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Potential Pathogens, Antimicrobial Patterns, and Genotypic Diversity of *Escherichia coli* Isolates in Constructed Wetlands Treating Swine Wastewater

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Constructed wetland for removal of swine waste

Abstract

E. coli populations originating from swine houses through constructed wetlands were analyzed for potential pathogens, antimicrobial susceptibility patterns, and genotypic diversity. *E. coli* isolates (n = 493) were screened for the presence of the following virulence genes: *stx*1, *stx*2,and eae (Shiga toxin-producing *E. coli* [STEC]), heat-labile enterotoxin (LT) genes and heat stable toxin STa and STb (enterotoxigenic *E. coli* (ETEC), cytotoxin necrotizing factors 1 and 2 (*cnf*1 and *cnf*2 [necrotoxigenic *E. coli*- NTEC]), as well as O and H antigens, and the presence of the antibiotic resistance genes bla_{TEM}, bla_{SHV}, bla_{CMY}-2, *tet* A, *tet* B, *tet* C, *mph*(A), *aadA*, StrA/B, *sul*1, *sul*2, and *sul*3. The commensal strains were further screened for 16 antimicrobials and characterized by BOX AIR-1 PCR for unique genotypes. The highest antibiotic resistance prevalence was for tetracycline, followed by erythromycin, ampicillin, streptomycin, sulfisoxazole, and kanamycin. Our data showed that most of the isolates had high distribution of single or multi drug-resistant (MDR) genotypes. Therefore, the occurrence of MDR *E. coli* in the wetland is a matter of great concern due to possible transfer of resistance genes from nonpathogenic to pathogenic strains or vice versa in the environment.

Introduction

Constructed wetlands can offer significant benefits to human populations in both developed and developing countries (Kadlec and Knight, 1996; Ibekwe et al., 2003, Ibekwe et al., 2007). Benefits include water-quality improvement, water reclamation, conservation of habitat for species, flood control, recreational and education activities. In the swine production system wastes are traditionally flushed into an anaerobic lagoon and then later sprayed on agricultural

fields. Waste from large swine farms has been blamed for polluting surface waters, contaminating wells, creating noxious odors, and discharging ammonia into the air. During hurricane Floyd in 1999, torrential rains and flooding caused many North Carolina swine waste lagoons to overflow polluting many surface water systems. Surface waters and large areas of land became contaminated with fecal and chemical wastes from compromised septic and municipal sewage systems, and livestock waste lagoons (Casteel et al., 2006). This brought international attention to the swine industry in the state and to waste management in particular.

The immediate effect of this storm (Casteel et al., 2006) on the swine industry was the development of new technologies for treating waste from swine operations in the state. One such research project is the use of constructed wetlands for the removal of waste constituents. This system uses natural wetland plants and soil as substrate to remove nitrogen (N), phosphorus (P), solids, and chemical oxygen demand (COD) from treated swine wastewater, therefore, preventing the overloading of nutrients on agricultural land to which the effluent is applied (Reddy et al., 2001). Similar wetlands were previously used for treatment of dairy waste in southern California, USA (Ibekwe et al., 2003). These authors (Ibekwe et al., 2003) concluded that, the wetland effluent was more suitable for on-site reuse and reduced the amount of contaminants entering groundwater supplies as a result of percolation of wastewater stored in ponds and sprayed on disposal lands. In a related study, Ibekwe et al. 2007 provided evidence that wetlands with 50% plant cover may promote the growth of diverse microbial communities that facilitate decomposition of organic pollutants in surface water and improve water quality.

The main contaminants from swine waste may include nutrients, salts, microbes, and pharmaceutically active compounds, and their removal involves complex physical, chemical, and biological processes. Some of the contaminants may include indicator bacteria like *E. coli* and

antimicrobials. *E. coli* are widely used as indicators of fecal contamination of waterways in most urban and rural areas. They have diverse genotypes and phenotypes, and some characteristics are shared among strains exposed to similar environments due to selection pressure (Ishii and Sadowsky, 2008). One method for identifying the diverse genotype in *E. coli* is the use of BOX AIR-1 PCR method (Dombek et al., 2000; Lyautey et al., 2010; Chandran and Mazumder, 2015). This technique is able to identify *E. coli* isolates with unique fingerprints as well as determine their diversity in complex environments. The unique fingerprints can further be used to identify host sources, and to determine the quality of water in a large watershed (Chandran and Mazumder, 2015). The level of selective pressure exerted on these bacteria in any environment may be a useful criterion in the evaluation of the quality of that environment as well. One such tool to assist in examining the selection pressure on *E. coli* is assessing their antimicrobial susceptibilities (Graves et al., 2002; Graves et al., 2007).

