) were evaluated for ARGs. A total of 5909 *E. coli* isolates were evaluated for phenotypic expression of resistance to various concentrations of the following antibiotics: erythromycin, neomycin, oxytetracycline, streptomycin, tetracycline, cephalothin, apramycin, trimethoprim, and rifampicin. About 96% of the

isolates displayed multiple antibiotic resistances. Genotypic evaluation indicated the presence of *aadA, strA, strB, tetA, tetB, tetC, sul1, sul2, sul3*, and *Aac(3)IV* ARGs in all the sources of isolates. Resistance genes that code for resistance to spectinomycin (*aadA*), low level streptomycin (*str*), tetracycline (*tetA*) and sulfonamide (*sul1*) were present in highest frequency. The database developed from antibiotic resistance patterns (ARP) and antibiotic resistant genes (ARGs) of *E. coli* isolates from the known sources were able to associate fecal bacteria in ground and surface water to lagoon effluent and to livestock respectively. The results clearly show that the presence of ARGs does not always result in phenotypic expression of the resistance and vice versa. The results will increase awareness on the widespread of antibiotic resistance in both agricultural systems and environmental pools within confined swine farms. Even though good management practices for swine waste management (anaerobic lagoon systems) are effectively mitigating fecal pollution, there is a need for prudent and responsible use of antibiotics especially with those prone to induce resistance.

Antibiotic Resistance and Water Quality: Land Application of Swine Lagoon Effluent as a Potential Source of Antibiotic Resistant Genes in Surface Water

by
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A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Soil Science

Raleigh, North Carolina

2009

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BIOGRAPHY

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ACKNOWLEDGEMENTS

I would like to express the deepest gratitude to my adviser Dr. Alexandria Graves for her effort for mentoring me as a researcher. She facilitated my participation in many scientific conferences to present my work that resulted into fruitful discussions with other researchers. I would also like to thank her for all her support with the research protocols some of them which were new to me. I would also like to thank my co-advisers, Dr. Daniel W. Israel and Dr. Eric van Heugten without whom my thesis and presentations would be just an abstract idea. I would like to thank Dr. Jeff Thompson for his advice in statistical analysis for my research data. Brad Robinson, research specialist in our lab, for the technical and physical support in the course of this project as we went through the woods of six run's creek together. I also acknowledge Joie Lubbers, Daniel Vaal, and Charles Cahoon for the technical assistance during the entire period of the project.

I would like to thank the management of Water Resource Research Institute of the University of North Carolina for supporting this project financially and the entire Soil Science Department at NCSU, faculty, staff, and students for their accommodative spirit and understanding during my study. It would not have been easier to study away from home, family and friends without them all.

Lastly, I would like to thank my wife, Jayne and my family members, especially my brother George who inspired me and not forgetting my late parents Desiderio and Evarista for the hard working spirit they instilled in me which helped me to get to this level.

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LITERATURE REVIEW

INTRODUCTION

Antibiotic resistance is a worldwide problem and well known in both human and veterinary medicine (Maynard et al, 2003; Teuber, 2001). The National Antimicrobial Resistance Monitoring System (NARMS) was established in 1996, as a branch of United States Centers for Disease Control and Prevention (CDC) to monitor the development of antibiotic resistance. The program focuses on the surveillance of antibiotic resistance in indicator bacteria and zoonotic agents which are the normal intestinal flora of animals (e.g., *Escherichia coli* and *Enterococcus spp*.). The problem with antibiotic resistance may be aggravated by antibiotic use in the agricultural industry. Antibiotic use in the agricultural industry is widespread among livestock producers such as those responsible for cattle and swine production (Khachatourians, 1998). As with other livestock industries, antibiotics are use in swine facilities because of their efficiency to reduce disease and increase swine growth. About half of all antibiotics used in the United States are for animal production, the major compounds being penicillin and tetracycline (Khachatourians, 1998). Ten percent of these antimicrobials are administered to treat infectious disease while the remaining 90% are for promotion of growth or as prophylactic agents (Khachatourians, 1998). Many antibiotics used in animal agriculture are not readily absorbed in the animal gut. Approximately 25% to as much as 75% of antibiotics given to stall-feed animals could be released undigested in feces (Edmund et al, 1971; Feinman and Matheson, 1978) and these may be present in soil after land application (Donoho, 1984; Gavalchin and Katz, 1994). The production of waste by livestock and poultry in the United States annually is close to 180 million tons as dry mass (Haapapuro et al., 1997), this waste may be a potential source of residues of these antibiotics and bacteria resistant to antibiotics when they enter into the environment through land application of sludge and/or by spraying lagoon effluent onto fields

(Chee-Sanford et al, 2009). Additionally, there is also the concern of lagoon waste seeping into groundwater beneath the pits. The fate of antibiotic residues, as well as, the survival and mobility of antibiotic resistant bacteria in soil leads to concern about livestock waste entering the environment. Livestock waste entering the environment may result in possible exposure of humans and animals to antibiotic residues and antibiotic resistant bacteria.

Problems associated with antibiotic resistance

Impacts of antibiotic resistance on human health

There are several implications related to antibiotic resistance on human health. According to CDC (1998) antibiotic resistant bacteria cause 2 million deaths each year particularly among individuals with compromised immune systems, including children and the elderly. Antibiotic resistant infections result in longer hospital stay and require the use of more expensive drugs to treat infections (McGowan, 2001). This suggests that in addition to human health impacts, there are also economic impacts due to loss of income from missing days of work and extra costs associated with more appropriate drugs (McGowan, 2001). Many enteric pathogens of swine including bacteria such as *Salmonella, Yersinia,* and *Campylobacter* are also infectious to humans through contact with farm workers and the food chain. Among these bacteria, the prevalence of resistance varies widely, from 0% to 90%, depending on the antimicrobial tested, animal species, and country of origin (McEwen, 2006).

Salmonella and *Escherichia coli* are economically important pathogens of livestock that result in high morbidity and mortality rates. These pathogens are responsible for an estimated 1.4 million cases of food borne illness, resulting in 600 deaths each year in humans (CDC, 1998). Reports from 2004 and 2005 indicated human diseases believed to have originated from swine have proven to be very difficult to manage. For instance, Pneumonia caused by methicillin resistant *staphylococcus aureus* (MRSA) was often found in pig and cattle farmers (van Loo, 2007) and has been recovered from humans in

35 cases by surveillance in the Netherlands, in 2006. These cases were geographically associated with pig farming.

The threat of diseases caused by antibiotic resistant microorganisms is complicated further because antimicrobial drugs used for human therapy often have significant structural similarities to those used for animals. For instance, resistance to apramycin, a veterinary medicine, might induce resistance to gentamycin, a structurally similar compound that is specifically used in humans (Mathew et al., 2003). The concern about antibiotic resistance has escalated in recent years because the rate at which antimicrobial medicines are being developed is much slower than the rate at which the current antimicrobials are being rendered ineffective. This leads to concern that deaths due to bacterial infections will once again become a real threat to substantial numbers of children, the elderly and immunocompromised persons (Gilchrist et al., 2007).

Impacts of antibiotic resistance on animal health and food safety

Antibiotic resistance is most clearly a problem when it leads to therapeutic failure or the need to use more costly, toxic, or expensive drugs. It can also be a problem when it increases the frequency, duration, or severity of infection. According to Bischoff et al (2004), there are two principal concerns associated with an increase in antimicrobial resistance among enteric bacteria (includes, *Escherichia, Klebsiella, Enterobacter, Serratia, Citrobacter, Proteus, Pseudomonas & Salmonella*). One concern is that fewer tools will be available for the livestock producers to manage diseases due to antibiotic resistant pathogens. A second concern involves the potential transmission of antibiotic resistant bacteria to humans via the food chain. The healthy risks associated with animal health with respect to antibiotic resistance pathogens of animal origin are related to those of humans even though there is lack of knowledge on its magnitude. (Cassella, 2006). Antibiotic resistance exists in important animal pathogens, such as *Escherichia coli and Salmonella* and the occurrence of resistance vary widely as with pathogens that infect humans (McEwen, 2006). In a study by Jacks et al. (1981) on cephamycin C treatment to an induced swine salmonellosis, they observed that 25% of the 12 pigs orally medicated

with 300 mg of tetracycline died, and had diarrhea during 54% of the days of observation. Consumers are becoming more selective on agricultural and meat products due to knowledge that antibiotic resistance bacteria are transmitted through the food chain. Normanno et al., (2007) conducted a 3-year survey on the occurrence of *S. aureus* in meat and dairy products between 2003 and 2005, out of 1634 samples analyzed, 209 (12.8%) were contaminated with *S. aureus* and 68.8% of the strains expressed resistance to at least one of antibiotics tested. Mayrhofer et al., 2006 evaluated antimicrobial resistance in *Escherichia coli* isolated from retail meat of various animal species in Austria, where they recovered resistant isolates mostly in pork (76%) followed by poultry (63%) and beef (40%). During a one-year survey in Italy by Pesavento et al. (2005), 42 strains of *S. aureus* were isolated from 176 samples of raw meat (poultry, pork and beef). They challenged strains with twelve antimicrobials to check for antibiotic resistance and found that the strains were resistant to three or more antibiotics indicating the prevalence of multi-antibiotic resistance in the microorganisms. In a survey of antimicrobial resistance and virulence of *E.coli* in foods mostly sold at the market in Vietnam, *E coli* was present in 180 samples of raw meat, poultry and shellfish (Van et al., 2008). Out of the ninety-nine *E. coli* isolates recovered from all the sources, 84% of the isolates were resistant to one or more of the 15 antimicrobial agents used in the investigation (Van et al., 2008). In response to concerns about human resistance to antibiotics, some meat retailers have altered their meat-purchasing policies. In June 2003, McDonald's Corporation announced that it would prefer to buy meat from suppliers that use fewer medically important antibiotics for growth promotion (Osterberg and Wallinga, 2004). In December 2003, Bon Appétit, a company that offers food service to corporations, universities, and many clients in 21 states, announced its policy of preferential purchase of meat from producers who use fewer antibiotics of medical importance for any non therapeutic purposes (Osterberg et al 2004). Gilchrist et al (2007) suggested that more comprehensive and explicit product labeling should be implemented on antibiotic-free food and give consumers a chance to identify these products and make selections according to their

market value. Such improvements in labeling could be an integral part of an overall quality assurance program that would drive the producer to reduce antibiotic use. Ultimately, the lack of appropriate drugs, to control diseases, leads to economic loss for the producer due to death of farm animals, loss of productivity as well as loss of revenue due to public perception of the role the livestock industry in its contribution to antibiotic resistance. On the other hand, the welfare of the animals may be compromised when antibiotic use is reduced.

Antibiotics and the environment

Veterinary drugs are introduced into the environment through a number of ways like direct applications as in aquaculture, excretions of treated animals through application of manure and/or slurry to agricultural fields and through disposal of wastes during the production processes. Recent studies indicate that residual concentrations of some antibiotics also result in the development of antibiotic resistance in bacteria from small wild animals in proximity of swine farms exposed to these antibiotics through contact with animal feed through access into barns and feed storage areas than those in natural areas (Kozak, 2009). The increased level of resistance suggests that levels of antibiotics as low as those present in the environment may cause biological effect hence a risk for the selection of resistant bacteria (Kleiner et al. 2007).

There have been reports of the presence of drugs and their metabolites in the environment. For instance, low levels (<1 μg/L) of antibiotic residues have been observed in surface water samples collected from sites considered susceptible to contamination both in Germany and the US (Hirsch et al. 1999). The fate of antibiotics in the environment depends on their physico-chemical characteristics, soil and water properties. Tetracycline, an antibiotic often used in the swine industry tends to strongly adhere to soil particles hence cannot be detected in surface and groundwater samples, while sulfonamides are rapidly transported to surface waters. However, accumulation of these antibiotics in the environment depends on the rate of degradation in the different types of media. For instance, in cattle, poultry and swine slurry tylosin degrades much faster than

chlortetracycline, tilmicosin and lincomycin (Zilles et al., 2005). Although under the Clean Water Act, the federal government is mandated to regulate water quality associated with animal agriculture, the EPA admits that compliance to current Clean Water Act regulations has not been satisfactory and suggests that the regulations themselves need revision (Osterberg et al., 2004). The new rules concerning the largest CAFOs imposed by the EPA in early 2003 required these CAFOs to acquire permits for future water discharges. The quantity of pollutants discharged from the production area must be accompanied by an equivalent or greater reduction in the quantity of pollutants released to other media and/or land application areas for all manure, litter, and process wastewater at on-site and off-site locations. The final rule indicates that fecal coliforms do not exceed MPN of 400 per 100 mL at any time (USEPA, 2003). Non compliance to this rule attracts a penalty to the polluter. However, these rules do not address concerns over antibiotic residues since there are no set limits for residual antimicrobials levels present in the discharged effluent.

Antibiotic resistance and lagoon systems in agriculture

The development of antibiotic resistance

Antibiotic use in the swine industry is common due to their efficiency in reducing disease pressure and increasing swine production efficiency. These chemicals treat animal diseases (therapeutic) when used at high levels and prevent diseases when used at intermediate levels. Antibiotics are also used at low sub therapeutic levels in feeds to improve growth rate and feed utilization efficiency, reduce mortality and morbidity, and improve performance in reproduction (Cromwell, 2002). However, the long-term use of a single antibiotic (>10 days) selects for bacteria that are resistant not only to that antibiotic, but to several others within the same family (Levy and Marshall, 2004). According to Levy (1998), when an antibiotic is administered to a group of bacteria, the most susceptible cells will die while those that survive via mutation or exchange of genes will proliferate, especially if small amounts of the drug are given to kill the bacteria. The

selection for antibiotic resistance may occur in the normal microflora of swine, the lagoon and/or the soil environment. The most common mechanisms of resistance are those that enhance the production of enzymes that degrade the antibiotic (Wilke et al., 2005). Bacteria may also alter their cell surface and effectively reduce the affinity of a drug for its target site or develop antibiotic efflux mechanisms, which rapidly pump the antibiotic out of the cell before it has a chance to interfere with cellular processes (McAllister et al., 2001). Bacteria also produce specific enzymes which attach additional chemical structures to the antibiotic, hence rendering it inactive (e.g., O-phosphorylation of the antibiotic erythromycin) (Wilke et al., 2005).

The transfer of antibiotic resistant genes among bacteria

Propagation of resistant non pathogenic bacteria increases the reservoir of resistance traits in the overall bacterial population resulting in an increase in the probability of gene transfer to pathogens. Drug resistance genes may be spread from a bacterium to another via plasmids, bacteriophages, naked DNA or transposons (Levy and Marshall, 2004). Transposons may be simple or complex (contain integrons). These integrons have a site that integrates expression of different antibiotic resistance genes and other gene cassettes in proximity from a single promoter. Originally integrons were found among Gram-negative bacteria, but now they are also found in Gram-positive commensal flora (Levy 2004). Transformation is the uptake of free and naked DNA present in the environment (Ochman et.al, 2000). The lagoon environment makes it conducive to gene transfer for this type of gene transfer to occur after microbial die off. New genetic material may also be introduced into a bacterium by a bacteriophage via transduction. Bacteriophages package random DNA fragments or the DNA adjacent to the phage attachment site during infection. Conjugation is due to physical contact between bacterial cells. DNA is transferred from a donor to a recipient strain through a self-transmissible or mobilizable plasmid (Ochman et al, 2000).

The efficiency of anaerobic fermentation process for treatment of animal wastes

The fate of antibiotics in the environment depends on their physico-chemical characteristics, soil and water properties. Tetracyclines, tend to strongly adhere to soil particles while sulfonamides are rapidly transported to surface waters. However, accumulation of these antibiotics in the environment depends on the rate of degradation in the different types of media (Zilles et al. 2005). Fresh manure may contain up to 100 million fecal coliform bacteria per gram, as well as other microorganisms that can contaminate soil and water in high concentrations (Cochran et al, 2000). Adsorption of bacteria onto soil particles is a result of interception, sorption, and sedimentation (Guangming et al, 2005). The use of anaerobic fermentation ponds has been employed in the temperate regions of the USA mainly to reduce the levels of nitrates and organic matter to acceptable levels for application to crop land. Even though the digestion of wastes in surface storage anaerobic lagoons can reduce or destroy many pathogens effectively, reasonable numbers of microbial pathogens still remain in treated animal waste (Burkholder et al., 2007). However, the effectiveness of the fermentation process to kill pathogens is unknown. Reports from studies indicate that pathogens can persist in swine lagoon liquid and/or sludge, in manure piles, and in waste litter (Plym-Forshell 1995; Radtke and Gist 1989). Pathogens may survive better in systems that do not heat manure to a high temperature enough to kill pathogens, such as liquid or moist waste and in sludge or lagoon treatments. According to Hill and Sobsey (2003), standard swine waste lagoons could achieve reductions of enteric microbes of approximately 1 to 2 $\log_{10}$ per lagoon system for *Salmonella* and for most of the microbial indicators of fecal contamination. However, efficiency of anaerobic fermentation process for treatment of animal waste depends on season. The levels of *Salmonella* and enteric bacteria mostly increase in swine waste lagoon systems over the winter months as opposed to summer months (Hill and Sobsey, 2003). The pathogens that might be present in the final effluent may move into the ground water system. Some pathogens may be transported to surface waters in surface run-off during rainfall events.

The transport of microbes through soil and water

Lagoon effluent is an important soil fertility replenishment component in the mixed crop-livestock farming systems and has traditionally been used as an important source of nutrients, especially nitrogen (N), phosphorus (P) and potassium (K) as they improve crop yields considerably (Mafongoya, 2006). However lagoon effluent has the potential to introduce large amounts of microorganisms on the soil surface. Although soil can mitigate bacterial movement or leaching, some bacteria applied on to soil or released within the soil may still be transported through and travel in the vadose zone to groundwater (Guber et al., 2005). The application of animal wastes to saturated soils may cause contaminants to move into surface waters due to runoff and leaching through permeable soils to vulnerable aquifers. However, this may happen even at recommended application rates. Furthermore, concentrated livestock feeding operations located in areas prone to flooding with a shallow water table increases the potential for environmental contamination (Burkholder, 2007). When animal waste is applied to agricultural lands followed by surface runoff, over irrigation or rainfall, leaching may occur resulting in contamination of water resources by fecal bacteria (Entry et al., 2000). The rate at which bacteria can travel in soil depends on type and texture of soil, hydraulic conductivity, and degree of saturation and length of time. However, the rate may vary from a few meters to 830 m (Guber et al., 2005). According to McMurry et al. (1998), 1 cm h^{-1} of irrigation or rainfall water can transport bacteria in poultry manure to a depth of 32.5 cm in silt loam soil. They also reported that preferential flow can occur in well structured soils under unsaturated condition.

Microorganisms in the soil may move in the vertical or horizontal direction. The horizontal movement may occur when the soil is saturated or impermeable in nature (Mawdsley et al., 1995). A soil leaching experiment by Gagliardi and Karns (2000), demonstrated that *E. coli* O157:H7 in soil replicates and is transported in high numbers when water flows vertically through the soil. *Escherichia coli* is able to travel long distances underground due to its negative surface charge and relatively low die-off, hence

a useful indicator of fecal contamination in groundwater (Foppen and Schijven, 2005). Bacterial movement through soils is facilitated by flow of soil solution and mostly determined by effects of advection and dispersion. *Escherichia coli* are somewhat attached to manure at slow flow velocities than at higher flow velocity (Guber et al., 2005). The retention of bacteria on soil particles is caused by interception, sorption, and sedimentation (Guangming et al., 2005). The transport of microorganisms may be limited by the pore size and the size of the microorganism, however, a certain percentage of heterogeneous grain size distribution of pore diameters can interfere with bacteria transport ie ≥10% in sand (Pekdeger and Matthess, 1983). Sedimentation may also indirectly affect microbial transport; bacteria attach themselves actively and irreversibly on the surfaces of solid particles. According to Pekdeger and Matthess (1983), the adsorption of viruses and bacteria is quite fast (i.e. within 2h and 24h, respectively) however, their desorption rates are unknown. The amount of organic matter in the soil has more influence on the adsorption of microorganisms to soil particles. Guber et al. (2005) reported that *E.coli* attachment to soil decreased with increased manure content probably due to increased competition for attachment sites. The survival time of bacteria and viruses in ground water varies with species and environment. Microorganisms are impacted by the effect of physical, biological and chemical conditions in the environment. However biological factors such as bacteriaophages and protolytic bacteria are the most important for survival of pathogenic bacteria and viruses (Pekdeger and Matthess, 1983).

Prevalence of antibiotic resistant genes in the environmental pools

Swine production in North Carolina is concentrated in a small geographical area. The intensive livestock production poses a concern because bacteria with antibiotic resistant genes mostly believed to be the causative agents for human diseases such as Pneumonia (*acinetobacter baumannaii*) and Tuberculosis (*Mycobacterium tuberculosis* (XDR-TB) are associated with swine (CDC, 1998). Manure management or the lack thereof can contribute potential environmental problems.

In 1998, the Centers for Disease Control and Prevention (CDC) investigated pathogen levels in ground and surface waters near large-scale hog operations in the state of Iowa and found that pathogen levels were highest in samples collected from anaerobic lagoons, 87% out of 23 samples, tested positive for *Enterococcus*, 78% for *E. coli*, and 9% for *Salmonella*. In addition, many of the pathogen isolates demonstrated antibiotic resistance (CDC, 2000). In a recent study by microbiologists from the University of Illinois, samples from soil and groundwater under two hog farms were analyzed in the state of Illinois. The results from this study indicated that bacteria in the soil and groundwater of these farms had tetracycline resistant genes related to bacteria from pigs' guts that were present in lagoon. The result showed that people at both sites were drinking the affected groundwater. This study concluded that the presence of these genes may contribute to antibiotic resistance and the problem may be widespread (Ananthaswamy, 2001). A study was carried out North Carolina to quantify the magnitude of antibiotic-resistant *E. coli* transport from swine farms into groundwater. Two swine farms and two control crop farms with known groundwater flow paths were evaluated for *E. coli*. The results of this study demonstrate that antibiotic-resistant *E. coli* strains are present in groundwater of swine farms with a typical lagoon and land application system for waste management (Anderson, 2006). Some monitoring studies recently have indicated the possibility of pollution in groundwater through seepage from waste lagoons (Krapac et al., 1998). However, this may occur if the lagoons are not properly lined. Krapac and coworkers found indicators like *enterococci* of fecal origin at high levels in Illinois groundwater samples obtained up to 100 m downstream of swine waste lagoons. These indicators were present over a period of years indicating the occurrence long-term impacts and environmental movement of contaminants (Krapac et al., 1998; Krapac et al., 2000).

