

Report as of FY2009 for 2007NC76B: "Antibiotic Resistance and Water Quality: Land Application of Swine Lagoon Effluent as a Potential Source of Antibiotic Resistant Genes in Surface Water"

Publications

- Other Publications:
 - ◆ Liwimbi, L.C. 2009. Antibiotic Resistance and Water Quality: Land Application of Swine Lagoon Effluent as a Potential Source of Antibiotic Resistant Genes in Surface Water., MS Thesis, Dept. of Soil Science, NC State University, Raleigh, NC.

Report Follows

Title

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

Problem

The use of antibiotics in animals is suspected to be a major route of the transference of antibiotic resistant bacteria to humans, even when different antibiotics are used in animals than in people. Mathematical models have been used to evaluate the medical impacts of simultaneously using the same antibiotic in food animals and human medicine. Analysis from the mathematical models demonstrates that animal antibiotic use may hasten the appearance of antibiotic resistance and decrease the efficacy of antibiotic used in humans. A number of reports have specifically linked antibiotic use in livestock with the spread of antibiotic resistant pathogenic bacterial to humans. North Carolina is the home of our Nation's second largest swine industry. Most of this swine production is restricted to a small geographical area in southeastern North Carolina. This high concentration of swine production may increase the risk of antibiotic resistant bacteria from swine operations reaching the nearby surface waters. If antibiotic resistance and the presence of antibiotic resistant genes are occurring at an elevated level in swine waste, then it logically follows that antibiotic resistant genes found in bacteria are potentially discharged during land application of swine lagoon effluent and have the potential to reach nearby surface waters. The goal of this study is to evaluate the association of antibiotic resistance genes found in *E.coli* isolated from swine with the actual phenotypic expression of the resistance. Additionally to develop an antibiotic resistance database for *E. coli* isolates from a commercial swine facility and assess its efficacy for tracking movement of bacteria from swine confinement houses to surface waters. The appearance of swine-manure derived bacteria in shallow groundwater near the stream or in the stream would document the need for improved mitigation strategies. To establish that swine manure-derived bacteria are discharged to surface waters, source tracking methods will be used.

The predominant manure management choice for swine is the lagoon system. Anaerobic lagoons are widely used in temperate climates in the United States for the treatment of swine manure. They are simple to manage and very effective in reducing organic matter and nutrients when properly designed and operated (Bicudo et al, 1999). Anaerobic lagoons store, treat and minimally dilute the waste from concentrated animal feeding operations (CAFO). Lagoons, however, were not designed to control pathogens, despite the fact that swine manure contains as high as a billion protozoa, fungi and bacteria per gram.

Previous studies showed that pathogens can persist in swine lagoon liquid and sludge, in manure piles, and in waste litter (Plym-Forsell 1995; Radtke and Gist 1989). Pathogens are more likely to persist in liquid or moist waste, and in sludge or lagoon treatments, which do not heat manure to a high enough temperature to kill pathogens (Kudva et al. 1998). Hog manure may contain pathogens like *Cryptosporidium* and *Salmonella*, which can cause diarrhea in normal healthy adults, but can be fatal in children, the elderly and other groups at risk. (Sobsey et al, 1999). Raw hog waste applied to crops can contain 100 to 10,000 times the number of pathogens that is allowed in treated human waste (Sobsey et al, 1999). However, since raw hog waste is rarely if ever applied to crop land in North Carolina, the level of human pathogens in effluent from treatment lagoons applied to crops is likely to be lower than that reported for raw manure (Sobsey, et al. 1999) Nevertheless, since pathogens move easily through air and water, there is potential for transmission from swine operations to humans.

Research Objectives

The goal of this study is to evaluate the association of antibiotic resistance genes found in *E.coli* isolated from swine with the actual phenotypic expression of the resistance. Additionally to develop an antibiotic

resistance database for *E. coli* isolates from a commercial swine facility and assess its efficacy for tracking movement of bacteria from swine confinement houses to surface waters.