There are at least 17 classes of antimicrobials approved for use in food animals in the United States (Anderson et al., 2003). These antimicrobials provide benefits such as improved animal health, higher productivity, and in some cases, reduction in foodborne pathogens (Mathew et al., 2007), and other pathogens of public health significance. However, use of antibiotics for agricultural purposes, particularly for growth enhancement, has come under much scrutiny throughout the world, as it has been shown to contribute to the increased prevalence of antibiotic-resistant bacteria of public health significance (Mathew et al., 2007). In 2003, the FDA directly addressed the issue of risks associated with use of antibiotics in food animals with the release of the Guidance for Industry 152 (www.fda.gov/cvm), which outlined steps for risk assessment in the evaluation of new animal drugs in terms of microbial food safety (FDA 2003).

The presence of antibiotics in the environment at certain concentrations has been associated with chronic toxicity and the prevalence of resistance to antibiotics in bacterial species has been recognized as a major threat to public health (Schwartz et al., 2006; Kummerer, 2009). It is now recognized that antibiotics constitute a new class of water contaminants of emerging concern with adverse effects on aquatic life (Kummerer**,** 2009; Fatta-Kassinos et al., 2011; Kolpin et al., 2002; Michael et al., 2013). The number of studies focusing on the use of constructed wetlands for the removal of antibiotics or antibiotic resistant genes is very limited (Sidrach-Cardona and Bécares, 2013). These authors (Sidrach-Cardona and Bécares, 2013) concluded that hydraulic design and presence of plants were extremely important in reducing total numbers of bacteria, which was related to the total numbers of antibiotic resistant bacteria. Therefore, higher bacterial removal in constructed wetlands may result in lower antibiotic resistant bacteria loadings to the environment. Unfortunately, many countries focus monitoring of antibiotic resistance in the clinical environments such as hospitals, with special emphasis on pathogenic bacteria, while aquatic ecosystems receive much less attention, although they might be the reservoirs of the resistances (Servais and Passerat, 2009).

The present study looks at potential *E. coli* pathogens, antimicrobial susceptibilities, and genetic diversity of isolates in surface flow constructed wetland systems used for treating swine waste. Our main objectives were to examine the presence of potential pathogens of *E. coli* and quantify antibiotic resistant *E. coli* from the wetland system, and describe their diversity in different configurations of constructed wetlands (Fig S1). The overall goal of this constructed wetland system is to have final effluent water that is suitable for on-site reuse and with reduced amounts of contaminants entering the environment.

Materials and methods

Experimental site

The experimental site was a constructed wetland located at a swine research facility at North Carolina Agricultural and Technical State University farm in Greensboro, NC, USA. The wetland has six cells 40 m long by 11 m wide and was constructed in 1995 (Fig. 1A). Each cell consisted of 11 m by 10 m marsh at both influent and effluent and 11 m by 20 m pond section separating the marshes and planted with *Typha latifolia* L. (broadleaf cattail) and *Scirpus amer*icanus (bulrush) in March 1996 (Reddy et al., 2001). The marsh and pond sections of wetlands (cells 1-4 Fig. 1A) have previously been described (Reddy et al., 2001). In 2003, modifications on cells 5 and 6 (Forbes et al., 2010) were done to achieve a continuous marsh system with a slope of 0.33%, from inlet to outlet end and planted with giant bulrushes (*Scirpus californicus*) (Fig.1B). This study focused on the evaluation of the modifications with the aim of understanding the role of microorganisms in the constructed wetland with continuous marsh. Waste flow from the swine houses was flushed with recycled water into a two-stage anaerobic lagoon, and the flow from the lagoon was pumped into a storage tank as described before (Dong and Reddy, 2010). The wastewater from the storage tank was discharged by gravity into the wetland cells, and the final effluent from the wetland was discharged into a storage pond for recycling into the swine house or application on land.

During the study, the number of pigs used from January 2007 to January 2012 ranged between 65 and 115. Furthermore, the following antibiotics were used for sub-therapeutic and therapeutic treatments and were always added through water supply: penicillin, tylosin tartrate, lincomycin hydrochloride, ceftiofur hydrochloride, and liquamycin. Feeding regiment included crude protein, lysine, crude fat, crude fiber, calcium, phosphorus, salt, selenium, and zinc at different ratios during different production stages (growers, finishers, gestation, and lactation).