Koike et al. (2006) monitored the distribution of resistance genes into the environment in Illinois. They determined the occurrence of tetracycline resistance genes in groundwater beneath two swine confinement operations. Elevated concentrations of tetracycline genes

were observed in the wells located down-gradient of the lagoon. Comparative analysis showed that the affected groundwater had gene sequences closely related to those in the lagoon by 99.8%, however these genes were not present in background libraries. New clones and unique native resistance gene populations were also present in the groundwater. Meaning that antibiotic resistance genes in groundwater are affected by swine manure, but they were related to the native gene population. According to Guerra et al. (2003), Lanz et al. (2003) and Boerlin et al. (2005), resistances to tetracycline, sulfonamides and streptomycin or spectinomycin are the most common among *E. coli*. Several studies have recently attempted to assess the dissemination of the resistance genes for the above named major antibiotics in *E. coli* populations of animal origin (Bischoff et al., 2005, Bryan et al., 2004). Much more research remains to be done to draw valid comparisons between *E. coli* isolates from different animal populations. For example, ongoing research conducted in Dr. Graves' laboratory showed that molecular fingerprints and the phenotypic antibiotic resistance fingerprints of swine and cattle differ significantly (paper in preparation). Such differences may be attributed to difference in antibiotic use, the clonal nature of some *E. coli*, as well as sampling bias.

Association of antibiotic resistance genes and phenotypic expression of antibiotic resistance

The fate of antibiotics in the environment and their link to the emergence of resistant genotypes found in the environment has received limited research attention. Evaluation of the impact of antibiotic use on populations of bacteria in natural waters has been the main focus of recent studies (Goni-Urriza et al., 2000). Antibiotic resistance analysis has been used to identify sources of fecal pollution (Graves et al., 2002, Hagedorn et al., 1999; Harwood et al., 2000). Phenotypic and genotypic are the two major categories in microbial source tracking. Traditional cultivation and phenotypic testing is still relied upon to determine the representative phenotypes in the environment. This method has been used with success by Parveen et al. (1999) and Carson et al. (2001), who were able to correctly classify 97 and 95% of animal, and 67 and 99% of human-derived isolates

respectively. These methods require cultivation and depend on the phenotypic expression of resistance. The use of techniques such as PCR and molecular gene probe analysis have emerged and allow sensitive detection of specific genes in the environment in the absence of cultivation (Chee-Sanford et al, 2001). The collection of genes used for such analyses has been extended to specific metabolic genes (Flanagan et al., 1999) and, recently, to antibiotic resistance genes (Aminov et al., 2001). The connection between the presence of an antibiotic resistant gene and the phenotypic expression of that gene has not been rigorously investigated. Hence, a major research objective described in this thesis was to evaluate of the occurrence of antibiotic resistant genes in *E.coli* and their phenotypic expression of antibiotic resistance. An additional objective was to evaluate the use of antibiotic resistance analysis (ARA), phenotypic resistance and antibiotic resistant genes (ARGs), genotypic indication of resistance, to identify sources of fecal pollution of ground and surface water.

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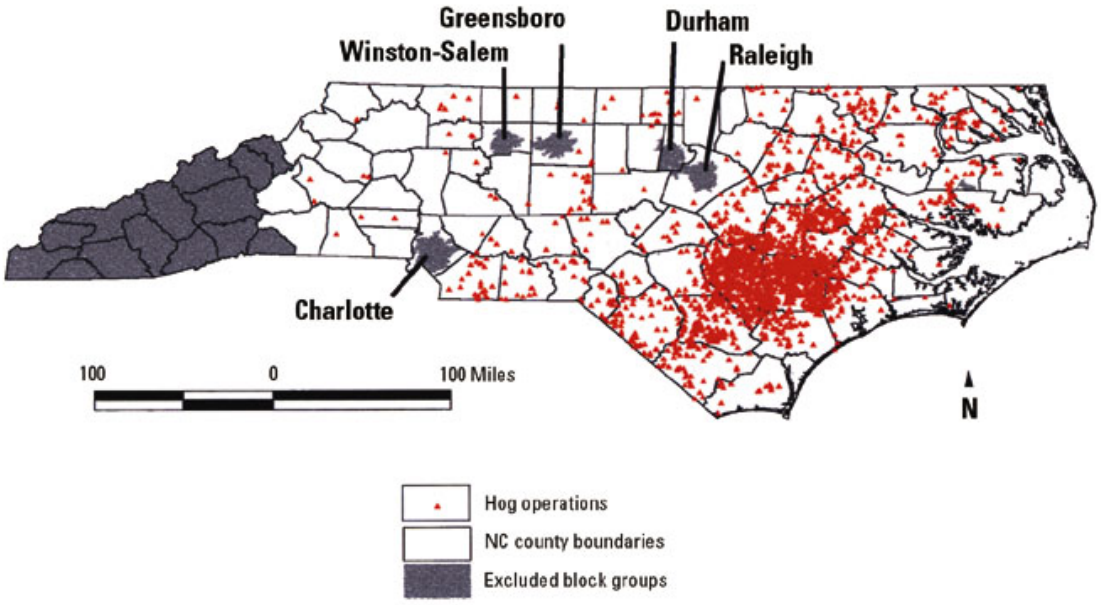

Figure 1.1. Map of North Carolina showing the concentration of swine facilities in south eastern part of the state (www.ehponline.org).

CHAPTER 2

A SURVEY OF ANTIBIOTIC RESISTANCE GENES IN *ESCHERICHIA COLI* RECOVERED FROM SWINE FECES, SWINE LAGOON LIQUID, AND SOIL FROM SPRAY FIELDS

ABSTRACT

Antibiotic resistant bacteria may be released into the environment through land application of swine lagoon effluent, resulting in the potential increase of antibiotic resistant bacteria in environmental pools. The goals of this study were 1) to characterize ten different types of antibiotic resistant genes in *E. coli* isolates from swine feces, swine lagoon effluent and soils that received lagoon effluent applications 2) to evaluate how the level of ARGs vary with season in lagoon effluent 3) to compare the presences of ARGs with the actual phenotypic expression of the resistance. The 10 ARGs were *aadA, strA, strB, tetA, tetB, tetC, sul1, sul2, sul3,* and *Aac(3)IV* were evaluated using polymerase chain reaction (PCR). Phenotypic expression of 616 *E. coli* isolates from swine feces, lagoon effluent, and soil in the application field were evaluated against battery of 9 antibiotics. Each of the 10 ARGs was detected with significant differences among the sources. The percentage of *E.coli* with *sul3* (23.9%) and *Aac(3)IV* (32.1%) genes recovered from the lagoon effluent were greater while *tetA* (85%) and *tetC* (20.9%) were lower than in *E.coli* recovered from swine feces. *E.coli* that possessed the *aadA* (97.2%), *tetA* (93.8%), *tetB* (82.6%) and *tetC* (34%) genes were greater whereas, those that possessed *sul2* (31.3%), *sul3* (2.1%) and *Aac(3)IV* (6.3%) genes in soil were lower than from lagoon effluent. The percentage of *E.coli* that possessed *aadA* (93.4%), *strA* (76.3%), *strB* (72.4%), *tetC* (13.2%), *sul2* (67.1%), *sul3* (31.6%) and *Aac(3)IV* (63.2%) genes in the lagoon effluent were greater during winter, than in fall while *tetA* (65.8%) was lower than in fall. In spring, *tetA* (96.7%), *tetC* (63.3%) and *sul3* (76.7%) were greater than in winter. In summer percentage of isolates with *strA* (96.7%), *strB* (100%) and *sul1* (90%) genes were greater than in spring while percentage of isolates with *sul2*

(16.7%) and *sul3* (13.3%) were lower in winter than in spring and no *Aac(3)IV* genes were recovered during summer. Thirty eight percent, 8% and 84% of *E.coli* isolates from swine feces, lagoon effluent, and soil, respectively, demonstrated the phenotypic expression of antibiotic resistance in the absence of the associated ARG. The results from this study suggest that environmental conditions in the lagoon and soil may have an impact on the survival and expression of resistance by *E.coli* composed of specific ARGs or that these isolates may have a gene that codes for resistance to a given antibiotic resistant gene not accounted for by these primers.

INTRODUCTION

Antibiotic resistance enables a microorganism to withstand the effects of antimicrobials and is a worldwide problem well known in both human and veterinary medicine (Maynard et al, 2003; Teuber, 2001). The use of antibiotics in livestock may be suspected as one of the routes for the transfer of antibiotic resistant bacteria to humans, even though antibiotics used for animals differ from those in humans (Khachatourians, 1998). About half of all antibiotics used in the United States are for animal production, only 10% of these drugs are given to treat infectious disease; the remaining 90% are given to promote growth, reduce mortality and morbidity, and improve reproductive performance (Khachatourians, 1998; Cromwell, 2002).

The long-term use of a single antibiotic (>10 days) selects for bacteria that are resistant not only to that antibiotic, but to several others within the same family (Levy and Marshall, 2004). According to Levy (1998), when an antibiotic is administered to a population of bacteria, the most susceptible will die while those that survive via mutation or gene exchange will proliferate, especially if too little of the antibiotic is given to kill the bacteria. The selection for antibiotic resistance may occur in the normal microflora of monogastric animals such as swine or in the lagoons and/or soil environment.

Antibiotic resistance genes may be spread from one bacterium to another by different mechanisms such as plasmids, bacteriophages, naked DNA or transposons (Levy and Marshall**,** 2004). This is a major concern when these genes are transferred to pathogens.

The threat of diseases caused by antibiotic resistant pathogens is enormous because antimicrobial drugs used for human therapy often have significant structural similarities to those used for animals. For example, resistance to apramycin, a veterinary medicine, might foster resistance to gentamycin, a structurally similar compound that is specified for use in humans (Mathew et al., 2003). The increased level of resistance has raised concerns since the rate at which drugs are being developed is much slower than the rate at which the current drugs are being rendered ineffective. Many antibiotics used in animal agriculture are not readily absorbed in the animal gut. Approximately 25% to as much as 75% of antibiotics given to stall-feed animals could be released undigested in feces (Edmund et al., 1971; Feinman and Matheson, 1978) and can persist in soil after land application (Donoho, 1984; Gavalchin and Katz, 1994), typically by spraying lagoon effluent to fields. The annual production of livestock and poultry waste in the United States exceeds 200 million tons (dry weight basis) (Aillery et al., 2006), this waste may potentially introduce into the environment an enormous amount of antibiotic residues and antibiotic-resistant bacteria.

Anaerobic lagoons used for treatment of animal wastes are not designed to control bacteria although they can effectively reduce or destroy many pathogens. According to Hill and Sobsey (2003), standard swine waste lagoons could achieve reductions of enteric microbes of approximately 1 to 2 $\log_{10}$ per lagoon system for *Salmonella* and for most of the microbial indicators of fecal contamination. However, the efficiency of anaerobic fermentation process depends on the season since the concentrations of *Salmonella* and enteric microbial indicators tend to increase in swine waste lagoon systems during winter months than during summer months (Hill and Sobsey, 2003). Substantial densities of microbial pathogens still remain in this type of waste (Burkholder et al 2007). Studies have shown that pathogens can persist in swine lagoon liquid and sludge, manure piles, and waste litter (Plym-Forshell 1995; Radtke and Gist 1989). According to Himathongkham et al., 1999, *E. coli* can survive for 27-60 days at 4, 20 or 37^{o}C in slurry or liquid and 49–56 days at warm temperatures (20–37°C) in manure. While Jiang et al.,

2002 and Kudva et al. 1998 found that *E. coli* survived up to 56 and 99 days in manure, respectively.

Microbiologists from University of Illinois discovered that bacteria in the soil and groundwater near swine farms had tetracycline resistant genes. These bacteria were traced to pigs affiliated with the swine farms and the microbiologists speculated that movement of antibiotic resistance genes might contribute to widespread antibiotic resistance in the environment (Ananthaswamy, 2001). Several recent studies have attempted to evaluate the prevalence of the resistance genes for major antibiotics in *E. coli* populations of from animals (Bischoff et al., 2005, Bryan et al., 2004). The use of techniques such as PCR and molecular gene probe analysis have emerged and allow sensitive detection of specific genes in the environment in the absence of cultivation (Chee-Sanford et al, 2001). The collection of genes used for such analyses has been extended to specific metabolic genes (Flanagan et al., 1999) and, recently, to antibiotic resistance genes (Aminov et al., 2001).

The connection between the presence of an antibiotic resistant gene and the phenotypic expression of that gene has not been rigorously investigated. We hypothesize that the presence of a gene does not automatically indicate expression, but that the presence of given antibiotic resistant gene should be correlated with the phenotypic expression of resistance to that antibiotic. The objectives of the study were, 1) to identify antibiotic resistant genes in *E. coli* isolates from swine feces, swine lagoon effluent and from soils treated with lagoon effluent, 2) to evaluate the effect of season on the recovery of *E.coli* with ARGs from lagoon effluent and from soil before and after application of lagoon effluent and 3) to determine the relationship between the presence of antibiotic resistant genes and the phenotypic expression of the resistance.

MATERIALS AND METHODS

*Site Description***.** The study was conducted on a commercial swine farm in Sampson County, North Carolina with a herd of 4400 hogs (finishing 720-725 hogs per house) and 100 cattle, located in a 275 ha watershed along the upper part of Six Runs Creek. The site has two waste application fields and a stream which flows in a southerly direction. Adjacent to waste application field 1, the stream flows in a channel, while the segment adjacent to waste application field 2 is impounded by two beaver dams and forms an elongated pond. The stream flows in a channel below the lower beaver dam. The watershed has four swine operations with 23 swine houses (Fig 2.1). Swine feces from the swine houses were washed into the waste lagoon once per week. Water levels were maintained at three quarters full to allow storage of excess rain water and to create anaerobic conditions in the lower portion of the lagoon to maintain compliance with waste management regulations. Lagoon effluent was sprayed on to farmland cropped with bermuda grass managed for hay and grazing pasture for cattle. On average, spray events occurs 3 to 4 times during the warm season, once in late fall and once in early spring. Soil in the waste application field (WAF2) is a Wagram series (loamy, kaolinitic, thermic Arenic Kandiudults) (Fig. 2.1) (Israel et al., 2005).

Sample collection. Sampling of swine feces and lagoon effluent was conducted between October 2007 and March 2008, while soil samples were collected in September 2008. Samples of fresh swine feces (20) were collected from swine houses and lagoon effluent (13) from the swine lagoon. Six soil core samples were collected from the application field (WAF2, Fig 2.1) immediately before application, immediately after application and one day after the spray event. A total of 1104 *E. coli* isolates from the 3 sources which included 576 from swine feces, 384 from lagoon effluent and 144 from the soil were obtained from all the samples.

Isolation of E.coli. The soil core samples (10 cm) collected from the application field was divided into, top samples (0-3 cm) and lower samples (3-7 cm). A 10 ml aliquot of effluent sample or 10 g of fecal and soil samples were enriched in 90 ml of

BHI broth (EMD chemicals Inc., Gibbstown, Nj) for 24 h at 37° C to allow *E.coli* to recover from environmental stress prior to molecular characterization. Another 10 g of soil sample was diluted in 100 ml of sterile deionized water. One ml of each sample was then diluted into 9 ml of sterile water (10^{-1} dilution) and then serial dilutions of 10^{-2}-10^{-6} were made. A 100 µl aliquot of the diluted samples was cultured on m-FC agar (Becton Dickinson, sparks, Md.) and incubated at 44.5^{o} C for 24 h to obtain single colonies (Hurst and Crawford, 2002). Single dark blue colonies presumed as fecal coliforms were then transferred using a tooth pick to microwells with colilert broth and cultured for 24 h. Five µl from each microwell was transferred to a vial containing 1 ml of pre-enrichment broth (Becton Dickinson, sparks, Md.), mixed, incubated for 24 h at 37 ^{o} C and stored at 4 ^{o} C.

Antibiotic resistance analysis. Antibiotic resistance analysis (ARA) was performed to evaluate the expression of antibiotic resistance in *E.coli* collected from the swine feces, lagoon effluent and soil. Antibiotic resistance patterns of each *E.coli* isolate was comprised of 9 observations. A 48-prong replicator (Sigma) was used to transfer isolates onto 100 mm diameter tryptic soy agar plates amended with the following concentrations of antibiotics: Erythromycin, 100 µg/l; Neomycin, 15 µg/l; Oxytetracycline, Streptomycin and Tetracycline, 90 µg/l; Rifampicin, 25 µg/l; Cephalothin, 25 µg/l; Apramycin and Trimethoprim, 128 µg/l. Antibiotics stocks were prepared as described in Graves et al. (2002), Hagedorn et al.(1999) and Harwood et al. (2000). The plates were incubated at 37^{o} C for 18 to 20 h. The antibiotic and their concentrations were selected based on previous ARA studies and their common use in human and veterinary medicine (Mathew et al., 1999). Resistance to an antibiotic was determined by comparing growth of each isolate to the same isolate on the control plate. A one (1) was recorded for a round, mostly filled colony and a zero (0) for no growth. Each isolate was tested against the 9 antibiotics.

Genetic analysis. PCR assays were performed to determine which antibiotic resistant genes were present in antibiotic resistant *E.coli* isolates. Primers used for the

amplification of the antibiotic resistance genes were based on previous research (Boerlin et al., 2005; Lanz et al., 2003; Perreten and Boerlin, 2003) (Table 2.1). Isolates resistant to two or more antibiotics were randomly selected from each source and analyzed for presence of antibiotic resistant genes (ARGs) by polymerase chain reaction (PCR). PCR was conducted on a subset of 238 isolates from swine feces, 234 from lagoon effluent, and 144 from soil.

A loopful of bacteria was resuspended in 500 µl sterile water, homogenized and heated at 95 °C for 15 minutes to prepare a lysate. The mixture was cooled to room temperature and centrifuged for 3 minutes at 14000 rpm. A 5 µl volume of supernatant was then transferred to each microwell and used as a template for each 25 µl PCR mixture. Each isolate was evaluated by each of the 10 primer sets (Table 2.1). The PCR mixture was prepared in a reservoir from a 1300 µl aliquot of sybergreen master mix, 600 µl of nuclease free water, and 192 µl of primer mixture. The primer mixture was made from 24 µl (working solution) of forward primer to the same quantity of a corresponding reverse primer to produce a single primer set for a particular antibiotic. An aliquot of 48 µl for the four primer sets were then combined into a primer mixture. Twenty microlitres of the final PCR mix was dispersed into each microwell using a multichannel pipettor. The PCR microwells were capped and placed in a thermocycler for analysis. The following temperature cycles were used to run the PCR : 1 cycle of 4 min at 95 °C; 35 cycles, each consisting of 1 min at 95 °C, 1 cycle at annealing temperature followed by 1 min at 72 °C; and 1 cycle of 7 min at 72 °C. Quantifast SYBR Green PCR Kit (Qiagen, Valencia, CA) was used to detect the amplified product. A negative control (no *E.coli*) of 5 µl water was included in all PCR assays.

Statistical analysis. The percentage contribution of swine feces, lagoon effluent and soil to the total number of ARGs in this system and the percentage of each type of ARG in each source was calculated using Microsoft office Excel, 2007. SAS statistical software (8.0., SAS Inst., Cary, NC) was used to determine the effect of season and treatment/conditions represented by the different sources on the presence of resistant

genes in the isolates. Non parametric savage 1-way Anova (logistic) analysis was used as the data was a binary categorical response (1 or 0 response). This method was chosen because the antibiotic resistant gene in the *E.coli* isolates is a random characteristic and was not normally distributed.

RESULTS

Numbers of E.coli. Swine feces, lagoon effluent and soil had mean concentrations of *E.coli* at $5.5*10^6$ CFU/g and $1.4*10^7$ CFU/ml and $1.8*10^3$ CFU/g respectively (Table 2.2 and Table 2.3). The soil mean is an overall mean for samples collected before, immediately after and 1day after spraying. Prior to lagoon effluent application, the soil had a mean concentration of $3.8*10^2$ CFU/g. The mean concentration of *E.coli* in the soil immediately after application was $1.8*10^3$ CFU/g, whereas the mean concentration of *E.coli* the day after application was $3.3*10^3$ CFU/g. (Table 2.3)

Antibiotic resistant genes. All 144 *E.coli* isolates from the soil had more than one ARG present and ≤2% had only two ARGs. On average, 1% of the soil isolates from the swine feces (n=238) and 2% of isolates from lagoon effluent (n=234) had only 1 ARG. Three and 6 % of the isolates from swine feces and lagoon effluent had only two ARGs, respectively. Whereas, 96% of isolates from swine feces, 92% isolates from lagoon effluent and 98% of isolates from soil had three or more ARGs (Table 2.4).

The prevalence of the combined total of all 10 ARGs among the three sources (swine feces, lagoon effluent and soil) of *E.coli* was assessed. Based on a tukey's test, there were no significant differences ($P < 0.05$) in the number of ARGs among any of the sources (Table 2.5). However, significant differences were indicated based on the evaluation of individual genes among the three sources. The *aadA* and *tetA* genes were found in ≥90% of the isolates and the occurrence of these two genes were significantly greater than the occurrence of *tetC, sul2*, *sul3* and *Aac(3)IV* (14.3-36.3%). There were no significant differences in the occurrence of *aadA, tetA, strA, strB, tetB* and *sul1* genes. Among all the ARGs, those that code for streptomycin/spectinomycin (*aadA/strA* and *strB*), tetracycline

(*tetA* and *tetB*) and sulfonamide (*sul1*) were most common among these isolates (Table 2.5).

Three paired comparisons including swine feces-lagoon effluent, lagoon effluent-soil and swine feces-soil were performed to assess the impact of treatment/soil conditions on the occurrence of AGRs in these sources. Based on the comparisons of isolates from swine feces and lagoon effluent, genes coding for *sul3* (23.9%) and *Aac(3)IV* (32.1%) were significantly greater in *E.coli* isolated from lagoon effluent as compared to those from swine feces (16.8%) and (16.8%) respectively. While the genes coding for *tetA* (85%) and *tetC* (20.9%) were significantly lower in *E.coli* recovered from the lagoon effluent than swine feces (94.5%) and (39.9%) respectively. There were no significant differences among the other types of genes (Table 2.5).

The isolates from the soil had significantly greater numbers of genes coding for *aadA, tetA, tetB* and *tetC* (34.0 -97.2%) and significantly lower numbers of *sul2, sul3* and *Aac(3)IV* (2.1-31.3%) as compared to those in the lagoon effluent (20.9-90.6%) and (23.9-41.5%), respectively. However there were no significant differences among the other types of genes between these sources. Furthermore, soil isolates had significantly greater numbers of genes coding for *aadA,* and *tetB* (82.6-97.2%) and significantly lower numbers of *strA, sul3* and *Aac(3)IV* (2.1-54.9%) when compared to those in the swine feces, (46.6-86.1%) and (16.8-67.6%), respectively. There were no differences among other types of genes between these sources (Table 2.5).