1. Determine the relationship between presence of antibiotic resistance genes for tetracycline, sulfonamides, streptomycin and apramycin resistant genes found in *E. coli* strains from swine manure, lagoon effluent and nearby ground and surface waters with the actual phenotypic expression of the resistance.
2. Develop a database of antibiotic resistance patterns for *E. coli* isolated from swine manure, cattle manure, wildlife manure, human and pets.
3. Evaluate the usefulness of this database for assessing movement (or dispersal) of *E. coli* from a confined swine operation to a nearby stream.

The goal of this study is to identify and quantify *E.coli* isolates with antibiotic resistant genes in raw swine manure, lagoon effluent from a commercial swine facility and in nearby ground and surface waters. The appearance of swine-manure derived bacteria in shallow groundwater near the stream or in the stream would document the need for improved mitigation strategies. To establish that swine manure-derived bacteria are discharged to surface waters, source tracking methods will be used.

Methodology

The Soil Science Department has well equipped laboratories for molecular and microbiological analysis of manure, water/wastewater, and soil. Dr. Graves's laboratory is equipped with a Mastercycler ep *realplex* real-time thermal cycling system, eppendorf thermocycler for conventional PCR, agarose gel electrophoresis units, gel documentation systems, membrane –filter manifolds, centrifuges, water baths, incubators, refrigerators, -20°C and -80°C freezers. The lab also houses PC computers with internet access.

Study Site: The study site (Figure 1) is a commercial swine farm with a standing herd of 4400 finishing animals, located in a 275 ha watershed along the upper reach of Six Runs Creek, which flows in a southerly direction in eastern Sampson County, NC. The study site is approximately 18 km north of Clinton, NC. The study site has two waste application fields. The stream adjacent to waste application field 1 flows in a channel, but the segment adjacent to waste application field 2 is impounded by two beaver dams and forms an elongated pond. Below the lower beaver dam the stream flows in a channel as it exits the producer's property. Four swine operations with 23 swine houses are located in this watershed. Fields receiving swine-lagoon effluent (approximately 40 ha) and cropped with coastal bermuda grass managed for hay or as grazed pastures are situated on both sides of the creek. A forested riparian buffer of variable width (41 to 87m) is located between the waste application fields and the creek. Three transects of piezometers (wells) have been installed in each of two waste application fields and the adjacent forested riparian system on the west side of Six Runs Creek for sampling of shallow ground water. Each transect has four or five well nests positioned on the side slope of the field, at the field edge, in the riparian zone, and at the stream edge. In the waste application fields, wells within a nest have been placed 1 m apart and screened at three different depths: near top of water table, and at two greater depths below the water table (Israel et al., 2005).