Sampling design

To detect the spatial and temporal variation of *E. coli*, isolates from the wetland samples were collected from eight different points in the wetland in April, August, and November 2010. The first sample (S1) was the flow from the swine house into a two-stage anaerobic lagoon system consisting of a primary lagoon 1 (S2) with overflow into a secondary lagoon 2 (S3) that flows to the storage tank (S4). Wastewater from the 8000 L storage tank was discharged by gravity to each continuous wetland cell influent for sampling point S5. The final effluent samples (S6) from the continuous marsh cells were discharged into a storage pond (S7) where it was recycled for flushing of the swine production facility and for land application (S8). All samples were maintained on ice until arrival in the laboratory and then stored at 4 ^{o}C for further analysis. Samples were analyzed for ammonia (NH^{+}_{4}-N), nitrate (NO_3-N), total-phosphate (TP) and available-phosphate (PO_4^{3-}) using a flow injection analysis instrument (Lachat-QuikChem 8000, Loveland, CO, USA). Carbon (C) and nitrogen (N) concentrations were measured using the Perkin–Elmer 2400, CHNS/O series II Analyzer (Shelton, CT, USA).

Enumeration *E. coli*

Water samples were processed in the laboratory and analyzed by adding 100 mL of a water sample to a Colilert vessel (Westbrook, Maine, USA) and processing following the manufacturer's protocol. *E. coli* populations were enumerated and expressed as Most Probable Number (MPN/100 mL). For isolation of *E. coli* colonies from Colilert vessels, 100 µL liquid sample was removed from positive wells, then spread plated onto Chromagar ECC agar (CHROMagar Microbiology, Paris, France), and incubated at 37 °C for 24 h. Individual colonies of pure cultures that were isolated were stored at –80 °C for further characterization following the manufacturer's protocol in accordance with method 9223 (Eaton et al., 1998).

Isolation of potentially pathogenic *E. coli* from the wetland systems

One mL of environmental samples was added to 9 mL of PBS, vortexed briefly, serially diluted and plated for the enumeration of potential pathogenic *E. coli* on Harlequin cefixime-tellurite sorbitol MacConkey (CT-SMAC) agar with BCIG (5-bromo-4-chloro-3-indoxyl-β-D-glucuronide) (LAB M: IDG–Lancashire, UK). The plates were incubated at 37 °C for 16 h. Sorbitol-negative, translucent colonies were tested by multiplex PCR to determine the presence of *hyl*A, *stx*1, *stx*2, and *eae* gene (Paton and Paton, 1998). Additionally, isolates that were sorbitol positive or β-glucuronidase positive (red/pink colonies with a purple center or green colonies) were enumerated as non O157 or other *E. coli*, or presumptive pathogenic *E. coli* (Table1). The presumptive pathogenic *E. coli* isolates were tested at the *E. coli* Reference Center (The Pennsylvania State University, University Park, PA, USA) for presence of gene sequences encoding: heat labile toxin (LT), heat stable toxins a and b (STa and STb), Shiga toxins 1 and 2 (*stx1 and stx2*), cytotoxin necrotizing factors 1 and 2 (*cnf*1 and *cnf*2), intimin (*eae*), including O and H antigens (DebRoy and Maddox**,** 2001) (Table 1).

Susceptibilities of isolates against 16 antimicrobials

Antimicrobial susceptibility tests (phenotypes) of *E. coli* isolates were done using disk diffusion assays following CLSI standards (CLSI, 2006) for 16 antimicrobials (Table 2). Mueller-Hinton II agar (Difco) was used and cells were harvested from the surface of the medium with a cotton swab after 24 h growth at 37 °C. *E. coli* ATCC 25922 (American Type Culture Collection, Manassas, VA, USA) was included in each assay as a negative control strain. Antimicrobial agents were tested with BD BBL Sensi-Disc antimicrobial susceptibility test discs (Becton Dickinson & Co., Sparks, MD, USA) with the breakpoints (μg mL^{-1}) indicated as follows:

amoxocillin/clavulanic acid, 20/10 μg mL^{-1}, ampicillin, 10 μg mL^{-1}, azithromycin, 15 μg mL^{-1}, cefoxitin, 30 μg mL^{-1}, ceftriaxone, 30 μg mL^{-1}, ceftiofur 30 μg mL−1, cephalothin, 30 μg mL^{-1}, erythromycin, 15 μg mL^{-1}, gentamicin 120/10 μg mL^{-1}, penicillin, 10 μg mL^{-1}, kanamycin, 30 μg mL^{-1}, trimethoprim/sulfamethoxazole, 1.25/23.75 μg mL^{-1}, sulfisoxaxole, 250 μg mL^{-1}, ciprofloxacin, 5 μg mL^{-1}, streptomycin, 10 μg mL^{-1}, and tetracycline, 30 μg mL^{-1}.