ARGs in soil before and after a spray event. The type of ARGs recovered from soil isolates collected before, immediately after and 1 day after spraying the field with lagoon effluent was evaluated (Table 2.6). The isolates collected immediately after spraying, indicated a significant lower percentage of *strB* (36.7%) and *sul1* (46.7%) as opposed to the percentages prior to spraying (83.8%) and (73.8%), respectively. There were no significant differences in the occurrence of the other ARGs (Table 2.6). The isolates collected 1 day after spraying the field had a significantly greater incidence of *tetB* (91.2%) and *sul1* (82.4%) than immediately after spraying the field (70%) and (46.7%),

respectively and a significantly lower incidence of *tetA* (85.3%) than immediately after spraying the field (100%) (Table 2.6).

ARGs based on seasons. The effect of season was also evaluated on the presence of ARGs in the lagoon effluent. When the season changed from fall (October) to winter (December and January), all the types of ARGs except *tetB* were affected. The recovery of the genes *aadA, strA, strB, tetC, sul2, sul3* and *Aac(3)IV* were significantly greater from (2.0 - 82.6%) to (13.2 - 93.4%) in winter while the recovery of *tetA* was significantly lower from 94.9 to 65.8%. The change from winter to spring (March) only affected *tetA, tetC* and *sul3* with a significantly greater recovery in spring from (13.1 - 67.8%) to (63.3 - 96.7%). The shift from spring into summer (July) indicated a significantly greater recovery of *strA, strB* and *sul1* genes from (66.6 - 80.0%) to (90.0 - 100%), while the recovery of *sul2* and *sul3* were significantly lower from (31.6 - 83.0%) to (13.3 - 16.7%). No *Aac(3)IV* genes were recovered during summer (Table 2.7).

Phenotypic expression of resistance to antibiotics for the *E.coli* isolates from the three sources was evaluated and compared to the genotypic response in the analysis for the presence of resistant genes. The types of antibiotic resistant genes were grouped into four categories based on how they relate to specific antibiotics to which they confer resistance. The four categories include tetracyclines, sulphonamides, streptomycin/spectinomycin and apramycin/neomycin. A positive response indicates growth of a round, mostly filled colony of an isolate on a test media plate while a negative response indicates no growth or partially filled colony of an isolate on the test media plate. Forty seven percent and 36% of the 238 isolates from swine feces had *sul* and *aadA/str* resistance genes, respectively, but displayed a negative phenotypic response to trimethoprim and streptomycin/spectinomycin. While only 4 and 1% of the swine feces isolates had *tet* and *Aac(3)IV* resistant genes, respectively but displayed a negative response to tetracycline and apramycin. In contrast, 35, 3 and <1% of the 238 swine feces isolates displayed a positive phenotypic response to apramycin, trimethoprim, and tetracycline respectively but did not have the associated resistant genes. There were no

swine feces isolates that had a positive phenotypic expression to streptomycin and possessed the *aadA* or *str* genes.

Fifty three percent, 49% and 19% of the 234 lagoon effluent isolates had the *sul*, *Aac(3)IV* and *aadA* or *str* genes, respectively, but displayed a negative phenotypic response to trimethoprim, apramycin and streptomycin antibiotics. While <1% of the lagoon effluent isolates had the *tet* gene but displayed a negative phenotypic response to tetracycline. On the contrary, 6% of lagoon effluent isolates displayed a positive phenotypic response to apramycin and 2% to tetracycline antibiotics but did not have the associated *Aac(3)IV* and *tet* genes respectively. There was no lagoon effluent isolate which displayed a positive phenotypic response to streptomycin and trimethoprim and possessed *aadA* or *str* and *sul* genes.

Eighty three percent, 42% and 39% of the soil isolates had the *sul*, *tet* and *aadA* or *str* genes, respectively, but displayed a negative phenotypic response to trimethoprim, tetracycline and streptomycin antibiotics. There was no soil isolate which had the *Aac(3)IV* gene, and displayed a negative response to apramycin. Eighty four percent of isolates from soil that displayed a positive phenotypic response to apramycin antibiotic did not have the *Aac(3)IV* gene. There was no soil isolate which displayed a positive phenotypic response to spectinomycin/streptomycin, tetracycline and trimethoprim without possessing the *aadA* or *str, tet* and *sul* genes (Table 2.8).

DISCUSSION

The average number of *E.coli* was 3 to 4 log numbers lower in soil (1.8×10^3 CFU/g) than in swine feces (5.5×10^6 CFU/g) and lagoon effluent (1.4×10^7 CFU/mL). However statistical analysis was not performed. Stoddard et al. (1998) found *E.coli* counts in soil that were impacted by cattle to be 3.0×10^3 CFU/g of soil whereas, Hu et al. (2008) found the numbers of *E.coli* in surface water sediments impacted by livestock to be 1.7×10^4 CFU/g of sediments in summer and 3.0×10^3 CFU/g of sediments in winter. Chandler, et al. (1981) reported fecal coliform averages of 3.3×10^3 CFU/g of soil receiving swine lagoon effluent. Duriez and Topp (2007) reported *E.coli* averages of 1.2×10^4 CFU/g in

swine effluent. Whereas Boes et al. (2005) reported *E.coli* averages of 3log of soil. The results from the other studies are not different from this study.

As indicated by the prevalence of antibiotic resistant genes among the isolates in the three sources (swine feces, lagoon effluent and soil), genes which code for spectinomycin, streptomycin, tetracycline and sulfonamide (*aadA, str, sul* and *tet*) resistance were the most common. Spectinomycin, streptomycin, tetracycline and sulfur drugs are more commonly used in both swine and cattle than the other antibiotics (Chee-Sanford, 2009). The more frequent occurrence of these ARGs in the *E.coli* isolates from these sources may be an indication that spectinomycin, streptomycin, tetracycline and sulfonamide antibiotics are being used at this farm.

Kozak et al. (2008), Guerra et al. (2003) and Duriez and Topp (2007) reported the prevalence of 83%, 60% and 84% for ARGs in *E.coli* isolated from swine feces respectively. Data on ARGs in *E.coli* isolated from lagoon effluent and soil is not readily available. The findings from these studies are different from ours because the prevalence is based on the number and types of ARGs evaluated. In this study, we evaluated 10 genes while most researchers evaluated 3-6 genes. Variations of specific types of ARGs in each source were evaluated based on treatment (fermentation lagoon) and environmental conditions (soil). The comparison of genes in these sources suggests that the fermentation process in the anaerobic lagoon system and the different environmental conditions that exist within the sources may have an impact on the ARGs possessed by the *E.coli* isolates. The overall observation that the genes coding for *sul3* and *Aac(3)IV* were recovered in significantly greater numbers of *E.coli* isolated from lagoon effluent than those from swine feces indicates that the fermentation process had no negative effect on *E.coli* that possessed these types of genes or even selected against isolates that did not have these genes. Guerra et al. (2003) and Hammerum et al. (2006) found 14% and 11% of *sul3* genes respectively in *E.coli* isolates from swine feces. Guerra et al. (2003) found 60% *Aac(3)IV* genes in *E.coli* isolates from both human and swine. However, we could not find data on the prevalence of these genes in lagoon effluent. The findings for sul3 by

other researchers were similar to our findings while those of *Aac(3)IV,* were different since the isolates were not reported separately but a mixture of source. Our findings may suggest that *E.coli* that harbors these particular ARGs may be more resistant to degradation by the fermentation process, hence continues to multiply under these lagoon conditions.

The overall observation that genes coding for *tetA* and *tetC* in *E.coli* were recovered in significantly lower numbers from the lagoon effluent as compared to swine feces may suggest that *E.coli* that possessed these genes were more sensitive to the fermentation process. Jindal et al. 2006 found <1% of *tet*A and *tetC* genes in *E.coli* from lagoon effluent. Zhang et al. (2009) in their waste water treatment experiment removed 97.7% of *tet*A and 98.3% of *tetC* genes with activated sludge. In a manure study, both *tet*A and *tetC* genes were 4.32 log in composted manure and 8.4 log in untreated swine manure (Yu et al., 2005). These findings are similar to this study in that these studies show that these genes are vulnerable to degradation or easily removed under biochemically active environments.

The increase in the number of *aadA, tetA, tetB* and *tetC* genes in *E.coli* recovered from the soil may suggest that the environmental conditions in the soil that promote the survival and replication of *E.coli* that possessed these genes are favorable. Jindal et al. (2006) found <1% of *tet*A and *tetC* genes in *E.coli* from soil samples collected twice between Dec.-Jan. and April-June. Srinivasan et al. (2008) found *aadA* 5.4%, 21.4% *tet*A, 5.4% *tetB* and *tetC* 0% genes in *E.coli* from dairy farm soil samples collected 3 days after a spray event. These findings are different from those obtained in our study in that our site had two types of manure (lagoon effluent and cattle) entering the soil and samples were collected only once 1 day after a spray event, there might be an effect of sampling period.

In the case of seasonal effects, the cool season might be the period when the genes are most stable due to dormancy of the microorganisms. At low temperatures, microbial growth and multiplication slows down, hence the transfer of these genes may also be reduced. In lagoon effluent, the decrease of *tetA* in winter from fall *sul2* and *sul3* and

Aac(3)IV in summer from spring suggest that *E.coli* that harbor genes may be sensitive to cool and elevated temperatures. In lagoon effluent, the increase in *tetA, tetC* and *sul3* in spring from winter may be associated with warmer temperatures, high microbial activity and multiplication which may increase gene transfer. In addition to temperature sensitivities, the recovery of various ARGs in *E.coli* may be a reflection of the initial numbers of antibiotic resistant *E.coli* that have been shed by the animals. The differences may also be associated with mobile genetic elements.

Sunde and Norström (2005) indicated that the presence of *aadA* gene cassettes affect the accuracy in classifying *E.coli* resistance to streptomycin. This is because distributions of integrons having the aadA cassettes interfere with the determination of an epidemiological cut-off value during surveillance because increased levels of *aadA*. The gene *aadA,* which codes for resistance to spectinomycin, may also cause low-level resistance to streptomycin. This may be the major source of resistance to streptomycin resistance observed in the soil since *strA* and *strB* are present at relatively low levels. The observation that tetracycline resistant genes (*tetA* and *tetB*) were at elevated levels and sulfonamide resistant genes (*sul2* and *sul3*) were at lower levels in the soil is in agreement with the findings by other researchers. Byrne-Bailey et al., (2008) found 23 % of *sul1*, 18 % *sul2* and 9 % *sul3* in *E.coli* isolated from manured agricultural soils. Tetracycline tends to strongly adhere to soil particles while sulfonamides are rapidly transported to surface waters (Zilles et al. 2005), as a result the accumulation of tetracycline in the soil may also be inducing resistance in the *E.coli* isolated from the soil. These results are in agreement with Lanz et al., (2003) and Sengolov et al., (2003) who found high levels of *tetA* (87% and 71%) while Marshall et al., (1983) and Levy et al., (1985) found high levels of *tetB* (76% and 82%) resistant genes in swine feces isolates.

Class 1 integrons are the most frequently detected among *Enterobacteriacea* (Guerra et al., 2003). Intergrons are gene expression systems that incorporate gene cassettes and make them functional. *Sul1* is an example of a class 1 intergron and may be an indicator of why *sul1* was present at high levels in this study.

The results from this study suggest that the phenotypic expression to resistance is not always correlated with genetic presence of an ARG. Generally, because of these differences, it may not be appropriate to predict phenotypes by only using occurrence of ARGS. According to Bochner (2003), it is not known how the biology of the cell or the whole organism is affected by catalytic or regulatory activity. It is difficult to define a single phenotype when several genes are present in that organism since these may also affect gene expression; nevertheless it's still possible to define at least one phenotype for each gene identified. This might be the reason why some isolates expressed resistance in the absence of the gene responsible while others did not express resistance despite the responsible gene being present. Bochner (2003) stated that in order to fully understand the function of a gene, we must be able to predict, relate and define all the phenotypes that may develop from the wild-type and mutants of that gene. Furthermore, a gene that codes for a particular antibiotic may also be linked to genes that code for resistance to other antibiotics. For example, gentamicin is linked to genes coding for resistance to tetracycline, sulfonamide, and penicillin, which may be used in rations of animals although its generally not used in swine (Dunlop, 1998). The researchers at University of Illinois found that the movement of genes across species or into new environment may either dilute or amplify the genes that confer antibiotic resistance (http://www.sciencedaily.com/releases/2007/08/070821153926.htm). There- fore it's also possible that these changes in the levels of the various genes are due to transfer from one species to another.

CONCLUSIONS

Even though the numbers of *E.coli* isolates were low in soil, there was an indication of high prevalence of multiple resistances in all sources and this may allow the bacteria

to expand its ecological niche and proliferate even in the presence of certain noxious compounds such as antimicrobial residues, ammonia, sulfur and nitrous oxides as shown by this research in lagoon effluent. The most frequently occurring antibiotic resistance genes are those that code for resistance to spectinomycin (*aadA*), streptomycin (*str*), tetracycline (*tet*) and sulfonamide (*sul*). The results also demonstrated that anaerobic fermentation process had no major effect on the population of *E.coli* that contained any of the 10 ARGs. Bacteria with certain types of genes respond to environmental stress conditions differently leading to more survival of *E.coli* of specific ARGs. The conditions during the spring season were more favorable for the isolates of *E.coli* to maintain all the 10 genes within their population as indicated by the 100% representation of ARGs in *E.coli*. There is also an assurance that 80% or more of the ARGs will be maintained in the *E.coli* regardless of season. Dynamics of gene prevalence and ARG expression are complex and depend on many factors which may include location and interactions such gene pairing and mode of action. The presence of a gene does not always result in the expression of antibiotic resistance a phenomenon commonly observed with resistance to streptomycin, spectinomycin and sulphonamides. Conversely, the expression of antibiotic resistance does not mean that the gene is present. An example of this is resistance to apramycin and neomycin.

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Table 2.1. Primer sequences used to detect antibiotic resistant genes in *E.coli.*

Gene	Primer name	Primer sequence	Anneal (°C)	Fragment size (bp)
aadA	4F[a]	GTGGATGGCGGCCTGAAGCC	68	525
	4R[a]	AATGCCCAGTCGGCAGCG		
strA	2F[a]	CCTGGTGATAACGGCAATTC	55	546
	2R[a]	CCAATCGCAGATAGAAGGC		
strB	3F[a]	ATCGTCAAGGGATTGAAACC	55	509
	3R[a]	GGATCGTAGAACATATTGGC		
tetA	TetA-L[b]	GGCGGTCTTCTTCATCATGC	64	502
	TetA-R[b]	CGGCAGGCAGAGCAAGTAGA		
tetB	TetB-L[b]	CATTAATAGGCGCATCGCTG	64	930
	TetB-R[b]	TGAAGGTCATCGATAGCAGG		
tetC	TetC-L[b]	GCTGTAGGCATAGGCTTGGT	64	888
	TetC-R[b]	GCCGGAAGCGAGAAGAATCA		
sul1	Sul1-L[b]	GTGACGGTGTTCGGCATTCT	68	779
	Sul1-R[b]	TCCGAGAAGGTGATTGCGCT		
sul2	Sul2-L[b]	CGGCATCGTCAACATAACCT	66	721
	Sul2-R[b]	TGTGCGGATGAAGTCAGCTC		
sul3	Sul3-F[c]	GAGCAAGATTTTTGGAATCG	51	880
	Sul3-R[c]	CATCTGCAGCTAACCTAGGGCTTTGGA		
aac(3)IV	Aac4-L[a]	TGCTGGTCCACAGCTCCTTC	59	653
	Aac4-R[a]	CGGATGCAGGAAGATCAA		

Boerlin et al.[a], 2005; Lanz et al.[b], 2003; Perreten and Boerlin[c], 2003

Table 2.2. Numbers of *E.coli* in swine feces and lagoon effluent samples collected from swine houses and an anaerobic lagoon over a sampling period of six months.

Month	**Number of *E.coli* isolates**	
	Swine feces (CFU/g)	**Lagoon effluent (CFU/ml)**
October 07	$1.5*10^5$	$3.5*10^6$
December 07	$1.6*10^7$	$2.1*10^6$
January 08	$5.0*10^6$	$4.7*10^7$
March 08	$1.7*10^6$	$1.4*10^6$
July 08	-	N/A
Mean	**$5.5*10^6$**	**$1.4*10^7$**

Table 2.3. Number of *E.coli* in soil samples collected from the application field at different time periods/treatments.

Time period/treatment	**Number of *E.coli* isolates (CFU/g)**		**Mean**
	0 to 3cm	**3 to 10cm**	
Before spray	3.3×10^2	4.3×10^2	3.8×10^2
Immediately After Spray	3.0×10^3	6.5×10^2	1.8×10^3
1 Day After Spray	4.3×10^3	2.2×10^3	3.3×10^3
Mean			**1.8×10^3**

Table 2.4. The prevalence of multiple resistant genes in *E.coli* isolated from swine feces, lagoon effluent and soil samples from the application field.

Source	Percentage of isolates with a specific number of ARGs		
	1 gene	**2 genes**	**3 or more genes**
Swine feces(n=238)	3 (1.3%)	6 (2.5%)	229 (96.2%)
Lagoon effluent(n=234)	5(2.1%)	14 (6.0 %)	215 (91.9%)
Soil before spray(n=62)	0(0.0)	2(3.2%)	60 (96.8%)
Soil immediately after spray(n=48)	0(0.0)	2(4.2%)	46 (95.8%)
Soil 1 day after spray(n=34)	0(0.0)	0(0.0)	34 (100%)

Table 2.5. Comparison of total number and types of antibiotic resistant genes in *E.coli* isolated from swine feces, lagoon effluent and soil samples from the application field.

Gene Type	Percentage of ARGs in each source			Mean
	Swine Feces (n=238)	Lagoon Effluent (n=234)	Soil (n=144)	
aadA	205(86.1[a])	212 (90.6[a])	140 (97.2[b])	**91.3[a]**
strA	161 (67.6[a])	139 (59.4[ab])	79 (54.9[a])	**60.6[abc]**
strB	165 (69.3[a])	149 (63.7[a])	93 (64.6[a])	**65.9[abc]**
tetA	225 (94.5[b])	199 (85.0[a])	135 (93.8[b])	**91.1[a]**
tetB	111 (46.6[a])	124 (53.0[a])	119 (82.6[b])	**60.7[abc]**
tetC	95 (39.9[b])	49 (20.9[a])	49 (34.0[b])	**31.6[cd]**
sul1	178 (74.8[a])	177 (75.6[a])	101 (70.1[a])	**73.5[ab]**
sul2	86 (36.1[ab])	97 (41.5[b])	45 (31.3[a])	**36.3[cd]**
sul3	40 (16.8[b])	56 (23.9[c])	3 (2.1[a])	**14.3[d]**
Aac(3)IV	40 (16.8[b])	75 (32.1[c])	9 (6.3[a])	**18.4[d]**
Mean	**54.85[a]**	**54.57[a]**	**53.69[a]**	

[a]Means without a common superscript letter are statistically different (Tukey's test P <0.05)

[b]The numbers in parenthesis are percentages of each type of gene within the source

[c] Percentages without a common superscript letter in each row are statistically different (Tukey's test P <0.05)

Table 2.6. Total percentage of antibiotic resistant genes in *E.coli* isolates from the soil collected before, immediately after and 1 day after spraying the field with lagoon effluent.

Gene Type	Percentage of ARGs			Mean
	Before spray (n=80)	Immediately after spray(n=30)	1 day after spray(n=34)	
aadA	77(96.3[a])	30(100[a])	33(97.1[a])	**97.8**
strA	46(57.5[a])	17(56.7[a])	16(47.1[a])	**53.7**
strB	67(83.8[b])	11(36.7[a])	15(44.1[a])	**54.8**
tetA	76(95.0[ab])	30(100[b])	29(85.3[a])	**93.4**
tetB	67(83.8[ab])	21(70[a])	31(91.2[b])	**81.6**
tetC	26(32.5[a])	10(33.3[a])	13(38.2[a])	**34.7**
sul1	59(73.8[b])	14(46.7[a])	28(82.4[b])	**67.6**
sul2	32(40.0[b])	7(23.3[ab])	6(17.6[a])	**27.0**
sul3	3(3.8[a])	0(0.0[a])	0(0.0[a])	**1.3**
Aac(3)IV	7(8.8[a])	1(3.3[a])	1(2.9[a])	**5.0**
Mean	**57.5**	**47.0**	**50.6**	

[a]The numbers in parenthesis are percentages of each type of gene within the source

[b] Percentages without a common superscript letter in each row are statistically different (Tukey's test $P < 0.05$)

Table 2.7. Comparison of the effect of seasonal changes on the distribution of antibiotic resistant genes in *E.coli* populations from lagoon effluent.

Gene Type	**Percentage of ARGs**			
	Fall (n=98)	**Winter (n=76)**	**Spring (n=30)**	**Summer (n=30)**
aadA	82(83.7[a])	71(93.4[b])	29(96.7[b])	30(100[b])
strA	28(28.6[a])	58(76.3[b])	24(80.0[b])	29(96.7[c])
strB	40(40.8[a])	55(72.4[b])	24(80.0[b])	30(100[c])
tetA	93(94.9[b])	50(65.8[a])	29(96.7[b])	27(90.0[b])
tetB	44(44.9[a])	45(59.2[ab])	21(70.0[b])	14(46.7[a])
tetC	2(2.0[a])	10(13.2[b])	19(63.3[c])	18(60.0[c])
sul1	80(81.6[b])	50(65.8[a])	20(66.7[a])	27(90.0[c])
sul2	16(16.3[a])	51(67.1[b])	25(83.3[b])	5(16.7[a])
sul3	5(5.1[a])	24(31.6[b])	23(76.7[c])	4(13.3[a])
Aac(3)IV	5(5.1[a])	48(63.2[b])	22(78.7[b])	0(0.0[a])

[a]The numbers in parenthesis are percentages of each type of gene within the season
[b] Percentages without a common superscript letter in each row are statistically different (Non parametric1way savage scores $P<0.05$)

Table 2.8. The relationship between phenotypic expression of antibiotic resistance and presence of antibiotic resistant genes in *E.coli* isolates from swine feces, lagoon effluent and soil from an application field.