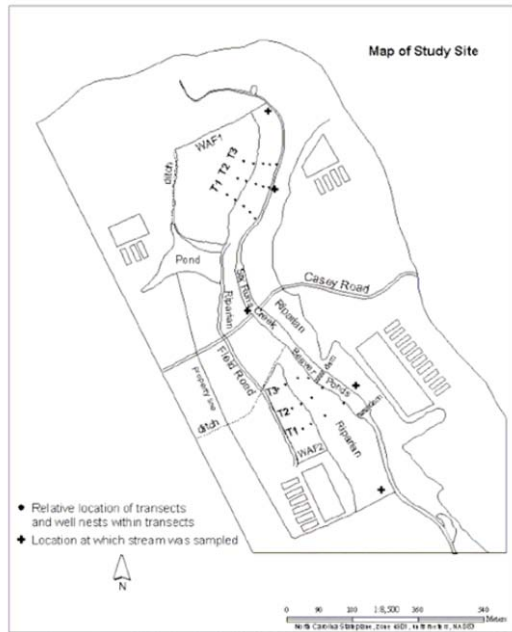


Figure 1. Map of study site. Figure found in Israel et al., 2005

Procedures for Objective 1: A combined total of 300 *E. coli* isolates from swine houses, lagoons, ground and surface waters will be evaluated for antibiotic resistance genes and phenotypic expression of antibiotic resistances. Shallow groundwater will be sampled from wells in the sprayfield and at the stream edge and the stream will be sampled upstream, adjacent to and down stream of the swine operation. Swine manure and lagoon effluent samples will be serially diluted (surface water and groundwater samples will not be diluted) and filtered on membrane filters. Filters will be transferred to plates and incubated at 44.5°C. After 24 h single colonies will be picked and transferred to liquid media and incubated at 37°C. After 24 h an aliquot of each culture will be taken for PCR analysis and another aliquot will be transferred to micro-well plates for the antibiotic resistance evaluations.

PCR detection of resistance genes. Bacterial lysates will be used as templates for the PCR reactions. Lysates will be prepared by resuspending a loopful of bacteria from a fresh overnight culture on a blood agar plate will be resuspended in 500µl of water, homogenized and heated at 95°C for 15 min. After cooling to room temperature, suspensions will be centrifuged for 3 min at maximum speed in a microcentrifuge. A 1-µl volume of the supernatant will be used as a template for each 25-µl PCR mixture. The primers and protocols for major resistance genes for tetracycline (*tetA*, *tetB*, and *tetC*), sulfonamides (*sul1*, *sul2*, and *sul3*), streptomycinspectinomycin (*strA/strB*, *aadA*), and apramycin [*aac(3)IV*] are described in Table 1. All polymerase chain reactions will be completed with the following temperature cycling: 1 cycle of 4 min at 95°C; 35 cycles, each consisting of 1 min at 95°C, 1 min at annealing temperature given in Table 1 followed by 1 min at 72°C; and 1 cycle of 7 min at 72°C. SYBR Green I (Applied Biosystems) will be used to detect the amplified product. The product will be run through gel electrophoresis to confirm fragment location.

Table 1. Single PCR conditions and control strains

Gene	Primer name	Primer sequence	Anneal (°C)	Fragment size (bp)	Positive control
<i>aadA</i>	4Fa	GTGGATGGCGGCCTGAAGCC	68	525	AMR-002d
	4Ra	AATGCCCGAGTCGGCAGCG			
<i>strA</i>	2Fa	CCTGGTGATAACGGCAATTC	55	546	AMR-009d
	2Ra	CCAATCGCAGATAGAAGGC			
<i>strB</i>	3Fa	ATCGTCAAGGGATTGAAACC	55	509	AMR-009d
	3Ra	GGATCGTAGAACATATTGGC			
<i>tetA</i>	TetA-Lb	GGCGGTCTTCTTCATCATGC	64	502	RO8d
	TetA-Rb	CGGCAGGCAGAGCAAGTAGA			
<i>tetB</i>	TetB-Lb	CATTAATAGGCGCATCGCTG	64	930	PB#11d
	TetB-Rb	TGAAGGTCATCGATAGCAGG			
<i>tetC</i>	TetC-Lb	GCTGTAGGCATAGGCTTGGT	64	888	PB#02d
	TetC-Rb	GCCGGAAGCGAGAAGAATCA			
<i>sul1</i>	Sul1-Lb	GTGACGGTGTTCGGCATTCT	68	779	AMR-130d
	Sul1-Rb	TCCGAGAAGGTGATTGCGCT			
<i>sul2</i>	Sul2-Lb	CGGCATCGTCAACATAACCT	66	721	AMR-130d
	Sul2-Rb	TGTGCGGATGAAGTCAGCTC			
<i>sul3</i>	Sul3-Fc	GAGCAAGATTTTTGGAATCG	51	880	RL0044c
	Sul3-Rc	CATCTGCAGCTAACCTAGGGCTTTGGA			
<i>aac(3)IV</i>	Aac4-Ld	TGCTGGTCCACAGCTCCTTC	59	653	AMR-075d
	Aac4-Rd	CGGATGCAGGAAGATCAA			

a Reference: Boerlin et al., 2005; b Reference: Lanz et al., 2003; c Reference: Perreten and Boerlin, 2003; d Reference: Boerlin et al., 2005.