Source	Percentage of isolates with gene present no resistance expressed				Percentage of isolates without gene but with resistance expressed			
	aadA/str	***tet***	***sul***	***Aac(3)iv***	***aadA/str***	***tet***	***sul***	***Aac(3)iv***
Swine feces (n=238)	85 (35.7)	9(3.8)	112(47.1)	3 (1.3)	0(0)	1(0.4)	6(2.5)	84(35.3)
Lagoon (n=234)	44 (18.8)	1(0.4)	124(53.0)	114(48.7)	0(0)	4(1.7)	0(0)	14(6.0)
Soil(n=144)	56 (38.9)	60(41.7)	120(83.3)	0(0)	0(0)	0(0)	0(0)	121(84.0)

[a]The numbers in parenthesis are percentages of each type of gene within the source

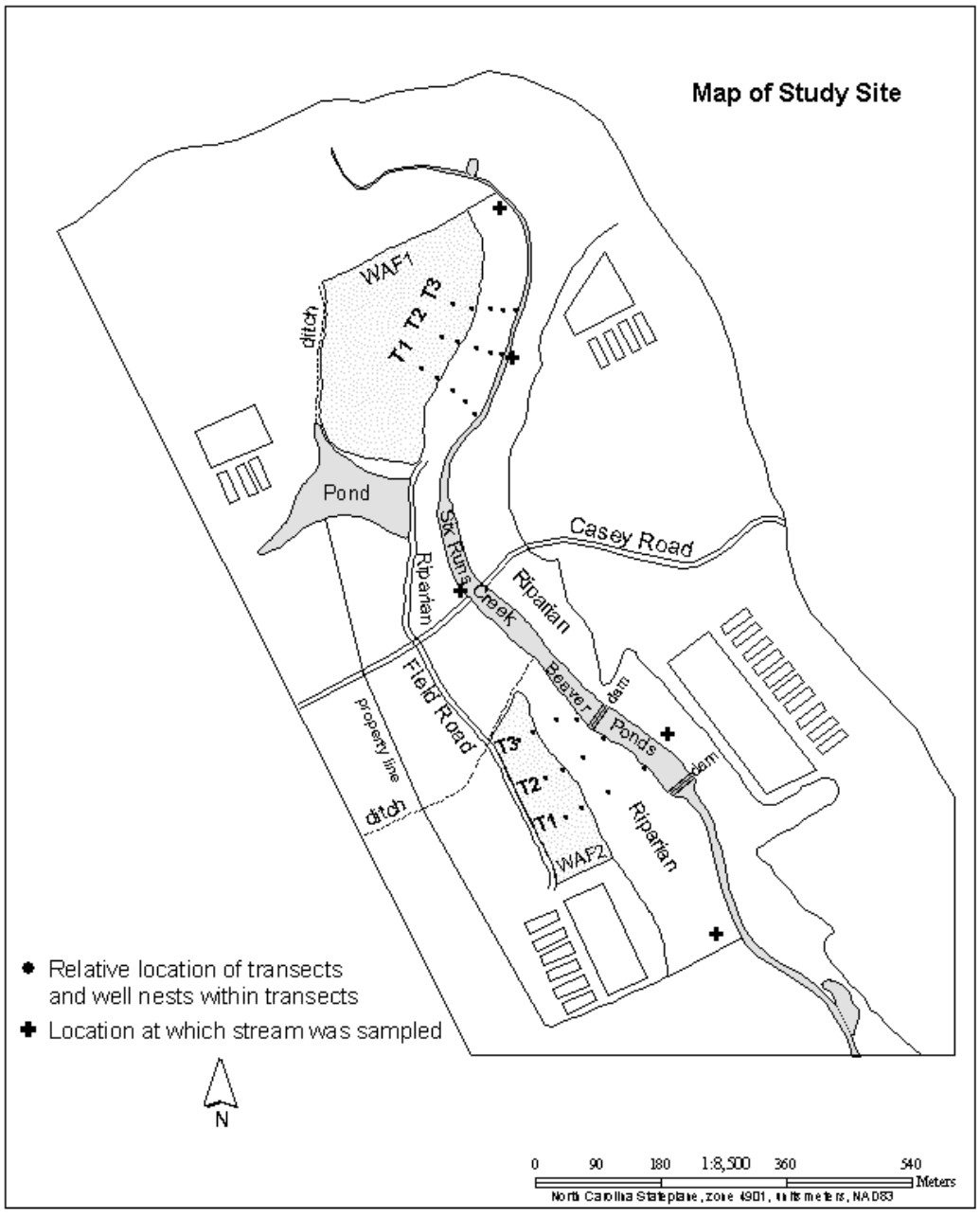

Figure 2.1. Map of the study site, Six Runs Creek in eastern Sampson County, NC**.** (Israel et al., 2005).
T1= Transect 1; T2= Transect 2; T3= Transect 3
WAF2 = Waste application field 2

CHAPTER 3

MICROBIAL SOURCE TRACKING IN A RURAL WATERSHED DOMINATED BY SWINE

ABSTRACT

Sources of fecal pollution in complex environments such as watersheds are often not known. Antibiotic resistance analysis (ARA) a phenotypic method is a common tool used to determine sources of fecal pollution in such environments. The objectives of this study were 1) to use antibiotic resistance patterns (ARPS) of *E.coli* isolates from fecal sources and lagoon effluent to determine sources of fecal pollution in ground water and in a segment of a nearby stream (six runs creek), 2) to evaluated the effect of season on pollution levels/source in ground and surface water. ARA patterns of 3,349 environmental isolates and 1,937 known source isolates (swine feces, lagoon effluent, cattle, deer, nutria, raccoon, beaver, bird and dog) were determined with 9 antibiotics at 38 different concentrations. The database of known isolates was screened by elimination of duplicated patterns and including only isolates clustered into a specific group at > 80% resulting in an average rate of correct classification (ARCC) of 94.7%. The unique pattern library of 470 *E.coli* isolates was then used to classify the isolates from environmental samples into the 9 possible source categories. Lagoon effluent was the major contributor responsible for 45.6% of the 1,388 *E.coli* isolates and 61.4% of the 1,961 *E.coli* isolates in ground and surface water respectively. Of the *E.coli* isolates in ground water, cattle contributed the most fecal pollutants during winter (52.8% of 97 isolates), spring (46.4% of 466 isolates), summer (50.0% of 17 isolates) and in the fall, lagoon effluent contributed 63.3% of 808. In surface water, lagoon effluent was predominant for summer (64.7% of 355 isolates) and fall (74.1% of 564 isolates). Cattle were predominant in winter and spring and contributed 40.9% of 407 isolates and 52.8% of 389 isolates respectively. However, it's important to note that the average numbers of *E.coli* found in groundwater was low (21.1CFU/100 mL), thus indicating that management

practices were effective. Although the Six Runs creek appears to be contaminated because the numbers of *E.coli* in surface water at an average of (272.1CFU/100 mL) was 10 fold higher than in ground water, this is not alarming in comparison with other polluted systems. However, this contamination may be related to other swine farms within the watershed.

INTRODUCTION

Surface water can be polluted with fecal bacteria from both point and nonpoint pollutant sources within agriculture, forestry, wildlife and urban systems (Simpson et al., 2002). The production of livestock and poultry waste on dry weight basis for the United States exceeds 200 million tons per year (Aillery et al., 2006). Livestock manure contains more than 150 pathogens, those that are mostly associated with human health risks, may include *Campylobacter spp.*, *Salmonella spp.* and *Escherichia coli* O157:H7. These pathogens account for over 90% of food and waterborne diseases in humans (USEPA, 2003). Infiltration of water through soil and run-off from agricultural fields during a rainfall event, as well as, free access of livestock and wildlife to water bodies contributes to water pollution (Simpson et al., 2002).

Typically lagoon systems are used for swine waste nutrient management. However reasonable levels of bacteria still persist since lagoons were not designed to control bacteria (Burkholder et al 2007). As a result, the land application of lagoon effluent gives rise to concern because land application has the potential to introduce many microorganisms to the soil surface of which some may reach surface and ground water. There is potential for survival and mobility of pathogens in soil resulting in possible exposure to humans and other animals. Soil has the potential to prevent bacterial movement or leaching, however, soils may still transport a reasonable amount of bacteria applied to it or released within. Even at recommended application rates, bacteria may travel in the vadose zone to groundwater (Guber et al., 2005). Bacteria being small in size (diameter 0.2-5μm) and negatively charged are repelled by the negatively charged soil

particles and remain in soil water which moves through the macropores (Pekdeger and Matthess, 1983)

Once fecal bacteria enter a water body, counts can be determined, however, source identification is not immediately known by fecal counts alone. Microbial Source Tracking (MST) is one approach to determining the sources of fecal pollution and pathogens affecting water bodies in complex environments such as a watershed. MST methods currently in use include genotypic and phenotypic methods which are based on the assumption that specific markers or strains of bacteria can be identified as originating from specific animal species (Amor et. al, 2000). These techniques are either library dependent or library independent. A "library" is a collection of microorganisms from different potential sources within the watershed under study. MST methods in use include antibiotic resistance analysis (ARA) which uses the antibiotic resistance patterns (ARPs) of fecal coliforms, *E.coli* or enterococci as phenotypic fingerprints and fecal bacteria species composition. Antibiotic resistance patterns reflect exposure of microflora to antibiotics due to the selective pressure imposed on the commensal gastrointestinal flora of animals and humans by antibiotic use (Harwood, 2000). Research has shown that isolates obtained from humans, chickens, and dairy cows have higher resistance levels than strains obtained from wild animals (Krumperman et.al, 1983).

The ARA approach is useful for tracking sources of fecal coliforms in small watersheds, relatively inexpensive and simple to perform. Discriminant analysis, a multivariate statistical tool is used to classify the subjects into categories based on a defined number of test variables (Huberty, 1994). The success of MST methods depends on several validity assumptions in that the species displays some degree of host specificity and geographical structure. The DNA sequence of the species isolated from soil and water are similar to the DNA sequence of the species in the host populations contributing fecal pollution to the environment. Generally, the DNA sequence of populations become stable with time, hence the same clones can be recovered from the same area or host populations for extended periods (Gordon, 2001). In separate studies to determine sources of fecal

pollution, Hagedon et al. (1999) achieved an average correct classification rate of 87% with isolates from humans, dairy cattle, beef cattle, chickens, deer, and waterfowl while Wiggins et al. (1999) and Harwood et al. (2000) reported 74% and 63.9% respectively. The key to successful ARA studies involves stringent statistical analyses of the database with regard to accuracy and representativeness (Graves et al., 2007).

The objectives of this study were to use ARA to determine sources of fecal pollution in the six runs creek and determine if seasonal changes have an influence on the host source of *E. coli* recovered from environmental waters.

MATERIALS AND METHODS

Site Description. The study was conducted on a commercial swine farm Sampson County, North Carolina with a standing herd of 4400 hogs (finishing 720-725 hogs per house) and 100 cattle, located in a 275 ha watershed along the upper part of Six Runs Creek. The site has two waste application fields (WAFs) and a stream which flows in a southerly direction. Adjacent to waste application field 1(WAF1), the stream flows in a channel, while the segment adjacent to waste application field 2(WAF2) is impounded by two beaver dams and forms an elongated pond. The stream flows in a channel below the lower beaver dam. The watershed has four swine operations with 23 swine houses located on both sides of the stream (Fig 2.1). Swine feces from the swine house were washed into waste lagoons once per week. Water levels were maintained at three quarters full to allow storage of excess rain water and create anaerobic conditions in the lower portion of the lagoon to maintain compliance with waste management regulations. Lagoon effluent was sprayed on farmland cropped with bermuda grass managed for hay and grazing pasture for cattle. On average, spray vents occur 3 to 4 times during the warm season, once in late fall and early spring. Soil in waste application field was a Wagram series (loamy, kaolinitic, thermic Arenic Kandiudults) (Israel et al., 2005).

Known source isolates. Samples from known sources were collected from the site every other month between October 2007 and March 2008. Liquid effluent samples were

collected from the lagoon, fresh feces from swine houses, cattle manure from the application field, dog manure from the field, bird and deer manure from the riparian, nutria, beaver and raccoon from the intestine of trapped animals. A total of 2,080 *E. coli* isolates from 9 known sources was used to develop the database of all possible sources of *E. coli* within the watershed. The database included 576 isolates from swine feces, 593 isolates from lagoon effluent, 480 isolates from cattle manure and 48 isolates each from deer feces, nutria, raccoon, beaver, bird and dog. The sources were then divided into two categories which included livestock and wildlife.

The soil core samples (10 cm) collected from the application field was divided into, top samples (0-3 cm) and lower samples (3-7 cm). A 10 ml aliquot of effluent sample or 10 g of fecal and soil samples were enriched in 90 ml of BHI broth (EMD chemicals Inc., Gibbstown, Nj) for 24 h at 37° C to obtain more *E.coli* isolates for the microbial source tracking study. Another 10 g of soil sample was diluted in 100 ml of sterile deionized water. One ml of each sample was then diluted into 9 ml of sterile water (10^{-1} dilution) and then serial dilutions of 10^{-2}-10^{-6} were made. A 100 µl aliquot of the diluted samples was cultured on m-FC agar (Becton Dickinson, sparks, Md.) and incubated at 44.5^{o} C for 24 h to obtain single colonies (Hurst and Crawford, 2002). Single dark blue colonies presumed as fecal coliforms were then transferred using a tooth pick to microwells with colilert broth and cultured for 24 h. Five µl from each microwell was transferred to a vial containing 1 ml of pre-enrichment broth (Becton Dickinson, sparks, Md.), mixed, incubated for 24h at 37 ^{o} C and stored at 4 ^{o} C.

Random fecal samples collected from swine pens were combined into a representative composite sample for each swine house occupied by animals. Two samples from the inlet and only outlet of the lagoon were collected in sterile glass jars and placed on ice before being transported to the laboratory for analysis. Random fecal samples from cattle and dog were collected from the application field and wildlife samples collected from the riparian. The soil core samples (10 cm) collected from the application field was divided into, top samples (0-3 cm) and lower samples (3-7 cm). A

10 ml aliquot of effluent sample or 10 g of fecal and soil samples were enriched in 90 ml of BHI broth (EMD chemicals Inc., Gibbstown, Nj) for 24 h at 37° C to allow *E.coli* to recover from environmental stress prior to molecular characterization. One ml of samples was then diluted into 9 ml of sterile water (10^{-1} dilution) and serial dilutions to 10^{-2}-10^{-6} were made. A 100 µl aliquot of the dilute sample was cultured on m-FC agar (Becton Dickinson, sparks, Md.) and incubated at 44.5^{o} C for 24 h to obtain single colonies (Hurst and Crawford, 2002). Single dark blue colonies presumed fecal coliform were then transferred using a tooth pick into micro wells with colilert broth and cultured for 24 h. Five µl from each microwell was transferred to a vial containing 1ml of pre-enrichment broth (Becton Dickinson, sparks, Md.), mixed, incubated for 24 h at 37 ^{o} C and stored at 4 ^{o} C.

Water isolates. Water samples were collected from the site on a monthly basis when conditions permitted between October 2007 and December 2008. Surface water samples were collected from a total of 5 stream sampling sites. Sampling sites consisted of upstream (above WAF 2, see Figure 2.1) and midstream site in relation to the WAF 2. Ground water samples were collected from the wells arranged in transects from WAF 2 to the stream. These wells were previously used in a nutrient study (Israel et al., 2005). Two wells were located at each sampling point at different depths. The sampling regime conducted on every other month was designed to cover all the four seasons. The wells (piezometers) were installed in waste application field and the adjacent forested riparian buffer on the west side of Six Runs Creek. Two transects had six well nests; 2 positioned in the field, 2 each at the field-riparian zone edge and at the stream edge (Fig.2.1). The third transect had only four wells since two wells in the waste application field could not be located. In the WAFs, wells in each nest were placed 1 m apart and screened at two different depths: near the top of water table, and at a depth below the water table. In a previous N movement study, water table elevation measurements demonstrated that ground water was moving from the WAF through the riparian area to the stream (Israel et al. 2005) Standing water was initially drained out of the well to allow the well to

recharge with fresh water. A sample was then drawn from the well using a bailing tube, into a 500ml sterile polystyrene bottle and placed on ice before being transported to the laboratory for processing. The samples were analyzed within 6 hrs of collection.

Isolates of *E. coli* were recovered by membrane filtration of 50ml and 100ml of sample from stream and wells, respectively through a 0.45µm membrane filter that was then placed on m-FC agar (Becton Dickinson, sparks, Md.) which is selective media for fecal coliforms. The plates were incubated for 24h in a 44.5°C water bath (Hurst and Crawford, 2002). Individual dark blue colonies were transferred using a tooth pick to 96-microwell plates containing 150µl colilert broth (IDEXX Laboratories Inc., Westbrook, Me.) which is selective media for *E. coli,* and incubated for 24 h at 37°C. Isolates were confirmed *E. coli* when the microwell fluoresced under UV light.

Antibiotic resistance analysis. Antibiotic resistance analysis (ARA) was performed to evaluate the expression of antibiotic resistance in *E.coli* collected from the swine feces, lagoon effluent, cattle, deer, nutria, raccoon, beaver, bird, dog, ground and surface water. A cumulative total of 5,909 *E coli* isolates from the site were evaluated. Antibiotic resistance patterns of each *E.coli* isolate was comprised of 38 observations. A 48-prong replicator (Sigma) was used to transfer isolates onto 100mm diameter tryptic soy agar plates amended with the following concentrations of antibiotics: Erythromycin, 60, 70, 90 and 100µg/l; Neomycin, 2.5, 5 and 10µg/l; Oxytetracycline, Streptomycin and Tetracycline, 2.5, 5, 7.5, 10 and 15µg/l; Rifampicin, 60, 75 and 90µg/l; Cephalothin, 15, 25 and 35µg/l; Apramycin and Trimethoprim, 8, 16, 32, 64 and 128µg/l (Table 3.1). These concentrations were chosen to include the minimum inhibitory concentration (MIC) for each antibiotic. This is the concentration of an antibiotic that result in inhibition of visible bacterial growth. Antibiotic stock solutions (Table 3.2) were prepared as described in Graves et al. (2002), Hagedorn et al. (1999) and Harwood et al. (2000). The plates were incubated at 37^{o}C for 18 to 20h. The antibiotic and concentrations were selected based on previous ARA studies and their common use in human and veterinary medicine (Mathew et al., 1999). Resistance to an antibiotic was determined by

comparing growth of each isolate to the same isolate on the control plate. A one (1) was recorded for a round, mostly filled colony and a zero (0) for no growth. This was repeated for every isolate on each of the 38 antibiotic plates.

Statistical analysis. Variables for the analyses included the number of antibiotics used and the degree of pooling of sources. ARA results for known sources were processed using SAS software to remove all results with duplicate patterns. The antibiotic resistance patterns (ARPs) for the unique sets were used to calculate the correct classification rates which were later used to establish both the classification rule and as test subjects (Harwood et al., 2000). Each analysis produced a classification rate for each known source of isolates. The rate of correct classification was determined by the number of isolates from a particular source that was allocated to the correct source category by discriminant analysis. In order to develop a more stringent database, all the isolates with the correct classification rate of less than 80% were removed from the database. The average rate of correct classification (ARCC) for the database was obtained by averaging the correct classification percentages for all sources (Harwood et al., 2000). A cross validation method whereby isolates from known sources were randomly removed from the data set and treated as test subjects was used as strong test to assess the power of the databases to identify sources (Harwood et al., 2000). To determine whether the known database was large enough or had ample representation, artificial clustering was used. Artificial clustering involves randomly assigning equal numbers of isolates from each source and applying discriminant analysis to determine the random ARCC. Our database contained 9 source types. The random ARCC was approximately 11% for each source. Thus, any misclassification percent significantly greater than the 11% ARCC indicates that the known source database was not representative. These measures made sure that the developed and validated database served as a good point of reference for identifying unknown source isolates collected from Six Runs Creek. The average rate of misclassification (ARMC) for each source category and a standard deviation were calculated, and this average and standard

deviation were used to develop a minimum detectable percentage (MDP) to make decisions about the significance of minor sources in water samples.

MDP = ARMC + (SD x Number of sources) = (0.68) + (0.92 x 9) = 9%

RESULTS

E.coli monitoring. Samples from both ground water in the swine lagoon effluent application field and nearby surface water body revealed the presence of *E. coli* from fecal sources. On average, the levels of *E.coli* in surface water were 272.1 CFU/100 mL, which exceeded the maximum standard for recreational water in the USA (200 CFU/100 mL). However, monthly measurements indicated changes in the level of fecal pollutants in both environmental water sources. Levels in surface water exceeded this minimum standard for almost three quarters of the year with only January, March and June below the threshold having 66.8, 87.3 and 90 CFU/100 mL respectively. While levels in ground water were constantly below the threshold with low levels of 1-10 CFU/100 mL in October 07, January, May, June, October 08 and December 08, moderate in July at 25.8 CFU/100 mL and high levels of 60.8 and 81.2 CFU/100 mL in March and September respectively (Table 3.3).

Antibiotic resistance analysis. Generally, isolates from all the sources within the site displayed resistance to one or more antibiotics under evaluation. On average, the isolates indicated the most resistance to Oxytetracycline 77.9%, Tetracycline 73.1%, Cephalothin 69.5%, Neomycin 68.4%, Streptomycin 67.7% and Erythromycin 59.9%. They also indicated intermediate levels of resistance to Apramycin at 40.6% and very low resistance to Trimethoprim 20.1% and Rifapicilin 1.6 % (Table 3.4)

Known source database. A total of 1,937 *E coli* isolates from the 9 known sources within the research site were used to build a database. One thousand three hundred eighty eight *E coli* isolates from the environmental sources (unknown) including ground and surface waters were evaluated against the database. The known sources were subdivided in two categories which included livestock and wildlife. ARA profiles with duplicate results

were identified and excluded from the database using a SAS program; the six sources (swine feces, lagoon effluent, cattle, bird, nutria and dog) lost approximately 50% of the isolates, while deer, beaver, and raccoon lost approximately 30, 70 and 92% respectively (Table 3.5). When discriminant analysis was conducted on these isolates to obtain the correct classification rate, the database produced an average correct classification rate of 85% with individual sources ranging from 70 -100% (Table 3.6). In an effort to develop a more stringent database, all isolates with correct classification rate of < 80% were removed with the exception of raccoon and beaver isolates because we had very few unique patterns for these sources. When the 80% threshold was applied, approximately 60% of isolates from swine feces and lagoon effluent, 40% from cattle, and 25% from bird and less than 10% of the isolates from dog were lost. The refined database of 470 isolates had an average correct classification rate (ARCC) 94.3%, with individual sources ranging from 75-100 %. The average rate of misclassification for the library was used to calculate the minimum detectable percentage (MDP) for the watershed. The average rate at which the isolates in the library were misclassified was 0.68 ± 0.92 SD. Nine times the standard deviation (0.92) of the average rate of misclassification added to the average MC of 0.68% was 9% for the MDP. With nine source categories, the probability of an isolate being assigned to any category by chance alone was 11%. However, 9% was taken as a more stringent lower limit for considering any one source category to be a significant contributor of fecal pollution (Harwood et al., 2000) (Table 3.7).

Source identification of E.coli from soil. In *E.coli* isolates from the soil, cattle (37.6%) were the predominant source contributor but not significantly greater than swine feces (29.5%) and lagoon effluent (20.7%). *E.coli* isolates from swine feces and lagoon effluent were not different from raccoon (20.4%) or wildlife sources combined (11.1%). *E.coli* isolates identified as lagoon effluent were not different from the other sources (<1%). Deer, bird, dog, nutria and beaver were all below the MDP (P=<0.0001) (Table 3.8).

Source identification of E.coli from ground water. In ground water, *E.coli* isolates identified as lagoon effluent (45.6%) dominated, but was not different from cattle (31.8%).

Cattle was not different from wildlife sources when combined but were significantly greater than the other sources which were <10 %. Swine feces, deer, bird, dog, raccoon, nutria and beaver were all below the MDP (P=<0.0001) (Table 3.9).

Source identification of E.coli from surface water. In surface water, *E.coli* isolates identified as lagoon effluent (61.4%) was significantly greater than cattle (27.1%). The isolates identified as cattle were significantly greater than all the other sources which were <5%. Swine feces, deer, bird, dog, raccoon, nutria, beaver and wildlife sources when combined were all below the MDP (P=<0.0001) (Table 3.10).

Source identification based on sampling period: ground water. In ground water, seasonal changes had various effects on the antibiotic resistance in the various sources. In winter, isolates from cattle (52.8 %) were significantly greater than isolates from lagoon effluent (21.4 %) and all the other sources at <10% which were not different (P=<0.0001). In spring, percentage of isolates from cattle (46.4%) was significantly greater than percentage of isolates from other sources except lagoon effluent (23.2%) and the percentage of isolates from all wildlife sources combined at 21.4%. However, percentage of isolates from lagoon effluent was not different from the percentages from beaver (9.4%), dog (5.5%), nutria (4.1%), bird (3.9%), swine feces (3.6%), deer (2.3%) and raccoon 1.8% (P=0.0001). In summer, there were no significant differences although percentage of isolates from cattle (50%) was predominant compared to bird (22.2%) and raccoon (16.8%). All wildlife sources combined contributed 44.4% of the isolates but all the other sources were <10 %(P=0.7509). In the fall, the percentage of isolates from lagoon effluent (63.3%) was significantly greater than the percentage from cattle (19.4%) which was also greater than the percentage for all the other sources except wildlife (9.6%) combined.(P=<0.0001) (Table 3.11).

Source identification based on sampling period: surface water. In surface water, winter effects were different from the other seasons. During this season, isolates from cattle (40.9%) were dominant but not different from lagoon effluent (34.5%). Percentage of isolates from lagoon effluent was greater than the percentages of isolates from all the

other individual sources except when all wildlife sources were combined (17%). In spring, percentage of isolates from cattle (52.8%) was significantly greater than the percentage from lagoon effluent (21.4%) and all the other sources which were <10%. However, percentage of isolates from lagoon effluent and the other sources were not different. In summer, percentage of isolates from lagoon effluent (64.7%) was greater than the percentage of isolates from cattle (21.2%) and the other sources (2.2-10.8%). Percentage of isolates from cattle was not different from the percentages of other sources except dog (0.4%) and nutria (0.2%) at P=<0.0001. In the fall, percentage of isolates lagoon effluent (74.1%) was greater than the percentage from cattle (12.4%). Percentage of isolates from cattle was also greater than percentages for the other individual sources all of which were <10% except when wildlife sources were combined at P=<0.0001 (Table 3.12).

DISCUSSION

The results indicated that *E.coli* counts in ground water were general low except in March 08 (60.8 CFU/100 mL), July 08 (25.8 CFU/100 mL) and September 08 (81.2 CFU/100 mL). Despite correlation with high rainfall during these months, the non-septic sampling procedure may have contributed some fecal bacteria. When opening the wells, some soil may have fallen into the wells thereby contributing to the fecal counts in the ground water. *E.coli* counts in the surface water (272.1 CFU/100 mL) within the watershed had counts above the 200 CFU/100 mL threshold for recreational waters in USA and 10 fold higher on average than that in ground water (21.1 CFU/100 mL). This may be due to the slow turnover of the water in the beaver pond as compared to a free flowing stream. As a result, there was an accumulation of nutrients and carbon as an energy source for the microorganisms to multiply resulting in elevated concentration of fecal coliforms. Fincher et al. (2009) reported fecal coliform counts of 373 CFU/100 mL and 1470 CFU/100 mL in rural and urban sections respectively of an impaired stream in a watershed in north central Indiana. The results from these other water sources were higher than those from our findings. This indicates that surface and ground water in the water shed evaluated

in this study does not have an exceptionally high level of fecal coliforms compared to other rural and urban water bodies.

The size of the database is one of the critical components in using MST techniques. Antibiotic resistance patterns frequently use large known-source libraries, consisting of about 1,000 to 6,000 isolates (Burnes, 2003). However many of the strains examined with these libraries are isolated from the same sample or source material, and hence the libraries may be biased due to the presence of multiple replications (clones) of the same bacterial genotype from the same animal source (Johnson et al., 2004). Likewise our database in this research was initially large and was scaled down through vigorous screening processes to obtain an accurate tool to serve as a point of reference to identify sources of *E.coli* collected from environmental pools.

The high rate of correct classification obtained with our database may be attributed to the stringent measures that were employed to achieve a database which is more accurate in predicting the sources. The action to remove all the isolates from the data which initially had duplicates enabled us to only use unique patterns of each isolate. Lower values of correct classification rate indicate that the probability to place an isolate into a specific group by chance is very high. To address this concern, the 80% threshold was used to allow only those isolates which are very closely related to be assigned to a specific group. As a result, differentiation between closely related isolates was achieved. Graves et al. (2007) found an ARCC of 95.4%, Hagedon et al. (1999) achieved an ARCC of 87%, and Booth et al. found an ARCC of 85.3% and Wiggins et al. (1999) reported 74%. The ARCC obtained in this study was similar to that found by Graves et al. (2007) but slightly greater than the findings of these other studies because the threshold used was also above these values.

Land use patterns and the geographical setting indicated that the dominant contributor of *E.coli* would be from livestock sources (swine and cattle). However, flocks of birds were often observed both within the application field and at the water source during some sampling trips. The presence of the beaver dam in the creek indicates that

there is a high population of beavers, hence a likely source of bacterial contamination. Most of the land within the watershed is dedicated for agricultural purposes, leaving significant distances to homes that could contribute to fecal loading due to failing onsite wastewater systems. The farm site had a herd of cattle that grazed in the application field, whose stocking rate varies during the year, however due to fencing the cattle at this site had no direct access to the creek.

ARA indicated that isolates from the soil were predominantly associated with swine feces. The residence time of the bacteria that exit the lagoon via spray events was not determined. However, the findings in our study may be a reflection of the inclusion of fresh fecal matter in lagoon effluent due to the high suction of the spray pumps. However, studies of source tracking involving soil isolates are very rare as microbial source tracking studies mostly involve identifying sources of fecal pollution in surface water.

Evaluation of ground water indicated that lagoon effluent was the predominant source of *E.coli* in the field (39.5%), riparian (46.9%) and stream wells (50.3 %). Evaluation of surface water indicated that lagoon effluent was the predominant source of *E.coli* in water samples above bridge (63.7%), below bridge (54.2%) and at locations adjacent to the application field (66.4%). Olivas and Faulkner (2007) in their study identified *E.coli* isolates as livestock at 48%. Graves et al. (2007) in their study in watershed dominated by cattle identified *E.coli* isolates as cattle at 52%. Booth et al. (2003) in their study in the Blachwater river identified *E.coli* isolates as livestock at 47.6%. In all these findings one common observation was that livestock was a dominant contributor. However, the magnitude of the impact depends on the type and intensity of the livestock population in these areas.

E.coli isolates from ground water were predominantly identified as cattle at 52.8, 46.4, 50.0 and 63.3% during winter, spring, summer and fall respectively. We were unable to find data from other researchers since MST studies in ground water are rare. In surface water, *E.coli* isolates identified as cattle were predominant in winter (40.9%) and spring

(52.8%). Whereas isolates identified as lagoon effluent were predominant in summer (64.7%) and fall (74.1%). Olivas and Faulkner (2007) in their study identified *E.coli* isolates as human at 56% in fall and livestock and human sources at similar rates (39 and 35%), (40 and 35%) and (43 and 37%) in winter, spring and summer respectively. In a watershed dominated by cattle, Graves et al. (2007) identified *E.coli* isolates as cattle at 60% in summer and human at 47% in winter. Booth et al. (2003) in their study in the Blackwater river identified *E.coli* isolates as livestock at 48.9% in summer and at 44.4% in winter. Results from other studies are similar to our findings except for the possible influence of human sources in the other studies which was not the case in our study. The large contribution of *E.coli* isolates identified as lagoon effluent in the summer and fall might be due to high inputs of effluent during this period, while elevated isolates from cattle in the winter and spring seasons may be due to lower lagoon effluent application.

Additionally warmer months tend to increase wildlife activity and there may have been transport of *E.coli* from the lagoon to the stream by wildlife (birds, turtles). The low application of lagoon effluent coupled with continuous grazing by cattle during the wetter cool season could result in longer survival and mobility of *E.coli* in the soil. Pathogens generally survive longer in water, followed by soil and manure. Under all these environments, they survive better at lower than at higher temperatures (Guan and Holley, 2003). The high contribution of bird and raccoon to *E.coli* isolates in ground water during summer may be a reflection of the small sample size during this season as compared to the other seasons. However the low contribution of *E.coli* isolates from lagoon effluent in ground water during summer may be associated with the high temperatures experienced during this season.

CONCLUSIONS

Using the ARPs for *E. coli* isolates from the known sources within this swine farm, we were able to develop a database which can be used for identifying the sources of *E. coli* within the watershed. However, it should be noted that this database is specific for this

particular watershed although the procedure can be adopted for use in other similar areas near swine facilities. In this watershed, there were multiple sources contributing fecal bacteria in the six run's creek. According to our results, the *E. coli* isolates present in the ground water were very much associated with cattle during the most of the year except in the fall when lagoon effluent dominated. In the surface water, *E.coli* isolates were associated more with lagoon effluent except in the winter when both cattle and lagoon effluent contributed equally. Good practices to manage swine wastes using anaerobic lagoon systems are effectively mitigating fecal pollution from animal wastes which resulted in low numbers of *E. coli* in samples collected from groundwater wells. When implementing waste management plans on farms, much focus is only on wastes from confined animals thereby undermining the impact of free range livestock thinking that having no direct access to water bodies is enough to control pollution from these sources. However farmers need to abide by the recommended effluent application rates during the period when they are allowed to spray the fields. During assessment of farms for compliance with effluent application regulations, considerations should be made for the role of non confined livestock such as cattle and wildlife transporting *E. coli* from lagoons to streams (Birds, turtles, etc.). We can also conclude that the movement of *E.coli* from spray field to surface water raises a possibility of pathogen movement. However, this does not prove that pathogens move to surface water in this system. Pathogen movement through the system must be evaluated directly to draw such a conclusion.

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Table 3.1. Types of antibiotics and concentrations used in antibiotic resistant analysis of *E.coli* isolates.

Antibiotics	Concentrations (µg/ml)	Volume Per 100ml	No. of Variables
Erythromycin	60, 70, 90, and 100	600, 700, 900, and 1000	4
Neomycin	2.5, 5.0, and 10	25, 50, and 100	3
Oxytetracycline	2.5, 5.0, 7.5, 10 and 15	25, 50, 75, 100 and 150	5
Streptomycin	2.5, 5.0, 7.5, 10 and 15	25, 50, 75, 100 and 150	5
Tetracycline	2.5, 5.0, 7.5, 10 and 15	25, 50, 75, 100 and 150	5
Cephalothin	15, 25 and 35	150, 250 and 350	4
Apramycin	8, 16, 32, 64 and 128	80, 160, 320, 640 and 1280	3
Trimethoprim	8, 16, 32, 64, and 128	80, 160, 320, 640 and 1280	5
Rifapicilin	60, 75 and 90	600, 750 and 900	3
Control	No antibiotic		2
Total			**40**

Table 3.2. Protocol for preparation of stock solutions for the different types of antibiotics

Antibiotics	Solvent	Stock Concentration (mg/ml)
Erythromycin	1:1 Water Ethanol	1g/100ml
Neomycin	Distilled water	1g/100ml
Oxytetracycline	1:1 Water Methanol	1g/100ml
Streptomycin	Distilled water	1g/100ml
Tetracycline	Methanol	1g/100ml
Cephalothin	Distilled water	1g/100ml
Apramycin	Methanol	1g/100ml
Trimethoprim	1:1 Water Ethanol	1g/100ml
Rifapicilin	Methanol	1g/100ml

Table 3.3. Number of *E.coli* (CFU/100mL) in ground and surface water samples collected over a 14 month period.

Month	Ground water			Surface water		
	No of samples	Mean CFU/100mL	Std Dev.	No of samples	Mean CFU/100mL	Std Dev.
October 07	8	3.6[b]	4.7	1	200.0[ab]	_
December 07	14	0	0	0	_	_
January 08	14	1.0[b]	3.7	8	66.8[b]	37.3
March 08	16	60.8[a]	62.3	3	87.3[ab]	44.2
May 08	16	6.4[b]	8.9	6	438.3[ab]	283.9
June 08	16	1.1[b]	4.0	6	90.0[ab]	98.0
July 08	12	25.8[b]	82.0	6	537.3[a]	1098.0
September 08	16	81.2[a]	87.5	6	496.7[ab]	153.3
October 08	16	4.6[b]	6.3	8	248.8[ab]	163.3
December 08	16	5.0[b]	6.1	8	283.8[ab]	296.3
Mean		21.1			272.1	
Std Dev.		**29.7**			**181.6**	

[a]Means with the same letter are not significantly different (Tukey-Kramer (HSD) test $P<0.05$)

Table 3.4. Percentage prevalence of phenotypic antibiotic resistance of *E.coli* isolates to antibiotic concentrations.

Concentration (µg/ml)	Type of antibiotic								
	OXY	**TET**	**CEP**	**NEO**	**STR**	**ERY**	**APR**	**TRIM**	**RIF**
2.5	82.4	76.4		88.7	82.3				
5.0	78.9	74.9		71.3	74.1				
7.5	76.7	73.2			66.5				
8							59.9	25.5	
10	76.0	71.7		45.1	60.3				
15	75.3	69.1	72.2		55.2				
16							35.5	21.7	
25			69.3						
32							39.5	20.3	
35			66.9						
60						67.2			**1.8**
64							40.8	17.4	
70						61.8			
75									**1.8**
90						55.0			**1.1**
100						55.4			
128							27.4	15.4	
Mean	**77.9**[a]	**73.1**[a]	**69.5**[a]	**68.4**[a]	**67.7**[a]	**59.9**[ab]	**40.6**[b]	**20.1**[c]	**1.6**[c]
Std Dev.	**2.9**	**2.8**	**2.7**	**21.9**	**10.8**	**5.8**	**12.0**	**3.9**	**0.4**

OXY= oxytetracycline TET= tetracycline CEP= cephalothin NEO= neomycin STR= streptomycin ERY= erythromycin, APR= apramycin, TRIM= trimethoprime RIF= rifampicilin

[a]Means with the same letters are not significantly different (Tukey –Kramer (HSD) test $P<0.05$)

Table 3.5. Number of *E. coli* isolates analyzed by Antibiotic Resistance Analysis and discriminant analysis.

Source	Location	Initial	Unique profiles	Isolates >80%
Lagoon effluent	Lagoon	593	322	131
Swine feces	Swine houses	576	284	102
Cattle	Field	480	242	137
Deer	Riparian	48	34	28
Bird	Riparian	48	25	19
Dog	Farm yard	48	22	20
Raccoon	Animal gut	48	4	4
Nutria	Animal gut	48	25	25
Beaver	Animal gut	48	4	4
Ground water	Field wells	367	367	367
Ground water	Riparian wells	548	548	548
Ground water	Stream wells	473	473	473
Surface water	Stream 1	618	618	618
Surface water	Stream 2	623	623	623
Surface water	Stream 3	474	474	474
Surface water	Above bridge	102	102	102
Surface water	Below bridge	144	144	144

Table 3.6. Nine-category database of *E.coli* isolates with no duplicate antibiotic resistant patterns using discriminant analysis for classification into source categories.

Source	Lagoon effluent		Cattle		Swine feces		Deer		Bird		Dog		Raccoon		Nutria		Beaver		Total (n_i)
	CC[a]	MC[b]	CC	MC	CC	MC	CC	MC	CC	MC	CC	MC	CC	MC	CC	MC	CC	MC	
Beaver		0		0		0		0		0		0		0		0	4		**4**
Bird		1		1		1		0	24			0		0		0		0	**27**
Cattle		15	183			17		0		1		0		0		1		0	**217**
Deer		0		1		0	34			0		0		0		0		0	**35**
Dog		0		1		1		0		0	22			0		0		0	**24**
Lagoon effluent	246			19		59		0		0		0		0		0		0	**324**
Nutria		0		0		0		0		0		0		2	23			0	**25**
Raccoon		0		0		0		0		0		0	2			1		0	**3**
Swine feces		56		29	204			0		0		0		0		0		0	**289**
Total	246	72	183	51	204	78	34	0	24	1	22	0	2	2	23	2	4	0	
Total isolates(n)																			**948**
%n CC ($100CC/n_i$)	75.9		84.3		70.6		97.1		88.9		91.7		66.7		92.0		100.0		**85.2**[c]
%n MC ($100MC/n-n_i$)	**8.2**		**5.7**		**9.0**		**0.0**		**0.1**		**0.0**		**0.2**		**0.2**		**0.0**		

[a] Number of isolates correctly classified(CC) by discriminant analysis. [c] Average rate of correct classification.

[b] Number of isolates misclassified (MC) by discriminant analysis. n= Total isolates n_i= Total source

Table 3.7. Nine-category database of *E.coli* isolates with no duplicate antibiotic resistant patterns and those with association of >80% using discriminant analysis for classification into source categories.

Source	Lagoon effluent		Cattle		Swine feces		Deer		Bird		Dog		Raccoon		Nutria		Beaver		Total (n_i)
	CC[a]	MC[b]	CC	MC	CC	MC	CC	MC	CC	MC	CC	MC	CC	MC	CC	MC	CC	MC	
Beaver		0		1		0		0		0		0		0		0	4		**5**
Bird		0		0		0		0	19			0		0		0		0	**19**
Cattle		1	136			0		0		0		0		0		1		0	**138**
Deer		0		0		0	28			0		0		0		0		0	**28**
Dog		0		0		0		0		0	20			0		0		0	**20**
Lagoon effluent	125			0		9		0		0		0		0		0		0	**134**
Nutria		0		0		0		0		0		0		1	22			0	**23**
Raccoon		0		0		0		0		0		0	3			2		0	**5**
Swine feces		5		0	93			0		0		0		0		0		0	**98**
Total	125	6	136	1	93	9	28	0	19	0	20	0	3	1	22	3	4	0	
Total isolates(n)																			**470**
%n CC ($100CC/n_i$)	95.4		99.3		91.2		100.0		100.0		100.0		75.0		88.0		100.0		**94.3[c]**
%n MC($100MC/n-n_i$)	**1.8**		**0.3**		**2.4**		**0.0**		**0.0**		**0.0**		**0.2**		**0.7**		**0.0**		

[a] Number of isolates correctly classified(CC) by discriminant analysis.
[b] Number of isolates misclassified (MC) by discriminant analysis.
[c] Average rate of correct classification.
n= Total isolates n_i= Total source

Table 3.8. Percentage contributions of the sources of *E.coli* isolates in the soil before a spray event, immediately after spray and 1 day after a spray event.

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Source	Percentage Classification										P-value
	Lagoon effluent	Cattle	Swine feces	Deer	Bird	Dog	Raccoon	Nutria	Beaver	Wildlife	
Before spray (n=62)	19.4	50.0	25.8	0.0	0.0	3.2	1.6	0.0	0.0	1.6	
Immediately after spray (n=48)	10.4	33.3	33.3	0.0	2.1	0.0	20.8	0.0	0.0	22.9	
1 day after spray (n=473)	32.4	29.4	29.4	0.0	0.0	0.0	8.8	0.0	0.0	8.8	
Mean	**20.7^{abc}**	**37.6^{a}**	**29.5^{ab}**	**0.0^{c}**	**0.7^{c}**	**1.1^{c}**	**10.4^{bc}**	**0.0^{c}**	**0.0^{c}**	**11.1^{bc}**	**<0.0001**

[a]Means with the same letters are not significantly different (Tukey's test P<0.05)

Table 3.9. Percentage contributions of the sources of *E.coli* isolates in ground water at different locations.

Source	Percentage Classification										P-value
	Lagoon effluent	**Cattle**	**Swine feces**	**Deer**	**Bird**	**Dog**	**Raccoon**	**Nutria**	**Beaver**	**Wildlife**	
Field (n=367)	39.5	32.7	2.7	2.7	2.5	9.5	0.5	0.5	9.3	15.6	
Riparian (n=548)	46.9	21.9	7.7	3.3	3.5	3.5	3.1	4.6	5.7	20.2	
Stream well (n=473)	50.3	40.8	1.9	3.2	0.2	0.9	1.3	0.0	1.5	6.2	
Mean	**45.6[a]**	**31.8[ab]**	**4.1[c]**	**3.1[c]**	**2.1[c]**	**4.6[c]**	**1.6[c]**	**1.7[c]**	**5.5[c]**	**14.0[bc]**	**<0.0001**

[a]Means with the same letters are not significantly different (Tukey's test P<0.05)

Table 3.10. Percentage contributions of the sources of *E.coli* isolates in surface water at different locations.

Source	Percentage Classification										P-value
	Lagoon effluent	**Cattle**	**Swine feces**	**Deer**	**Bird**	**Dog**	**Raccoon**	**Nutria**	**Beaver**	**Wildlife**	
Above Bridge (n=102)	63.7	27.5	2.0	0.0	0.0	2.0	2.9	0.0	2.0	4.9	
Below Bridge (n=144)	54.2	35.4	1.4	0.7	2.8	0.7	4.9	0.0	0.0	8.4	
Adjacent field (n=1715)	66.4	18.5	3.1	2.0	3.7	1.6	2.5	0.3	1.9	10.4	
Mean	**61.4[a]**	**27.1[b]**	**2.2[c]**	**0.9[c]**	**2.2[c]**	**1.4[c]**	**3.4[c]**	**0.1[c]**	**1.3[c]**	**7.9[c]**	**<0.0001**

[a]Means with the same letters are not significantly different (Tukey's test P<0.05)

Table 3.11. Percentage contributions of the sources of *E.coli* isolates in ground water at different seasons of the year.