Antibiotic resistance analysis of isolates. Various antibiotic concentrations will be used to determine antibiotic resistance patterns in target microorganisms (Table 2). The antibiotics/concentrations were selected based on previous success from other ARA studies and their common use in human and veterinary practice (Mathew et al., 1999). Each of the thirty-eight antibiotic/concentrations is added separately to flasks of autoclaved and cooled Trypticase Soy Agar (TSA, BBL) from stock antibiotic solutions to achieve the desired concentration, and then poured into sterile 15x100mm petri dishes. Control plates (no antibiotics) are included with each set. Isolates are transferred from the microwell plate using a stainless steel 48-prong replica plater (Sigma). The replicator is flame-sterilized (95% ethanol) after inoculation of each TSA plate. The inoculant is allowed to soak into the agar and the plates are then incubated for 48 hours at 37°C. Resistance to an antibiotic is determined by comparing each isolate to the growth of that isolate on the control plate. A one (1) is recorded if that isolate grew (a round colony, mostly filled) and a zero (0) is recorded for no growth. This is repeated for each isolate on each of the 30 antibiotic plates.

This information will allow correlation of occurrence of antibiotic resistance genes carried by

isolates with the expression of antibiotic resistances encoded by these genes. This will allow an assessment of the level of expression of antibiotic resistance genes in the *E. coli* population.

Table 2. Antibiotics and concentrations used in ARA.

Antibiotics	Concentrations (µg/ml)	No. of Variables
Erythromycin	60, 70, 90, and 100	4
Neomycin	2.5, 5.0, and 10	3
Oxytetracycline	8, 16, 32, 64, and 128	5
Streptomycin	8, 16, 32, 64, and 128	5
Tetracycline	8, 16, 32, 64, and 128	5
Cephalothin	16, 32, 64, and 128	4
Apramycin	16, 32, and 64	3
Sulfamethoxazole	64, 128, 256, and 512	4
Trimethoprim- Sulfamethoxazole	8, 16, 32, 64, and 128	5
Control	No antibiotic	2
Total		40

Procedures for Objective 2. Strains of *E. coli* will be isolated from known fecal waste samples to develop a known source library. No more than 10 isolates will be taken from a given sample of each manure source to build a database of 1000-1200 isolates. The known source categories will be composed of swine, cattle, wildlife and pets. Over 300 *E. coli* isolates from swine and 300 *E. coli* isolates from cattle have already been collected for database development. Antibiotic resistance analysis on 1000-1200 known isolates will be performed as described under Objective 1.

Statistical Analysis for ARA: Variables for the analyses include the number of antibiotics used and the degree of pooling of sources. Each analysis produces a classification set for every known source isolate. The correct classification rates are calculated using one set of antibiotic resistance patterns (ARPs) both to establish the classification rule and as test subjects (Harwood et al., 2000). The number of isolates from a given source that are placed in the correct source category by discriminant analysis is termed the rate of correct classification. The average rate of correct classification (ARCC) for the database is obtained by averaging the correct classification percentages for all sources (Harwood et al., 2000). The holdout method of cross validation, in which isolates from known sources are randomly removed from the data set and treated as test subjects, will be used as a more rigorous test of the predictive power of the databases (Harwood et al., 2000). To determine whether the known database are large enough or has ample representation, artificial clustering will be used. Artificial clustering involves randomly assigning equal numbers of isolates from each source and applying discriminant analysis to determine the random ARCC. Our database will contain 4 source types, swine, cattle, wildlife and pets. The random ARCC should be approximately 25% for each source. Thus, any percent significantly greater than the 25% ARCC indicates that the known source database is not representative. If the ARCC for a source segment of the database is found not to be representative, isolates will be added until the problem is corrected. By doing so, assures that the database will serve as a good point of reference for identifying unknown source isolates collected from Six Runs Creek. The development and validation of this database will allow determination of the source of unknown *E. coli* isolates obtained from the Six Runs Creek.

Procedures for Objective 3. Water samples will be collected from a total of 5 stream sampling sites, once a month for nine months. Sampling sites will consist of upstream (above waste application field 1, see Figure 1) and downstream sites in relation to the swine facility. The sampling regime is designed to capture possible seasonal variation in host sources contributing bacterial loading to Six Runs Creek. Ground water samples will be collected from the wells of transect two at each waste application fields (figure 1). Sampling from these sites will occur once every other month for nine months.

Isolation of *E. coli* will be performed by membrane filtration of a known volume of a water sample passed through a membrane filter that is then placed on media that is selective for the target microorganism. After incubation for 24 hr in a 44.5°C water bath, colonies will be transferred to 96-microwell plates containing 0.2 ml colilert broth specific for the target microorganism, and incubated for 24 h at 37°C. Twenty-four isolates from each water sample will be evaluated using antibiotic resistance analysis to determine its source. Antibiotic resistance analysis will be performed as described under objective 1. Isolates identified as swine will be evaluated for the presence antibiotic resistant genes using procedures described in objective 1.

Principal Findings

Microbial source tracking by means of antibiotic resistance analysis (ARA) and polymerase chain reaction (PCR) have been performed on *E. coli* recovered from groundwater and surface water. Out of a total of 192 groundwater well samples only 7 wells had *E. coli* counts greater than 250 cfu/100ml, representing 3.6% of the samples. Of the 3.6% of groundwater samples that had elevated levels of *E. coli*, MST indicated that both lagoon effluent and wildlife (bird, deer, and unknown wildlife sample) were the major contributors of this contamination.

Surface water samples had *E. coli* counts that were consistently higher than the recreational standard of 250 cfu/ 100 ml. Elevated surface water counts were not surprising as beavers had taken residence in the stream and built a dam. We recently made contact with a professional wildlife trapper and were able to collect feces from beaver, nutria and raccoon; ARA and PCR profiles from these three sources will be added to the database and all the data collected from the groundwater and surface water will be re-evaluated against the more comprehensive database.

To date, a total of 1,208 *E. coli* isolates from swine feces, lagoon effluent, cattle, wildlife and nearby ground and surface waters (n=238, 234, 192, 144, 200 and 200, respectively) have been evaluated for phenotypic expression of resistance to various concentrations of the following antibiotics: erythromycin, neomycin, oxytetracycline, streptomycin, tetracycline, cephalothin, apramycin, trimethoprim, and rifampicin. All the isolates displayed multiple antibiotic resistances. These isolates have also been evaluated for antibiotic resistance genes. 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.

- Swine feces and lagoon effluent isolates: Had high levels (*aad*, *strA*, *strB* *tetA* and *sul1*); intermediate (*tetB* and *tetC*); low (*sul2*, *sul3* and *Aac(3)IV*) (Figure 2).
- Cattle isolates: Had high levels (*aadA* and *tetA*); intermediate (*strA*, *strB*, *tetB* and *sul1*); while wildlife isolates had high levels (*aadA*, *strB*, *tetA* and *tetB*); intermediate (*strA* and *sul1*). Both sources had low levels of *tetC*, *sul2*, *sul3* and *Aac(3)IV* genes (Figure 2).
- Ground and surface water isolates: Had high levels (*aadA*, *strA*, *tetA*, *tetB* and *sul1*); intermediate (*strB*, *sul2* and *sul3*) and low (*Aac(3)IV*). Both *strB* and *tetC* genes increased in surface water (Figure 3).
- ARGs in isolates from swine feces, lagoon effluent and wildlife sources were not significantly different (Figure 4).
- ARGs in isolates from both ground and surface water were significantly greater than all the known sources (P<0.05) (Figure 4).