Season	Percentage Antibiotic Resistance in Ground water										P-value
	Lagoon effluent	Cattle	Swine feces	Deer	Bird	Dog	Raccoon	Nutria	Beaver	wildlife	
Winter (n=97)	21.4[b]	52.8[a]	6.8[b]	2.2[b]	2.8[b]	8.2[b]	1.1[b]	0.0[b]	4.7[b]	10.8[b]	<0.0001
Spring (n=466)	23.2[ab]	46.4[a]	3.6[b]	2.3[b]	3.9[b]	5.5[b]	1.8[b]	4.1[b]	9.4[b]	21.4[ab]	0.0001
Summer (n=17)	0.0a	50.0[a]	0.0[a]	5.6[a]	22.2[a]	5.6[a]	16.8[a]	0.0[a]	0.0[a]	44.4[a]	0.7509
Fall (n=808)	63.3[a]	19.4[b]	4.0[c]	3.6[c]	0.8[c]	3.7[c]	1.7[c]	0.7[c]	2.8[c]	9.6[bc]	<0.0001

[a]Means with the same letters are not significantly different (Tukey's test P<0.05)

Table 3.12. Percentage contributions of the sources of *E.coli* isolates in surface water at different seasons of the year.

Season	Percentage Antibiotic Resistance in Surface water										P-value
	Lagoon effluent	Cattle	Swine feces	Deer	Bird	Dog	Raccoon	Nutria	Beaver	wildlife	
Winter (n=407)	34.5^{ab}	40.9^{a}	5.3^{c}	2.8^{c}	7.9^{c}	2.3^{c}	0.6^{c}	0.3^{c}	5.5^{c}	17.0^{bc}	<0.0001
Spring (n=389)	21.4^{b}	52.8^{a}	0.8^{b}	0.3^{b}	4.4^{b}	2.0^{b}	0.0^{b}	0.3^{b}	0.8^{b}	5.8^{b}	<0.0001
Summer (n=355)	64.7^{a}	21.2^{b}	2.9^{bc}	2.5^{bc}	3.0^{bc}	0.4^{c}	2.9^{bc}	0.2^{c}	2.2^{bc}	10.8^{bc}	<0.0001
Fall (n=564)	74.1^{a}	12.4^{b}	4.0^{c}	2.4^{c}	0.0^{c}	1.7^{c}	4.8^{bc}	0.4^{c}	0.2^{c}	7.9^{bc}	<0.0001

[a]Means with the same letters are not significantly different (Tukey's test $P<0.05$)

CHAPTER 4

ANTIBIOTIC RESISTANCE GENES FOR DETECTION OF FECAL POLLUTION IN SURFACE AND GROUND WATERS

ABSTRACT

Water resources are mostly impacted by fecal pollution and this presents a potential health risk to humans who come in contact with those waters and to establish the sources of these pollutions is often difficult. The presence of resistant genes in *E.coli* can be related with the the sources mostly exposed to that particular antibiotic, hence assist in differentiation of bacteria to establish the source. The goals of this study were 1) to identify and quantify antibiotic resistant genes (ARGs) from *E.coli* isolates recovered from manure and feces of warm blooded animals within Six Runs Creek watershed, 2) to evaluate the effects of season on the levels and sources of ARGs, 3) to assess the possibility of using this data for detection of fecal pollution in surface and ground water within Six Runs Creek watershed. *Escherichia coli* isolates were recovered from manure and water samples by basic microbiological culture and IDEXX Colilert methods, respectively. Polymerase chain reaction (PCR) was used to identify antibiotic resistance genes from *E.coli* isolates. A total of 1,208 *E. coli* isolates from swine feces, lagoon effluent, cattle, deer, bird, dog and nearby ground and surface waters were recovered. All the *E. coli* isolates were screened for ARGs that confer resistance to erythromycin, neomycin, oxytetracycline, streptomycin, tetracycline, cephalothin, apramycin, trimethoprim, and rifampicin. The database with 808 isolates was reduced to 215 unique patterns by removing duplicated patterns and including only isolates associated to a specific group at >40%. This database achieved an average rate of correct classification of 47.2%. Due to low ARCC, the sources sources were combined into two major groups, livestock and wildlife. This approach took into consideration the overwrap observed among the closely related sources. Testing environmental isolates against the reference database, sources indicated that 87% of the isolates in groundwater were derived from livestock and 13% from wildlife. Ninety- two

percent of isolates from surface water were derived from livestock and 8% from wildlife. These results suggest that the host origin library based on ARGs can identify source of *E. coli* in both the ground and surface water when broader source groups are used. However, the reliability of correct classification achieved with ARGs is low as compared to the ARPs approach.

INTRODUCTION

Aquatic environments including ground and surface water are impacted by fecal pollution and this presents a potential health risk to humans who come in contact with those waters. Fecal bacteria from warm-blooded animals include pathogens such as *cryptosporidium parrvum*, *Giardia lamdia*, *salmonella* species and *Escherichia coli* 0157:H7 (Field, 2003). It is difficult to determine the contributing sources of fecal bacteria. Potential sources of these bacteria include livestock waste applied to land, wildlife with direct access to surface water, failing septic systems among other avenues of pollution (Hartel et al, 2002). Previous methods for detection of fecal pollution involved monitoring increases in nutrient concentration against background levels and use of microorganism such as fecal coliforms and fecal streptococci as indicators (Evenson and Strevett, 2006). However, total coliform and fecal coliform measurements can only estimate the quantity of fecal pollution levels, but can not specify the host source of microbial pollution (Carson et. al., 2001).

Genotypic methods which use DNA sequences that are unique to each microorganism have been used to identify the sources of fecal bacteria (Evenson and Strevett, 2006). Some genotypic methods used in MST include ribotyping, pulsed field gel electrophoresis, and PCR methods (Hartel, 2002). The genotypic profiles are much capable to establish the difference between different animal sources, because they are believed to be much more stable than phenotypic profiles (Simpson et al., 2002). When organisms are adapted to a particular environment and become established, the off spring produced by subsequent replications will be genetically identical. Therefore, over time, a group of organisms within

that particular host or environment should possess a similar or identical genetic sequence, different from those organisms adapted to a different host and environment (Scott, at.al 2002).

Molecular methods, such as ribotyping and DNA fingerprinting, that use single colonies to screen against a database produce accurate results but it's costly to achieve required numbers to obtain valid statistical results (Hagedorn et al. 1999). Specific genes may be detected in the environment by using techniques like PCR and molecular gene probe analysis which do not require cultivation (Chee-Sanford et al, 2001). The collection of genes used for such analyses has been extended to specific metabolic genes (Flanagan et al., 1999) and, recently, to antibiotic resistance genes (Aminov et al., 2001). One of the biomarkers used for source identification is *E. coli* toxin gene, which is related with the gut of warm-blooded animals and is based on pathogenic *E. coli* (Jiang et al., 2007). Other functional and virulence genes from *E. coli* have shown to be potential targets for assays in specific groups like cattle, pigs, humans, geese, ducks, and deer (Hamilton et al., 2006; Khatib et al., 2003).

Recently many studies have tried to assess the prevalence of the resistance genes for main antibiotics in *E. coli* populations from animals (Bischoff et al., 2005, Bryan et al., 2004) however, much more research is required to obtain meaningful results for comparisons between *E. coli* isolates from various animal populations. For example, ongoing research conducted in Dr. Graves' laboratory showed that molecular fingerprints and the phenotypic antibiotic resistance fingerprints of swine and cattle differ significantly (not published). Such differences may be attributed to difference in antibiotic use and /or the clonal nature of some *E. coli.* The presence of resistant genes in *E.coli* may be related to a source exposed to that particular antibiotic. In this research, we attempted to use antibiotic resistant genes coding for specific antibiotics in *E.coli* detected by Polymerase Chain Reaction (PCR) to identify their host sources.

The objectives of this study were, 1) to identify and quantify antibiotic resistant genes from *E.coli* isolates recovered from feces of livestock, wildlife and pets within six runs

creek watershed, 2) evaluate the effects of seasonal changes on ARGs in *E.coli* isolates and assess the use of ARGs patterns of environmental *E.coli* isolates to assign fecal pollution of surface and ground water to a specific animals and/or animal groups.

MATERIALS AND METHODS

Site Description. The study was conducted on a commercial swine farm in Sampson County, North Carolina with a herd of 4400 hogs (finishing 720-725 hogs per house) and 100 cattle, located in a 275 ha watershed along the upper part of Six Runs Creek. The site has two waste application fields and a stream which flows in a southerly direction. Adjacent to waste application field 1, the stream flows in a channel, while the segment adjacent to waste application field 2 is impounded by two beaver dams and forms an elongated pond. The stream flows in a channel below the lower beaver dam. The watershed has four swine operations with 23 swine houses (Fig 2.1). Swine feces from the swine houses were washed into waste lagoon once per week. Water levels were maintained at three quarters full to allow storage of excess rain water and to create anaerobic conditions in the lower portion of the lagoon to maintain compliance with waste management regulations. Lagoon effluent was sprayed on to farmland cropped with bermuda grass managed for hay and grazing pasture for cattle. On average, spray events occurs 3 to 4 times during the warm season, once in late fall and early spring. Soil in the waste application field (WAF2) is a Wagram series (loamy, kaolinitic, thermic Arenic Kandiudults) (Fig. 2.1) (Israel et al., 2005).

Known source isolates. Sampling from the site was conducted between October 2007 and December 2008. Samples of fresh swine feces (20) were collected from swine houses, houses, effluent from the lagoon; cow manure and dog feces from the application field and and surrounding areas, bird and deer manure from the riparian. A total of 1,793 *E. coli* isolates from the 6 known sources was used to identify and quantify antibiotic resistant genes in *E. coli* isolates from ground and surface water from the site. The isolates included

included 576 from swine feces, 593 from lagoon effluent, and 480 from cattle and 48 each each from deer, bird and dog.

Isolation of E.coli. Random fecal samples collected from swine pens were combined into a representative composite sample for each swine house occupied by animals. Two samples from the side where manure enters lagoon and the opposite side away from the inlet pipes of the lagoon were collected in sterile glass jars and placed on ice before being transported to the laboratory for analysis. Random fecal samples from cattle and dog were collected from the application field and wildlife samples collected from the riparian. A 10 ml aliquot of effluent sample or 10g of fecal sample was enriched in 90mls of BHI broth (EMD chemicals Inc., Gibbstown, NJ) for 24h at 37°C. The soil core samples (10 cm) collected from the application field was divided into, top samples (0-3 cm) and lower samples (3-7 cm). A 10 ml aliquot of effluent sample or 10 g of fecal and soil samples were enriched in 90 ml of BHI broth (EMD chemicals Inc., Gibbstown, Nj) for 24 h at 37° C to allow *E.coli* isolates to recover from environmental stress prior to molecular characterization. Another 10 g of soil sample was diluted in 100 ml of sterile deionized water. One ml of samples was then diluted into 9mls of sterile water (10^{-1} dilution) and then serial dilutions to 10^{-2}-10^{-6} were made. A 100µl aliquot of the diluted sample was cultured on m-FC agar (Becton Dickinson, sparks, Md.) and incubated at 44.5^{o}C for 24 h to obtain single colonies (Hurst and Crawford, 2002). Single dark blue colonies presumed as fecal coliform were then transferred using a tooth pick to micro wells with colilert broth and cultured for 24h. Five µl of each isolate was inoculated into a vial containing 1ml of pre-enrichment broth (Becton Dickinson, sparks, Md.), mixed, incubated for 24h at 37 ^{o} C and stored at 4 ^{o} C.

Water sample isolates. A total of 3,349 isolates from environmental samples which included 1,961 and 1,388 isolates from ground water and surface water respectively were collected from the site between October 2007 and October 2008. A subset of 200 isolates from ground water and 200 isolates from surface water were randomly selected and used for detection of fecal pollution in the environment based on the presence of ARGs. Surface

water samples were collected from a total of 5 stream sampling sites. These sampling sites sites consisted of upstream (above waste application field 2, see Fig.2.1) and midstream sites in relation to waste application field 2. Ground water samples were collected from wells arranged in transect to the waste application field. These wells were previously used used in a nitrogen movement study (Israel et al., 2005). Two wells were located at each sampling point at different depths. The sampling regime was designed to cover all the four four seasons. The wells (piezometers) were installed in waste application fields and the adjacent forested riparian system on the west side of Six Runs Creek. Two transect had six six wells nests positioned in the field two each at the field- riparian zone edge and at the stream edge (Fig. 2.1). The third transect had only four wells since two wells in the waste application field could not be located. In the waste application fields, wells were placed at at 1 m apart and screened at two different depths: near top of water table, and at a depth below the water table. In a previous nitrogen movement study, water table elevation measurements demonstrated that ground water was moving from the WAF through the riparian area to the stream (Israel et al. 2005). The standing water was initially drained out out of the well to allow the well to recharge with fresh water into the well using a bailing tube. A sample was then drawn from the well using a baling tube into a 500 ml sterile polystyrene bottle and placed on ice before being transported to the laboratory. The samples were analyzed within 6 hrs after collection

Isolates of *E. coli* were recovered by filtration of 50ml and 100ml of sample from stream and well samples respectively through a 0.45µm membrane filter that was then placed on m-FC agar (Becton Dickinson, sparks, Md.) which is selective media for fecal coliforms. The plates were incubated for 24 h in a 44.5°C water bath (Hurst and Crawford, 2002). Individual dark blue colonies were transferred using a tooth pick to 96-microwell plates containing 150µl colilert broth (IDEXX Laboratories Inc., Westbrook, Me.) which is selective media for *E. coli,* and incubated for 24 h at 37°C. Isolates were confirmed as *E. coli* if the microwell fluoresced under UV light.

Genetic analysis. PCR assays were performed to determine which antibiotic resistant gene was present in antibiotic resistant *E.coli* isolates. Primers used for the amplification of the antibiotic resistance genes were based on previous research (Boerlin et al., 2005; Lanz et al., 2003; Perreten and Boerlin, 2003). Isolates resistant to two or more antibiotics were randomly selected from each source and analyzed for presence of antibiotic resistant genes (ARGs) by polymerase chain reaction (PCR). PCR was conducted on a subset of 238 isolates from swine feces, 234 from lagoon effluent, 144 from cattle, 48 from deer, 48 from dog, 48 from bird and 200 each from ground and surface water.

A loopful of bacteria was resuspended in 500µl sterile water, homogenized and heated at 95°C for 15 minutes to prepare a lysate. The mixture was cooled to room temperature and centrifuged for 3 minutes at 14000 rpm. A 5µl volume of supernatant was then transferred to each microwell and used as a template for each 25 µl PCR mixture. Each isolate was evaluated with each of the 10 primer sets (Table 4.1). The PCR mixture was prepared in a reservoir from a 1300 µl aliquot of sybergreen master mix, 600µl of nuclease free water, and 192µl of primer mixture. The primer mixture was made from 24µl (working solution) of forward primer to the same quantity of a corresponding reverse primer to produce a single primer set for a particular antibiotic resistant gene. A fluorescent light which is detected by the thermocycler is produced when SYBR Green reacts with the double stranded DNA. An aliquot of 48µl for the four primer sets were then combined into a primer mixture. Twenty microlitres of the final PCR mix was dispersed into each microwell using a multichannel pipettor. The PCR microwells were capped and placed in a thermocycler for analysis. The following temperature cycles were used to run the PCR : 1 cycle of 4 min at 95°C; 35 cycles, each consisting of 1 min at 95°C, 1 cycle at annealing temperature followed by 1 min at 72°C; and 1 cycle of 7 min at 72°C. Quantifast SYBR Green PCR Kit (Qiagen, Valencia, CA) was used to detect the amplified product. A negative control (no *E.coli*) of 5 µl water was included in all PCR assays.

Statistical analysis. The percentage contribution of each source to the total number of ARGs in this system and the percentage of each type of ARG in each source was calculated using Microsoft office Excel, 2007. SAS statistical software (8.0., SAS Inst., Cary, NC) was used to remove isolates with similar patterns (duplicates) in reference to the presence/absence of the 10 antibiotic resistant genes. Discriminant analysis using jmp software was conducted to classify *E.coli* from known sources into source categories. A two-way source category since we were unable to achieve higher classification rates due to poor clustering of the source groups. Comparison of the different seasons and ranking of the sources in ground water, surface water were conducted using tukey's pair wise comparison.

RESULTS

Antibiotic resistant genes in animal sources. Comparisons among the sources of *E.coli* (swine feces, lagoon effluent, dog, deer, cattle and bird) in terms of abundance of ARGs, revealed that there were no significant differences between swine feces (54.9%), lagoon effluent (54.6%), dog (56.9%), deer (61.5%), cattle (41.3%) and bird (40.2%). On average, *aadA, strB, tetA* and *tetB* were the most abundant antibiotic resistant genes with means >60%. However, these genes were not significantly greater than *strA* and *sul1* means ranging from 50 to 60%. The AGRs *tetC, sul2, sul3* and *Aac(3)IV* were present at very low levels with their means (<30%) and were not significantly different from each other (Table 4.2).

The prevalence of the specific types of ARGs in *E.coli* from all the six fecal sources was assessed. Significant differences were observed among them based on a tukey's test ($P < 0.05$). The *aadA, strB*, *tetA* and *tetB* genes were found in ≥60% of the isolates and the occurrence of these two genes were significantly greater than the occurrence of *tetC, sul2*, *sul3* and *Aac(3)IV* (15.7-27.3%). The *strA* and *sul1* genes were present at 57.5% and 58.6% respectively. However, there were no significant differences in the occurrence of *aadA, tetA, strA, strB, tetB* and *sul1* genes. Among all the ARGs, those that code for

streptomycin/spectinomycin (*aadA/strA* and *strB*), tetracycline (*tetA* and *tetB*) and sulfonamide (*sul1*) were most common among these isolates (Table 4.2).

Antibiotic resistance genes in surface and ground water. Sixty three percent of 200 ground water isolates had ARGs and 68.8% of 200 surface water isolates had ARGs. In 200 *E.coli* isolates from ground water there was a high representation of *aadA, tetA, tetB* and *sul1* genes at levels >70% and a moderate representation of *strA, strB* and *sul3* ARGs at levels between 50 and 60%. The ARGs *tetC, sul2* and *Aac(3)IV* represented between 20 and 50% of the 200. In *E.coli* isolates from surface water, the highest representation of >70% was from *aadA, strA, strB, tetA, tetB* and *sul1*. The ARGs *tetC, sul2* and *sul3* had a moderate contribution to the total ARGs in surface water at levels of between 40 and 50%, while that of *Aac(3)IV* gene was only 10%. On average, *aadA, tetA, tetB* and *sul1* were significantly greater than *Aac(3)IV* in both sources, but not different from *strA, strB, tetC, sul2* and *sul3.* Furthermore, these genes were not significantly different from each other and from *Aac(3)IV* (Table 4.3).

Antibiotic resistant gene database from known sources. Duplicate patterns of the presence/absence of ARGs among the host sources were removed using SAS software. More than 60% of the bird and dog isolates and more than 50% of the swine feces and lagoon effluent isolates were removed from the database. The two sources cattle and wildlife lost 42 and 27% of their isolates respectively (Table 4.4). When discriminant analysis was conducted, the cattle was the only source out of the six with higher correct classification rate (61.6%) while those for dog, bird, lagoon effluent swine feces and deer were between 18 - 40%. The data also indicated that the isolates were related to two or more sources, resulting in high misclassification rates for lagoon effluent (25.3%), swine feces (24.7%) and cattle (15.6%). As a result, we achieved a very low (RCC) of 35.9 % (Table 4.5). When we tried to increase the RCC by using a 40% threshold, we lost more than 60% of the isolates for bird, dog and wildlife resulting in few unique isolates of these categories. Where as those from cattle, swine feces and lagoon effluent lost between 30-50% of their isolates. However, the RCC increased from 35.9 to 47.2 %. Despite the

increase in the RCC, most of the isolates from lagoon effluent were misclassified either as from swine feces or as from cattle and those from swine feces as from lagoon effluent or cattle. This is expressed by the high values of misclassification rates (MC) for those sources (Table 4.6).

As result we combined the isolates into a two-way library of livestock and wildlife. The livestock category included cattle, lagoon effluent and swine feces isolate. The wildlife included bird, dog and deer isolates. The two-way library had an ARCC of (74%). An unknown source isolate could be classified into either category at 50% by chance alone. Given the challenges associated with obtaining a high RCC for both categories, an MDP of 50% was selected. Any source with greater than 50% contribution was considered a significant contributor to fecal pollution (Table 4.7).

Sources of ARGs in environmental samples. Based on the Turkey's test, sampling location and time/treatment conditions had no significant effect on the host sources of antibiotic resistance genes found in the soil, ground water and surface water. The results for soil isolates indicated close association with livestock (97.9%), which was significantly greater than wildlife (2.1%) (P=0.0019) (Table 4.8). The results from the analysis of ground water isolates clearly indicated that livestock (86.8%) was the predominant source of isolates with ARGs in this watershed. This was significantly greater than the contribution of wildlife (13.2%) (P<0.0001) (Table 4.9). In the surface water, livestock (91.9%) was the most predominant contributor of isolates with ARGs and significantly greater than wildlife (8.1%) (P<0.0001) (Table 4.10).

In ground water, seasonal changes had various effects on the antibiotic resistance in the two major sources. In winter, livestock (60%) was significantly greater than wildlife (40%) though statistical analysis was not done since all ground water isolates except one were from a single sample source. However, the values indicate that these sources did not have different contributions. In spring, livestock (81.9%) was significantly greater than wildlife (18.1%) (P=0.0355). In summer, livestock (100.0%) was significantly greater than wildlife (0%) (P=<0.0001). In the fall, livestock (94.3%) was significantly greater than

wildlife (5.7%) (P=0.0067 (Table 4.11). In surface water during winter, percentage of isolates from livestock (92.5%) was significantly greater

than percentage from wildlife (7.5%) (P=0.0005). In spring, percentage of isolates from livestock (89.6%) was significantly greater than percentage from wildlife (10.4%). (P=0.0028). In summer, the percentage of isolates from livestock (92.2%) was significantly greater than percentage wildlife (7.8%) (P=0.0007). For the fall season, percentage of isolates from livestock (94.4%) was significantly greater than percentage from wildlife (5.6%) (P=0.0153) (Table 4.12).

DISCUSSION

Only a subset of the total number of isolates from the site was used for evaluation of antibiotic resistant genes for detection of fecal pollution because of the cost attached to the analysis and the small number of isolates collected in the case of deer, dog and bird.

Establishing a known source database with high correct classification rates based on the presence or absence of ARGs proved to be difficult. Initially the cross classification of lagoon effluent, swine feces and cattle led to misclassification rates (MC) of 25.3%, 24.7% and 15.6%, respectively. When only the isolates which were associated to a specific category at >40% were included, the MC of lagoon effluent, swine feces and cattle were 28%, 24.5% and 11.6%, respectively. These misclassifications may be attributed to cattle and wildlife ingesting grass from the application field sites. According to Chee-Sanford et al., (2009), 75% of antibiotics aren't adsorbed and fully metabolized by animals, thus antibiotic residues persist in the swine waste applied to grazing pasture for cattle. Overtime, it would likely be difficult to distinguish between the two because indirectly, cattle are ingesting the same antibiotics as swine. Campagnolo et al., (2002) reported that various classes of antibiotics were detected in swine waste storage lagoons at levels of >100 μg/l while Brown et al., (2005) detected lincomycin in 2 samples of dairy effluent at 700 and 6600 ng/L. These levels of antibiotics or residues are often released in the effluent being applied to agricultural fields.

In separate studies to determine sources of fecal pollution, Hagedon et.al (1999) achieved an average correct classification rate of 87% with isolates from humans, dairy cattle, beef cattle, chickens, deer, and waterfowl while Wiggins et.al (1999), reported 74% and Grave et al., (2007) reported 95.4% for a rural watershed dominated by cattle. Contrary to the results from livestock in this study, the diversity of ARGs within a wildlife host resulted in low CC rates. Given the small number of wildlife isolate sets included in the database, one would expect a higher level of similarity as what one would see when evaluating isolates for the phenotypic expression of resistance (ARA) (Wiggins et al 2003). However, the opposite seems to be true for wildlife in this study. Several factors may contribute to the diversity of ARGs within a specific wildlife host. These include (1) The migratory nature of wildlife which exposes them to various environments that can have an impact to the development of resistance for example (a) farm to farm movement (b) urban areas. (2) Access to waterways impacted by municipal wastewater treatment plants or septic systems. Kozak et al. (2009) found 20% prevalence of resistance in *E.coli* isolates from small mammals (mice, voles and shrews) from the farm environment, *aadA, sul1, tetB* and *tetC* being the most frequent resistant genes. Skurnik et al., (2006) found 49% resistance genes (*aadA* or *dfrA)* in *E.coli* isolates from wild animals habiting in urban areas with higher human density. The results on ARGs for wildlife in an urban setting were similar to our findings while those for agricultural settings were low. This may be due to the difference in the types of animals being evaluated.

To date, there are no reports on the use of ARGs to determine the sources of fecal pollution in water. However, there are a number of studies that have evaluated the phenotypic expression of resistance (ARA) as a tool for fecal source identification. Olivas and Faulkner (2008) conducted a study in a watershed with low resistance while Graves et al. (2007) conducted their study in a watershed dominated by cattle and Booth et al. (2003) in Virginia's blackish water river found that livestock was the major contributor to fecal pollution. Results from this study suggest that livestock are the major contributor to fecal pollution in ground and surface water in this watershed.

The fact that the overall average numbers of *E.coli* recovered were low in ground water (21.1 CFU/100 mL, chapter 1) indicates that the best management practices (BMPs) were effective at this study site. However we cannot rule out the potential for contamination from bailing procedure and soil particles getting into the well during sampling when removing the covers from the wells. Anderson and Sobsey (2006) in their survey in ground water on swine farms in North Carolina found an average of 5-32.7 CFU/100 mL on swine farms. While Sapkota et al. (2007) found 3-40 CFU/100 mL in ground water near swine farms. The results from this study are similar to these results. Since counts were similar to those of others, contamination was not a serious problem.

The high average numbers of *E.coli* counts observed in surface water (272.1 CFU/100 mL, chapter 1) can not totally be due to pollution contributed by the livestock in this watershed. There are several environmental factors which might be contributing to this high level of fecal coliforms. The surface water body had segments which had low water flow, stagnant water and algae growth indicating high nutrient levels which might promote microbial growth. The water body also had a lot of plant growth which could result in shading, thereby preventing UV light which kills bacteria from penetrating into the water. The high counts in the surface water may be related to the multiplication of the small amount of fecal pollution that enters this water body. The carbon sources from the nearby riparian and the decaying of aquatic plants growing in this water body serve as an energy source for the multiplication of the bacteria entering the water.

CONCLUSIONS

The results indicate that it may be difficult to establish a database of individual host source categories based on the presence or absence of antibiotic resistant genes. This difficulty may be a result of the high variability of ARGs within individual host source categories. The two-way source category approach with broader groups (such as livestock and wildlife) was partially able to overcome this challenge. The results indicated that livestock was the major source of fecal pollution in this part of the watershed and is in agreement with the results generated by antibiotic resistance analysis. However the ARG database still requires sufficient representation in terms of numbers of isolates with unique patterns for all the source categories in order to have a good point of reference for the identification of isolates from water sources. The fact that best management practices are effective at this farm, suggests that elevated levels of bacterial populations in surface water may be a result of proliferation due to presence of organic carbon and low flow conditions in Six Runs Creek.

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Table 4.1. A list of PCR primer sequences (5' to 3') for antibiotic resistant genes used for a each single PCR.

Gene	Primer name	Primer sequence	Anneal (°C)	Fragment size (bp)
aadA	4F[a]	GTGGATGGCGGCCTGAAGCC	68	525
	4R[a]	AATGCCCAGTCGGCAGCG		
strA	2F[a]	CCTGGTGATAACGGCAATTC	55	546
	2R[a]	CCAATCGCAGATAGAAGGC		
strB	3F[a]	ATCGTCAAGGGATTGAAACC	55	509
	3R[a]	GGATCGTAGAACATATTGGC		
tetA	TetA-L[b]	GGCGGTCTTCTTCATCATGC	64	502
	TetA-R[b]	CGGCAGGCAGAGCAAGTAGA		
tetB	TetB-L[b]	CATTAATAGGCGCATCGCTG	64	930
	TetB-R[b]	TGAAGGTCATCGATAGCAGG		
tetC	TetC-L[b]	GCTGTAGGCATAGGCTTGGT	64	888
	TetC-R[b]	GCCGGAAGCGAGAAGAATCA		
sul1	Sul1-L[b]	GTGACGGTGTTCGGCATTCT	68	779
	Sul1-R[b]	TCCGAGAAGGTGATTGCGCT		
sul2	Sul2-L[b]	CGGCATCGTCAACATAACCT	66	721
	Sul2-R[b]	TGTGCGGATGAAGTCAGCTC		
sul3	Sul3-F[c]	GAGCAAGATTTTTGGAATCG	51	880
	Sul3-R[c]	CATCTGCAGCTAACCTAGGGCTTTGGA		
aac(3)IV	Aac4-L[a]	TGCTGGTCCACAGCTCCTTC	59	653
	Aac4-R[a]	CGGATGCAGGAAGATCAA		

Boerlin et al., 2005[a]; Lanz et al., 2003[b]; Perreten and Boerlin, 2003[c];

Table 4.2. The comparison of the number and types of antibiotic resistant genes in *E.coli* isolated from the possible sources of fecal pollution within the watershed.

Gene	Percentage of specific type of resistant genes in each source						Mean
	Swine feces (n=238)	Lagoon effluent (n=234)	Cattle (n=192)	Deer (n=48)	Dog (n=48)	Bird (n=48)	%
aadA	205(86.1)	212(90.6)	116(60.4)	48(100.0)	47(97.9)	45(93.8)	**88.1a**
strA	161(67.6)	139(59.4)	110(57.3)	17(35.4)	41(85.4)	19(39.6)	**57.5abc**
strB	165(69.3)	149(63.7)	106(55.2)	46(95.8)	42(87.5)	25(52.1)	**70.6a**
tetA	225(94.5)	199(85.0)	137(71.4)	48(100.0)	48(100.0)	44(91.7)	**90.4a**
tetB	111(46.6)	124(53.0)	76(39.6)	36(75.0)	44(91.7)	40(83.3)	**64.9a**
tetC	95(39.9)	49(20.9)	52(27.1)	15(31.3)	5(10.4)	1(2.1)	**22.0bcd**
sul1	178(74.8)	177(75.6)	94(49.0)	35(72.9)	25(52.1)	13(27.1)	**58.6ab**
sul2	86(36.1)	97(41.5)	45(23.4)	22(45.8)	3(6.3)	5(10.4)	**27.3bcd**
sul3	40(16.8)	56(23.9)	6(3.1)	21(43.8)	17(35.4)	0(0)	**20.5cd**
Aac(3)IV	40(16.8)	75(32.1)	51(26.6)	7(14.6)	1(2.1)	1(2.1)	**15.7d**
Mean	**54.85**[a]	**54.57**[a]	**41.31**[a]	**61.46**[a]	**56.88**[a]	**40.22**[a]	

[a]Means with the same letters are not significantly different (Tukey's test P<0.05)

Table 4.3. The comparison of the number and percentage of *E.coli* with specific types of antibiotic resistant genes isolated from ground and surface water samples.

Type of gene	Ground water (n=200)	Surface water (n=200)	Mean %
aadA	183 (91.5)	197 (98.5)	**95.0**[a]
strA	120 (60.0)	174 (87.0)	**73.5**[ab]
strB	115 (57.5)	150 (75.0)	**66.3**[ab]
tetA	190 (94.5)	187 (93.5)	**94.0**[a]
tetB	151 (75.5)	187 (93.5)	**84.5**[a]
tetC	56 (28.0)	94 (47.0)	**37.5**[ab]
sul1	168 (84.0)	177 (88.5)	**86.3**[a]
sul2	95 (47.5)	89 (44.5)	**46.0**[ab]
sul3	116 (58.0)	101(50.5)	**54.3**[ab]
Aac(3)IV	69 (34.5)	20 (10.0)	**22.3**[b]

[a]Means with the same letters are not significantly different (Nonparametric1way Savage Scores P<0.05)

[b] the numbers in parenthesis represent percentages of *E.coli*

Table 4.4. The number of *E. coli* isolates analyzed by polymerase chain reaction and discriminant analysis.

Source	Location	Initial	Unique Profiles	Isolates >40%
Lagoon effluent	Lagoon	238	106	58
Swine feces	Swine houses	234	99	60
Cattle	Field	192	112	75
Deer	Riparian	48	35	13
Bird	Riparian	48	19	4
Dog	Farm yard	48	16	5
Ground water	Field well	80	80	80
	Riparian well	73	73	73
	Stream well	47	47	47
Surface water	Stream 1	93	93	93
	Stream 1	41	41	41
	Stream 1	36	36	36
	Above bridge	30	30	30

Table 4.5. Six-category database of *E.coli* isolates with no duplicate antibiotic resistant gene patterns using discriminant analysis for classification into source categories.

Source of isolates	Cattle		Dog		Bird		Lagoon		Swine		Deer		Total(n_i)
	CC[a]	MC[b]	CC	MC	CC	MC	CC	MC	CC	MC	CC	MC	
Cattle	69			3		7		37		31		1	112
Dog		1	3			0		2		0		2	16
Bird		3		2	7			3		5		3	19
Lagoon		23		5		2	35			28		12	106
Swine		12		0		0		21	28			4	99
Deer		4		3		3		8		7	13		35
Total CC or MC	69	43	3	13	7	12	35	71	28	71	13	22	
Total isolates(n)													387
%n CC into category (100CC/n_i)	61.6		18.8		36.8		33.0		28.3		37.1		35.9[c]
%n MC into category (100MC/(n-n_i)	**15.6**		**3.5**		**3.3**		**25.3**		**24.7**		**6.3**		

[a] Number of isolates correctly classified (CC) by discriminant analysis.

[b] Number of isolates misclassified (MC) by discriminant analysis.

[c] Average rate of correct classification.

n= Total isolates n_i= Total source

Table 4.6. Six-category database of *E.coli* isolates with no duplicate antibiotic resistant gene patterns and isolates with association of >40% using discriminant analysis for classification into source categories.

Source of isolates	Cattle		Dog		Bird		Lagoon		Swine		Deer		Total(n_i)
	CC[a]	MC[b]	CC	MC	CC	MC	CC	MC	CC	MC	CC	MC	
Cattle	59			0		2		26		21		0	108
Dog		1	2			0		1		0		2	6
Bird		0		0	2			1		1		0	4
Lagoon		9		2		0	14			15		3	43
Swine		6		0		0		14	22			1	43
Deer		0		1		0		2		1	7		11
Total CC or MC	59	16	2	3	2	2	14	44	22	38	7	6	
Total isolates(n)													215
%n CC into category ($100CC/n_i$)	78.7		40.0		50.0		24.1		36.7		53.8		47.2[c]
%n MC into category ($100MC/(n-n_i)$	**11.4**		**1.4**		**0.9**		**28.0**		**24.5**		**3.9**		

[a] Number of isolates correctly classified (CC) by discriminant analysis.

[b] Number of isolates misclassified (MC) by discriminant analysis.

[c] Average rate of correct classification.

n= Total isolates n_i= Total source

Table 4.7. Two-category database of *E.coli* isolates with no duplicate antibiotic resistant gene patterns and isolates with association of >40% using discriminant analysis for classification into source categories.

Source of isolates	Livestock		Wildlife		Total(n_i)
	CC[a]	MC[b]	CC	MC	
Livestock	187			11	193
Wildlife		6	11		22
Total CC or MC	187	6	11	11	
Total isolates(n)					215
%n CC into category (100CC/n_i)	97.0		50.0		73.5[c]
%n MC into category (100MC/(n-n_i)	**3.0**		**50.0**		

[a] Number of isolates correctly classified (CC) by discriminant analysis.

[b] Number of isolates misclassified (MC) by discriminant analysis.

[c] Average rate of correct classification.

n= Total isolates n_i= Total source

Table 4.8. Percentage contributions of the sources of *E.coli* isolates in the soil before a spray event, immediately after spray and 1 day after a spray event.

Location	Livestock	Wildlife	P-value
Before spray (n=32)	100.0	0.0	
After spray (n=48)	93.8	6.3	
1 Day after spray(n=64)	100.0	0.0	
Mean	**97.2[a]**	**2.1[b]**	**0.0019**

[a]Means with the same letters are not significantly different (Tukey's studentized range (HSD) test $P<0.05$)

Table 4.9. Percentage contributions of the sources of *E.coli* isolates in ground water at different locations.

Location	Livestock	Wildlife	P-value
Field (n=81)	86.4	13.6	
Riparian (n=67)	83.6	16.4	
Stream well (n=52)	90.4	9.6	
Mean	**86.8[a]**	**13.2[bc]**	**<0.0001**

[a]Means with the same letters are not significantly different (Tukey's studentized range (HSD) test P<0.05)

Table 4.10. Percentage contributions of the sources of *E.coli* isolates in surface water at different locations.

Location	Livestock	Wildlife	P-value
Above bridge (n=30)	90.0	10.0	
Stream 1 (n=93)	93.6	6.5	
Stream 2 (n=41)	95.1	4.9	
Stream 3 (n=36)	88.9	11.1	
Mean	**91.9[a]**	**8.1[b]**	**<0.0001**

[a]Means with the same letters are not significantly different (Tukey's studentized range (HSD) test $P<0.05$)

Table 4.11. Percentage contributions of the sources of *E.coli* isolates in ground water at different seasons of the year.

Season	Livestock	Wildlife	P - Value
Winter(n=20)	79.0	21.1	-
Spring(n=110)	81.9[a]	18.1[b]	0.0355
Summer(n=15)	100.0[a]	0.0[b]	<0.0001
Fall(n=70)	94.3[a]	57.7[b]	0.0067

[a]Means with the same letters are not significantly different (Tukey's studentized range (HSD) test P<0.05)

Table 4.12. Percentage contributions of the sources of *E.coli* isolates in surface water at different seasons of the year.

Season	Livestock	Wildlife	P - Value
Winter (n=43)	92.5[a]	7.5[b]	0.0005
Spring (n=40)	89.6[a]	10.4[b]	0.0028
Summer (n=40)	92.2[a]	7.8[b]	0.0007
Fall (n=47)	94.4[a]	5.3[b]	0.0153

[a]Means with the same letters are not significantly different (Tukey's studentized range (HSD) test $P<0.05$)

APPENDICES

Appendix A. Monthly water levels in wells for sampling ground water

LOCATION	October 07	December 07	January 08	March 08	May 08	June 08	July 08	September 08	October 08	December 08
Field 1 (18_20) '	15.4'	15.85'	15.7'	11.55'	11.7'	12.5'	0	14'	11.7'	11.7'
Field 1 (12_14) '	_	0	0	11.55'	11.8'	12.5'	0	13.8'	11.65'	11.6'
Field 2 (17_19) '	13.9'	14.3'	14'	9.9'	10.3'	11.0'	0	12.15'	10.35'	10'
Field 2 (12_14) '	_	0	0	9.45'	10.15'	10.9'	0	12'	10.25'	9.9'
Riparian1 (18_20) '	12.0'	12.15'	11.6'	9.25'	9.25'	9.9'	10.1'	9.9'	9.3'	8.95'
Riparian 1 (10_12) '	10.11'	11.1'	10.6'	8.4'	8.35'	9.0'	9.3'	9.55'	8.65'	8.35'
Riparian 2 (17_19) '	11.4'	11.6'	10.9'	8.3'	8.4'	9.1'	9.3'	9.4'	8.5'	8'
Riparian 2 (10_13) '	7.4'	11.65'	10.9'	7.9'	8.0'	8.65'	9.0'	9.25'	8.2'	7.7'
Riparian 3 (7_9) '	5.7'	5.3'	3.7'	1.6'	9.0'	3.8'	4.0'	2.35'	2.5'	1.4'
Riparian 3 (4_6) '	_	5.3'	3.4'	1.75'	5.5'	3.7'	4.0'	2.3'	2.5'	1.4'
Stream 1 (6_8) '	_	5.1'	4.6'	2.15'	2.0'	2.7'	2.8'	2.5'	2'	1.69'
Stream 1 (2_4) '	_	4.2'	3.6'	2.1'	2.4'	2.85'	3.0'	2.7'	2.2'	1.95'
Stream 2 (6_8) '	_	3.8'	3.4'	1.2'	1.3'	1.65'	1.65'	1.5'	1.15'	0.95'
Stream 2 (2_4) '	_	3.4'	2.7'	1.3'	1.25'	1.9'	2.0'	1.7'	1.35'	1.1'
Stream 3 (6_8) '	6.1'	5.85'	4.9'	2.6'	2.8'	3.7'	3.7'	2.75'	2.7'	2.2'
Stream 3 (2.5_4.5) '	_	4.8'	3.9'	2.4'	2.85'	3.65'	3.75'	2.75'	2.5'	2.3'

Appendix B. Rainfall data for the site during the study period.

Month	Rainfall
Aug-07	3.9'
Sep-07	2.7'
Oct-07	4.8'
Nov-07	0.2'
Dec-07	4.2'
Jan-08	1.7'
Feb-08	7.8'
Mar-08	5.2'
Apr-08	4.3'
May-08	3.8'
Jun-08	1.0'
Jul-08	5.6'
Aug-08	10.7'
Sep-08	10.6'
Oct-08	1.5'
Nov-08	5.2'
Dec-08	2.0'

Appendix C. *E.coli* counts in soil collected from the application field.

Treatment	Time sample collected	Depth	*E.coli* count CFU/g		Mean
			plate 1	plate 2	
Direct inoculation	Before spraying	0-3cm	2.0×10^2	4.5×10^2	3.3×10^2
		3+cm	3.0×10^2	5.5×10^2	4.3×10^2
	After spraying	0-3cm	5.3×10^3	6.5×10^2	3.0×10^3
		3+cm	1.1×10^3	2.0×10^2	6.5×10^2
	1 Day after spraying	0-3cm	1.1×10^3	7.6×10^3	1.8×10^3
		3+cm	2.3×10^3	2.2×10^3	2.2×10^3
Enriched samples	Before spraying	0-3cm	5.0×10^8	1.8×10^7	2.8×10^8
		3+cm	5.0×10^6	1.1×10^8	1.3×10^7
	After spraying	0-3cm	1.1×10^9	1.6×10^8	4.7×10^8
		3+cm	1.5×10^9	3.3×10^7	4.6×10^7
	1 Day after spraying	0-3cm	3.5×10^7	3.8×10^7	2.0×10^8
		3+cm	5.0×10^6	2.1×10^7	2.8×10^7

Appendix D. Most probable number and Plate counts of *E.coli* from water samples by IDEEX and Membrane Filtration methods

Location	Depth	Month	IDEEX			Membrane Filtration		
			Count/100ml	Lower limit	Upper limit	Actual count	Dilution factor	Count/100ml
Field 1	18_20	Oct. 07	0	0	0	3	1	3
Field 1	12_14	Oct. 07	_	_	_	_	_	_
Field 2	17_19	Oct. 07	0	0	0	12	1	12
Field 2	12_14	Oct. 07	_	_	_	_	_	_
Riparian 1	18_20	Oct. 07	1	0.1	5.5	1		10
Riparian 1	10_12	Oct. 07	1	0	3.7	0	1	0
Riparian 2	17_19	Oct. 07	0	0	0	1	1	1
Riparian 2	10_13	Oct. 07	0	0	0	0	1	0
Riparian 3	7_9	Oct. 07	0	0	0	1	1	1
Stream 3	6_8	Oct. 07	0	0	0	2	1	2
Stream	_	Oct. 07	104.6	74.6	142.1	20	2	200
Field 1	18_20	Dec. 07	0	0	0	0	1	0
Field 1	12_14	Dec. 07	_	_	_	_	_	_
Field 2	17_19	Dec. 07	0	0	0	0	1	0
Field 2	12_14	Dec. 07	_	_	_	_		_
Riparian 1	18_20	Dec. 07	0	0	0	0	1	0
Riparian 1	10_12	Dec. 07	0	0	0	0	1	0
Riparian 2	17_19	Dec. 07	0	0	0	0	1	0