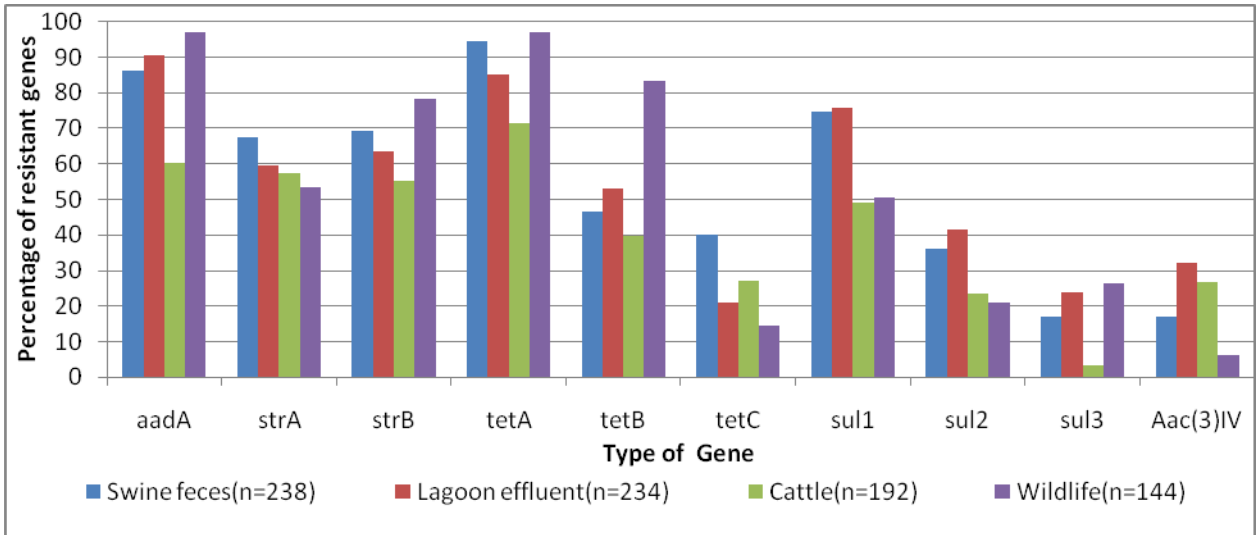


Figure 2: Distribution of antibiotic resistant gene types in known sources.