Appendix D. Continued

Riparian 2	10_13	Dec. 07	0	0	0	0	1	0
Riparian 3	7_9	Dec. 07	0	0	0	0	1	0
Riparian 3	4_6	Dec. 07	0	0	0	0	1	0
Stream 1	6_8	Dec. 07	0	0	0	0	1	0
Stream 1	2_4	Dec. 07	0	0	0	0	1	0
Stream 2	6_8	Dec. 07	0	0	0	0	1	0
Stream 2	2_4	Dec. 07	0	0	0	0	1	0
Stream 3	6_8	Dec. 07	0	0	0	0	1	0
Stream 3	2.5_4.5	Dec. 07	0	0	0	0	1	0
Field 1	18_20	Jan. 08	0	0	0	0	1	0
Field 1	12_14	Jan. 08	_	_	_	_	_	_
Field 2	17_19	Jan. 08	0	0	0	0	1	0
Field 2	12_14	Jan. 08	_	_	_	_	_	_
Riparian 1	18_20	Jan. 08	2	0.3	7.1	0	1	0
Riparian 1	10_12	Jan. 08	0	0	0	0	1	0
Riparian 2	17_19	Jan. 08	20.3	12.1	32.2	14	1	14
Riparian 2	10_13	Jan. 08	1	0	3.7	0	1	0
Riparian 3	7_9	Jan. 08	0	0	0	0	1	0
Riparian 3	4_6	Jan. 08	0	0	0	0	1	0
Stream 1	6_8	Jan. 08	0	0	0	0	1	0
Stream 1	2_4	Jan. 08	0	0	0	0	1	0
Stream 2	6_8	Jan. 08	2	0.3	7.1	0	1	0
Stream 2	2_4	Jan. 08	0	0	0	0	1	0
Stream 3	6_8	Jan. 08	0	0	0	0	1	0

Appendix D. Continued

Stream 3	2.5_4.5	Jan. 08	0	0	0	0	1	0
stream 1A	_	Jan. 08	8.5	3.9	15.6	14	2	28
stream 1B	_	Jan. 08	18.3	10.5	28.8	43	2	86
stream 2A	_	Jan. 08	3.1	0.7	8.9	15	2	30
stream 2B	_	Jan. 08	74.3	53	98.8	48	2	96
stream 3A	_	Jan. 08	198.9	141.8	273.3	61	2	122
stream 3B	_	Jan. 08	7.5	3.6	14.9	11	2	22
Above bridge	_	Jan. 08	39.9	26.9	56.5	30	2	60
Below Bridge	_	Jan. 08	162.4	119	215.7	45	2	90
Field 1	18_20	Mar. 08	214.2	152.7	294.4	17	2	34
Field 1	12_14	Mar. 08	88.8	65	117.5	0	1	0
Field 2	17_19	Mar. 08	549.3	402.3	724.5	49	2	98
Field 2	12_14	Mar. 08	378.4	262.4	526.2	5	2	10
Riparian 1	18_20	Mar. 08	501.2	357.3	687.9	71	2	142
Riparian 1	10_12	Mar. 08	141.4	103.5	187.8	24	2	48
Riparian 2	17_19	Mar. 08	721.5	542.6	941.5	92	2	184
Riparian 2	10_13	Mar. 08	601.5	440.6	804.5	40	2	80
Riparian 3	7_9	Mar. 08	12	6	20.3	0	1	0
Riparian 3	4_6	Mar. 08	36.8	24.8	52.2	11	2	22
Stream 1	6_8	Mar. 08	202.5	164.5	244.1	82	2	164
Stream 1	2_4	Mar. 08	6.3	2.9	13.7	0	1	0
Stream 2	6_8	Mar. 08	191.8	136.7	264.5	60	2	120
Stream 2	2_4	Mar. 08	71.7	51.1	97.5	16	2	32

Appendix D. Continued

Stream 3	6_8	Mar. 08	38.9	26.2	55.9	15	2	30
Stream 3	2.5_4.5	Mar. 08	27.2	17.3	40.2	4	2	8
stream 1A	_	Mar. 08	248.9	172.6	3502	41	2	82
stream 1B	_	Mar. 08	_	_	_	_		_
stream 2A	_	Mar. 08	1011.2	740.6	1323.5	67	2	134
stream 2B	_	Mar. 08	_	_	_	_		_
stream 3A	_	Mar. 08	5.2	2.3	11.9	23	2	46
stream 3B	_	Mar. 08	_	_	_	_		_
Field 1	18_20	May 08	21.6	12.9	33.7	8	2	16
Field 1	12_14	May 08	5.2	2.3	11.9	1	2	2
Field 2	17_19	May 08	7.4	3.2	14.4	10	2	20
Field 2	12_14	May 08	0	0	0	1	2	2
Riparian 1	18_20	May 08	2	0.3	7.1	1	2	2
Riparian 1	10_12	May 08	4.1	1.7	9.5	2	2	4
Riparian 2	17_19	May 08	1	0	3.7	1	2	2
Riparian 2	10_13	May 08	0	0	0	0	1	0
Riparian 3	7_9	May 08	0	0	0	14	2	28
Riparian 3	4_6	May 08	1	0	3.7	9	2	18
Stream 1	6_8	May 08	8.5	3.9	15.6	3	2	6
Stream 1	2_4	May 08	1	0	3.7	1	2	2
Stream 2	6_8	May 08	0	0	0	0	1	0
Stream 2	2_4	May 08	0	0	0	0	1	0
Stream 3	6_8	May 08	1	0	3.7	0	1	0

Appendix D. Continued

Stream 3	2.5_4.5	May 08	1	0	3.7	0	1	0
stream 1A	_	May 08	658.6	482.5	886.5	62	10	620
stream 1B	_	May 08	260.3	175.4	365.2	13	10	130
stream 2A	_	May 08	829.7	623.9	1108.7	33	10	330
stream 2B	_	May 08	1011.2	740.6	1323.5	91	10	910
stream 3A	_	May 08	306.3	248.3	378.5	40	10	400
stream 3B	_	May 08	243.6	188	305.1	24	10	240
Field 1	18_20	June 08	0	0	0	0	1	0
Field 1	12_14	June 08	0	0	0	0	1	0
Field 2	17_19	June 08	0	0	0	8	2	16
Field 2	12_14	June 08	0	0	0	0	1	0
Riparian 1	18_20	June 08	3	0.7	7.4	0	1	0
Riparian 1	10_12	June 08	1	0	3.7	1	2	2
Riparian 2	17_19	June 08	0	0	0	0	1	0
Riparian 2	10_13	June 08	0	0	0	0	1	0
Riparian 3	7_9	June 08	0	0	0	0	1	0
Riparian 3	4_6	June 08	0	0	0	0	1	0
Stream 1	6_8	June 08	0	0	0	0	1	0
Stream 1	2_4	June 08	0	0	0	0	1	0
Stream 2	6_8	June 08	0	0	0	0	1	0
Stream 2	2_4	June 08	0	0	0	0	1	0
Stream 3	6_8	June 08	0	0	0	0	1	0
Stream 3	2.5_4.5	June 08	0	0	0	0	1	0
stream 1A	_	June 08	193.5	130.4	279.5	23	10	230

Appendix D. Continued

stream 1B	_	June 08	78	55.6	103.8	20	10	200
stream 2A	_	June 08	26.6	16.9	39.2	2	10	20
stream 2B	_	June 08	37.3	25.2	53.3	5	10	50
stream 3A	_	June 08	10.9	5.6	19.5	2	10	20
stream 3B	_	June 08	20.1	12.4	31.8	2	10	20
Field 1	18_20	July 08	_	_	_	_	_	_
Field 1	12_14	July 08	_	_	_	_	_	_
Field 2	17_19	July 08	_	_	_	_	_	_
Field 2	12_14	July 08	_	_	_	_	_	_
Riparian 1	18_20	July 08	0	0	0	0	1	0
Riparian 1	10_12	July 08	4.1	1.2	9	1	10	10
Riparian 2	17_19	July 08	4.1	1.2	9	0	1	0
Riparian 2	10_13	July 08	0	0	0	0	1	0
Riparian 3	7_9	July 08	0	0	0	1	2	2
Riparian 3	4_6	July 08	0	0	0	0	1	0
Stream 1	6_8	July 08	0	0	0	0	1	0
Stream 1	2_4	July 08	0	0	0	0	1	0
Stream 2	6_8	July 08	0	0	0	2	2	4
Stream 2	2_4	July 08	0	0	0	0	1	0
Stream 3	6_8	July 08	0	0	0	4	2	8
Stream 3	2.5_4.5	July 08	0	0	0	143	2	286
stream 1A	_	July 08	76.3	58.8	96.2	25	2	50
stream 1B	_	July 08	286.3	232	343.5	28	10	280
stream 2A	_	July 08	184.7	149.7	223.6	34	2	68

Appendix D. Continued

stream 2B	_	July 08	1011.2	740.6	1323.5	277	10	2770
stream 3A	_	July 08	4.1	1.2	9	13	2	26
stream 3B	_	July 08	4.1	1.2	9	3	10	30
L1 1ml	_	July 08	499.6	404.9	618.9	_	_	_
L1 10ml	_	July 08	1011.2	740.6	1323.5	_	_	_
L2 1ml	_	July 08	499.6	404.9	618.9	_	_	_
L2 10ml	_	July 08	1011.2	740.6	1323.5	_	_	_
L3 1ml	_	July 08	437.4	354.6	526.2	_	_	_
L3 10ml	_	July 08	1011.2	740.6	1323.5	_	_	_
L4 1ml	_	July 08	362.3	293.7	439.5	_	_	_
L4 10ml	_	July 08	1011.2	740.6	1323.5	_	_	_
L5 1ml	_	July 08	317.4	263.5	378.5	_	_	_
L5 10ml	_	July 08	1011.2	740.6	1323.5	_	_	_
Field 1	18_20	Sept.08	115.3	82.2	158.1	31	10	310
Field 1	12_14	Sept. 8	47.1	31.8	66.6	6	10	60
Field 2	17_19	Sept.08	18.9	11.3	30.4	62	10	62
Field 2	12_14	Sept.08	248.9	172.6	350.2	2	10	20
Riparian 1	18_20	Sept.08	658.6	482.4	886.5	19	10	190
Riparian 1	10_12	Sept.08	238.2	165.2	340.8	5	10	50
Riparian 2	17_19	Sept.08	71.7	51.1	97.5	7	10	70
Riparian 2	10_13	Sept.08	62.7	44.7	85.3	12	1	12
Riparian 3	7_9	Sept.08	24.5	16	36.1	31	1	31
Riparian 3	4_6	Sept.08	870.4	654.5	1153.8	24	1	24

Appendix D. Continued

Stream 1	6_8	Sept.08	206.4	147.1	283.1	3	10	30
Stream 1	2_4	Sept.08	218.7	151.7	314.5	1	10	10
Stream 2	6_8	Sept.08	344.1	245.3	472.5	11	10	110
Stream 2	2_4	Sept.08	93.4	68.4	124.2	2	10	20
Stream 3	6_8	Sept.08	185	131.9	256.3	23	10	230
Stream 3	2.5_4.5	Sept.08	101.9	72.7	140.4	7	10	70
stream 1A	_	Sept.08	689.3	518.3	892.6	40	10	400
stream 1B	_	Sept.08	601.5	440.6	804.5	29	10	290
stream 2A	_	Sept.08	913.9	705	1174.6	47	10	470
stream 2B	_	Sept.08	960.6	703.6	1245.4	51	10	510
stream 3A	_	Sept.08	1011.2	740.6	1323.5	74	10	740
stream 3B	_	Sept.08	960.6	703.6	1245.4	57	10	570
L1 1ml	_	Sept.08	133.3	92.4	186.9	9	100	900
L2 1ml	_	Sept.08	73.3	53.7	96.2	25	100	2500
L3 1ml	_	Sept.08	79.5	59.8	103.6	21	100	2100
L4 1ml	_	Sept.08	61.1	45.9	79.5	14	100	1400
L5 1ml	_	Sept.08	73.8	54	96.2	19	100	1900
Field 1	18_20	Oct. 08	<1.0	0	3.7	4	2	8
Field 1	12_14	Oct. 08	1	0.1	5.5	0	1	0
Field 2	17_19	Oct. 08	2	0.3	7.1	3	2	6
Field 2	12_14	Oct. 08	<1.0	0	3.7	0	1	0
Riparian 1	18_20	Oct. 08	8.5	3.9	15.6	6	2	12
Riparian 1	10_12	Oct. 08	3.1	0.7	8.9	5	2	10

Appendix D. Continued

Riparian 2	17_19	Oct. 08	25	15.9	37.1	11	2	22
Riparian 2	10_13	Oct. 08	<1.0	0	3.7	0	1	0
Riparian 3	7_9	Oct. 08	1	0	5.5	0	1	0
Riparian 3	4_6	Oct. 08	1	0	5.5	1	2	2
Stream 1	6_8	Oct. 08	<1.0	0	3.7	4	2	8
Stream 1	2_4	Oct. 08	<1.0	0	3.7	0	1	0
Stream 2	6_8	Oct. 08	<1.0	0	3.7	3	2	6
Stream 2	2_4	Oct. 08	<1.0	0	3.7	0	1	0
Stream 3	6_8	Oct. 08	<1.0	0	3.7	0	1	0
Stream 3	2.5_4.5	Oct. 08	1	0	5.5	0	1	0
stream 1A	_	Oct. 08	39.9	26.9	56.5	1	10	10
stream 1B	_	Oct. 08	44.8	30.2	63.4	16	10	160
stream 2A	_	Oct. 08	313	217	439.5	39	10	390
stream 2B	_	Oct. 08	193.5	130.4	279.5	31	10	310
stream 3A	_	Oct. 08	148.3	105.7	199.9	19	10	190
stream 3B	_	Oct. 08	313	217	439.5	47	10	470
Below Bridge	_	Oct. 08	31.5	20.6	45.7	8	10	80
Above ridge	_	Oct. 08	183.5	134.4	243.1	38	10	380
Field 1	18_20	Dec. 08	5.2	1.8	10.8	3	2	6
Field 1	12_14	Dec. 08	<1.0	0	3.7	4	2	8
Field 2	17_19	Dec. 08	<1.0	0	3.7	1	2	2
Field 2	12_14	Dec. 08	5.2	1.8	10.8	11	2	22

Appendix D. Continued

Riparian 1	18_20	Dec. 08	5.2	2.3	11.9	3	2	6
Riparian 1	10_12	Dec. 08	1	0.1	5.5	0	1	0
Riparian 2	17_19	Dec. 08	7.4	3.2	14.4	0	1	0
Riparian 2	10_13	Dec. 08	6.3	2.9	13.7	5	2	10
Riparian 3	7_9	Dec. 08	<1.0	0	3.7	0	1	0
Riparian 3	4_6	Dec. 08	<1.0	0	3.7	0	1	0
Stream 1	6_8	Dec. 08	<1.0	0	3.7	2	2	4
Stream 1	2_4	Dec. 08	<1.0	0	3.7	0	1	0
Stream 2	6_8	Dec. 08	2	0.3	7.1	1	2	2
Stream 2	2_4	Dec. 08	9.8	4.7	18.4	6	2	12
Stream 3	6_8	Dec. 08	1	0.1	5.5	0	1	0
Stream 3	2.5_4.5	Dec. 08	<1.0	0	3.7	4	2	8
stream 1A	_	Dec. 08	689.3	518.3	892.6	91	10	910
stream 1B	_	Dec. 08	378.4	262.4	526.2	56	10	560
stream 2A	_	Dec. 08	90.9	66.6	121.6	8	10	80
stream 2B	_	Dec. 08	114.5	81.6	155.5	12	10	120
stream 3A	_	Dec. 08	70.3	50.1	94.6	12	10	120
stream 3B	_	Dec. 08	90.6	64.6	124.1	10	10	100
Below Bridge	_	Dec. 08	96	68.6	131.7	18	10	180
Above Bridge	_	Dec. 08	57.3	39.7	79.1	20	10	200

Appendix E. Values for Statistical analysis of the paired comparisons for swine feces, lagoon effluent and soil sources.

Gene	Swine feces - lagoon effluent		Lagoon effluent - soil	
	P-value	**Std. Dev**	**P-value**	**Std. Dev**
aadA	0.1311	3.71	0.0136	2.48
strA	0.0630	6.50	0.3862	6.06
strB	0.1937	6.24	0.8584	5.61
tetA	0.0007	3.46	0.0105	3.22
tetB	0.1680	7.54	<0.0001	5.59
tetC	<0.0001	8.54	0.0049	7.53
sul1	0.8306	5.39	0.2395	4.84
sul2	0.2368	8.18	0.0470	7.16
sul3	0.0548	8.73	<0.0001	7.52
Aac(3)IV	0.0001	8.69	<0.0001	7.58

Appendix F. Values for Statistical analysis of the paired comparisons of genes in lagoon effluent isolates per season.

Gene	Fall - Winter		Winter - Spring		Spring - Summer	
	P-value	**Std. Dev**	**P-value**	**Std. Dev**	**P-value**	**Std. Dev**
aadA	0.0193	2.31	0.6288	1.12	0.3173	0.50
strA	0.0125	3.36	0.2860	2.22	0.0462	1.32
strB	<0.0001	3.01	<0.0001	2.39	0.0105	1.22
tetA	0.3338	1.20	0.4412	1.41	0.3047	1.00
tetB	0.7605	4.33	0.0149	3.18	0.0691	2.46
tetC	0.0448	4.43	0.7509	3.28	0.7923	2.37
sul1	0.0008	2.95	0.0556	2.76	0.0296	1.80
sul2	0.9129	4.70	<0.0001	3.04	<0.0001	2.67
sul3	0.6800	3.00	<0.0001	3.45	<0.0001	2.79
Aac(3)IV	0.4611	3.83	<0.0001	3.58	<0.0001	2.94

Appendix G. Values for Statistical analysis of the paired comparisons of genes in soil isolates.

Gene	Before spray-After Spray		Before Spray -A Day After Spray		After Spray -A Day After Spray	
	P-value	**Std. Dev**	**P-value**	**Std. Dev**	**P-value**	**Std. Dev**
aadA	0.2844	0.77	0.8307	0.91	0.3476	0.50
strA	0.9376	3.01	0.3080	3.24	0.4464	2.72
strB	<0.0001	2.51	<0.0001	2.57	0.5479	2.96
tetA	0.2143	0.89	0.0800	1.37	0.0300	1.12
tetB	0.1100	2.08	0.2985	1.83	0.0316	1.72
tetC	0.9342	3.63	0.5566	3.77	0.6857	3.04
sul1	0.0077	2.69	0.3251	2.37	0.0029	2.32
sul2	0.1052	3.58	0.0211	3.78	0.5756	3.18
sul3	0.2844	2.71	0.2546	2.81	1.0000	0.0
Aac(3)IV	0.3321	3.37	0.2688	3.50	0.9289	2.34

Appendix H. Plate counts of *E.coli* from fecal samples from the known sources.

Date/Sample Number		***E.coli* counts(CFU/10g)**					
		Lagoon effluent	**Swine feces**	**Cattle**	**Wildlife**	**Dog**	**Bird**
Oct 07	1	$2.4x10^7$	5.6×10^6	7.5×10^7	1.7×10^8		
	2	4.5×10^7	4.8×10^5	4.7×10^7			
	3	1.1×10^6	9.0×10^3	7.0×10^6			
	4	5.0×10^6	1.2×10^4				
	5	1.0×10^5					
Dec 07	1		7.0×10^6	6.0×10^8			
	2		1.0×10^3	9.0×10^6			
	3		7.3×10^5				
	4		6.4×10^8				
Jan 08	1	9.0×10^8	3.1×10^7	3.0×10^7		5.5×10^6	6.3×10^6
	2	5.0×10^8	1.3×10^8	2.3×10^8			
	3	3.9×10^6	2.8×10^7	1.1×10^8			
	4		1.0×10^8				
	5		8.0×10^6				
	6		4.0×10^6				
Mar 08	1	$2.8 x10^7$	2.3×10^4	9.0×10^7			
	2	5.3×10^6	2.6×10^4	4.0×10^8			
	3	9.9×10^6	1.1×10^5	7.8×10^7			
	4		3.4×10^7	4.6×10^8			
	5		5.1×10^7				
Mean		$\mathbf{1.4 \times 10^7}$	$\mathbf{5.5 \times 10^6}$	$\mathbf{1.7 \times 10^7}$	$\mathbf{1.7 \times 10^8}$	$\mathbf{5.5 \times 10^6}$	$\mathbf{6.3 \times 10^6}$
Std dev		$\mathbf{2.9 \times 10^7}$	$\mathbf{1.5 \times 10^7}$	$\mathbf{2.0 \times 10^7}$			

Appendix I. Number of *E.coli* isolates collected from the site during the study (Oct. 07 - Feb. 09).

Date	Source												Sub total
	Swine feces	Lagoon effluent	Cattle	Bird	Dog	Deer	Raccoon	Nutria	Beaver	Surface water	Ground water	Soil	
Oct 07	144	96	144	0	0	0	0	0	0	36	60	0	**480**
Dec 07	48	48	48	0	0	48	0	0	0	0	0	0	**192**
Jan 08	144	96	96	48	48	0	0	0	0	205	21	0	**658**
Mar 08	240	144	192	0	0	0	0	0	0	101	418	0	**1095**
May 08	0	0	0	0	0	0	0	0	0	288	48	0	**336**
Jun 08	0	0	0	0	0	0	0	0	0	180	13	0	**193**
Jul 08	0	480	0	0	0	0	0	0	0	175	4	0	**659**
Sept 08	0	208	0	0	0	0	0	0	0	288	679	144	**1319**
Oct 08	0	0	0	0	0	0	0	0	0	310	69	0	**379**
Dec 08	0	0	0	0	0	0	0	0	0	378	76	0	**454**
Feb 09	0	0	0	0	0	0	48	48	48	0	0	0	**144**
Total	**576**	**1072**	**480**	**48**	**48**	**48**	**48**	**48**	**48**	**1961**	**1388**	**144**	**5909**

Appendix J. SAS code for the removal of isolates with duplicate patterns.

```
data E.coli;
input Source RIF60 RIF75 RIF90 OTC2 OCT5 OTC7 OTC10 OTC15 STR2 STR5 SRT7 STR10 STR15 CEP15 CEP25 CEP35 ERY60 ERY70
ERY90 ERY100 TET2 TET5 TET7 TET10 TET15 NEO2 NEO5 NEO10 APR8 APR16 APR32 APR64 APR128 TRI8 TRI16 TRI32 TRI64 TRI128;
Datalines;

;
Proc sort Data= E.coli out= Ecoli2 NODUPKEY;
By Source RIF60 RIF75 RIF90 OTC2 OCT5 OTC7 OTC10 OTC15 STR2 STR5 SRT7 STR10 STR15 CEP15 CEP25 CEP35 ERY60 ERY70 ERY90
ERY100 TET2 TET5 TET7 TET10 TET15 NEO2 NEO5 NEO10 APR8 APR16 APR32 APR64 APR128 TRI8 TRI16 TRI32 TRI64 TRI128;
Run;
Proc Print Data= Ecoli2;
Run;
data Ecoli;
input Source aadA STRA   STRB   TETA   TETB   TETC   SUL1   SUL2   SUL3   Aac;
datalines;

;
Proc sort Data= Ecoli out= Ecoli2 NODUPKEY;
By Source aadA   STRA   STRB   TETA   TETB   TETC   SUL1   SUL2   SUL3   Aac;
run;
Proc Print Data= Ecoli2;
Run;
```