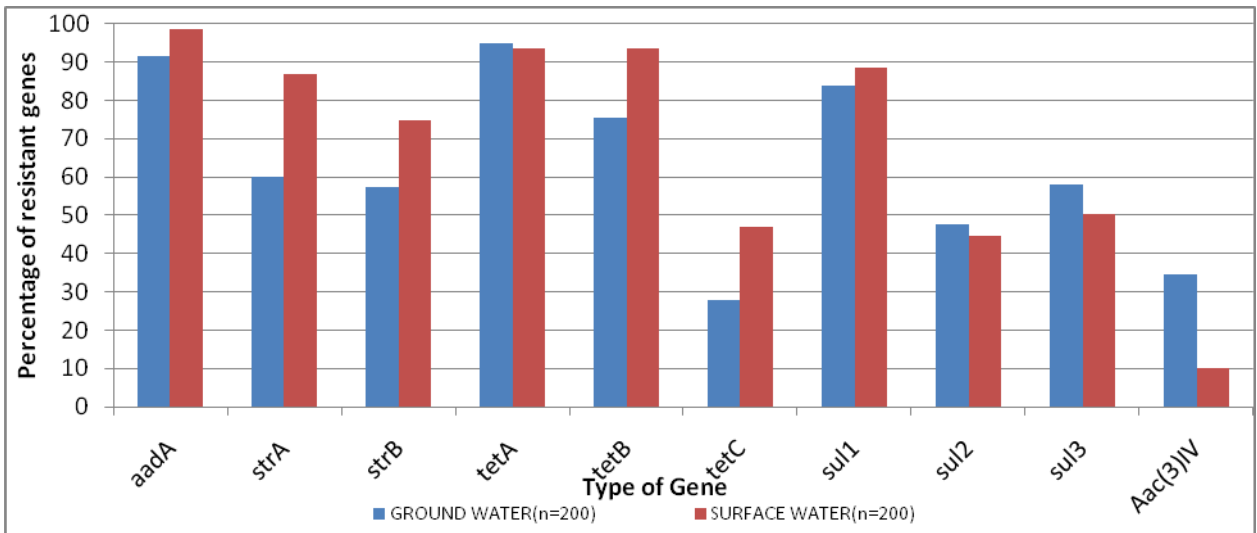


Figure 3: Distribution of antibiotic resistant gene types in environmental sources.

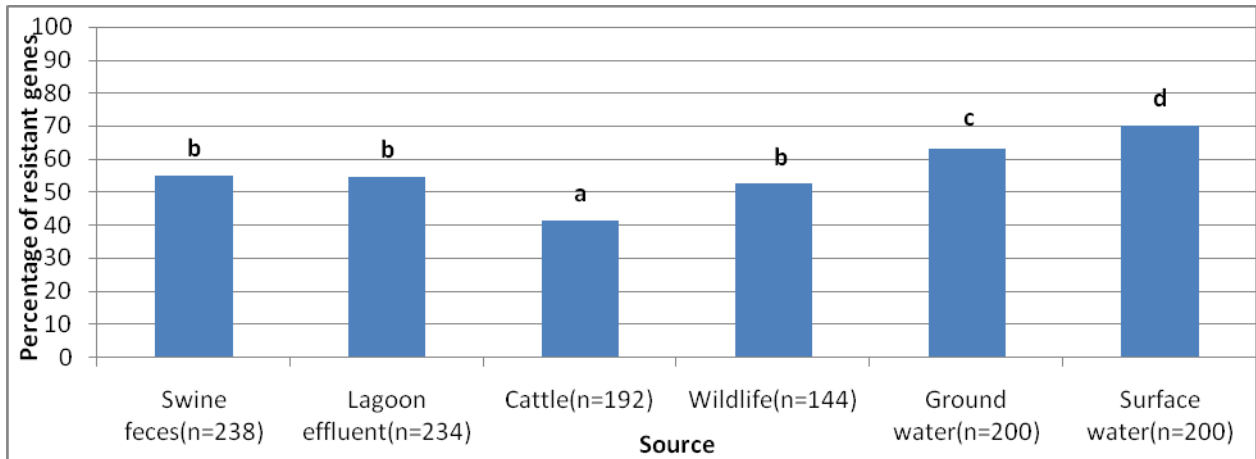


Figure 4: Distribution of antibiotic resistant genes among the sources ($P < 0.05$).

Significance

Microbial resistance to antibiotics is spreading fast; incidence of vancomycin resistance has increased from less than 1% to 17% within a span of 10 years (Pfaller et al., 1998). This study is intended to evaluate the association of antibiotic resistance genes found in *E.coli* isolated from swine with the actual phenotypic expression of the resistance. Additionally to develop an antibiotic resistance database for *E. coli* isolates from a commercial swine facility and assess its efficacy for tracking movement of bacteria from swine confinement houses to surface waters. Quantitative polymerase chain reaction will provide robust, sensitive and highly discriminant data. The results of this research will provide important information regarding the role of land application of lagoon effluent in spreading of bacteria with antibiotic resistance genes to surface waters. Early diagnosis of the problem will allow for the development of improved technologies and mitigation strategies.

This work can be used to track sources responsible for fecal pollution in the environment and also to make decisions based on scientific evidence to establish if waste management systems are working properly. The study can also provide timely answers to questions about antibiotic resistance. Swine production is not the major source of fecal pollution in the creek but multiple sources are responsible. This might also be the case for other similar locations dominated by swine. However, considerations should be made for the role of wildlife in transporting *E. coli* from lagoons to the streams, such as birds, turtles, etc. They were surprised that some resistant genes were more pronounced in wildlife than the livestock where antibiotics are mostly used.

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