Antimicrobial resistance gene detection

Multiplex PCR screens were performed on the *E. coli* isolates targeting sequences of genes encoding for ampicillin resistance (*bla*$_{TEM}$, *bla*$_{SHV}$, bla$_{CMY}$-2), tetracycline resistance (*tet*A, *tet*B, and *tet*C), streptomycin resistance (*aadA*, StrA/B), erythromycin (*mph*(A)), and sulfisoxazole (*sul*1, *sul*2, and *sul*3). Details of primers, annealing temperatures, and amplicon sizes are as previously provided (Kozak et al., 2009; Nguyen et al., 2009). The multiplex PCR screens were performed using 25 μl mixtures and Ready-To-Go PCR beads (Pharmacia Biotech Inc. NJ).

Typing of *E. coli* using BOXAIR-1

Genomic DNA fingerprinting of *E. coli* isolates was performed using procedures described previously by others (Dombek et al., 2000; Lyautey et al., 2010; Chandran and Mazumder, 2015). Box-PCR fingerprints were obtained by using primer BOX AIR (Versalovic et al., 1998; Rademaker and de Bruijn, 1997). Following amplification, the PCR amplicons were electrophoresed, and the gel images were obtained using a quality one gel imaging system (Bio-Rad Lab., Hercules, CA, USA). Comparison of restriction enzyme digestion patterns and cluster analysis was performed with the BioNumerics software, version 5.0 (Applied Maths, Austin, TX, USA). Fingerprints were clustered using the Jaccard similarity coefficients evaluated by the unweighted pair-group method using arithmetic averages (UPGMA).

Analysis of *E. coli* genotyping

E. coli isolates were analyzed temporally and spatially as previously described (Goto and Yan**,** 2011; Chandran and Mazumder, 2015) using BOX AIR -PCR DNA fingerprinting. Briefly, the total number of unique *E. coli* genotypes was calculated, the distribution of the genotypes, Shannon diversity indices (*H*'), and the occurring frequencies in the wetland samples were determined using Jaccard similarity coefficients and UPGMA. The BOX AIR -PCR DNA fingerprints of *E. coli* isolates at all sampling sites over the sampling period were used in a single clustering analysis to identify unique genotypes and Shannon diversity indices (*H*'). The level of reoccurrence of particular genotypes at the same sampling sites at different sampling dates was determined. To construct genotype accumulation curves, the unique genotypes and their abundances (i.e., how many isolates share the same genotypes) were calculated and analyzed.

Statistical analysis

All analyses were performed in duplicate, and analysis of variance (ANOVA) was conducted with $\log_{10}$-transformed density of *E. coli* bacteria using SAS version 9.1 (SAS, 2009) to determine statistically significant differences, and Tukey's studentized test range (HSD) was used for mean separation. Shannon diversity index (*H'*) was used to calculate genetic diversity as previously described (Chandran and Mazumder, 2015):

$$H' = -\sum_{i=1}^{S} p_i \ln p_i$$

where S is the number of unique genotypes and p_i is the number of isolates sharing the same genotype, i, over the total number of isolates.

Results

E. coli isolates from wetland samples.

To assess the spatial and temporal variations of *E. coli* populations, isolates were obtained from effluent from the swine house (S1) and wetland effluent (S2-S8) during March, August, and November from a surface flow constructed wetland (Fig. S1A) with continuous marsh (CM-Fig.1B). *E. coli* populations were significantly (P = 0.022) higher in November than in March and August (Fig.2) in the lagoon 1 &2 (S2 & S3), and storage tank (S4). However, no significant differences were found in other cells. Spatially, there was a significant drop in *E. coli* populations from wetland influent (S1- swine house) to the final effluent (S8 - final effluent).

Potential pathogenic *E. coli*

A total of 72 isolates (Table 1) were screened with Harlequin cefixime-tellurite sorbitol MacConkey (CT-SMAC) agar with BCIG (5-bromo-4-chloro-3-indoxyl-β-D-glucuronide) to enumerate potentially pathogenic *E. coli* isolates, and these isolates were rescreened by PCR for various virulence factors. These isolates were sent to Pennsylvania State University *E. coli* Reference Center for complete typing (Table 1). Only 15% of the isolates (11) carried the *stx*2 gene, none carried *stx*1 and 20% of the isolates (15) carried *eae* genes. However, none of the isolates with *eae* genes co-carried *stx*2 genes. The rest were classified as other pathotypes, such as enterotoxigenic *E. coli* (ETEC) due to the presence of heat labile or heat stable genes.

Furthermore, 72% of the isolates carried either/or the heat stable toxins a and b (*sta*/*stb*) genes reflecting that these are enterotoxigenic strains.

When the 72 isolates were analyzed for the presence of resistance genes in relation to various virulence factors, 66 isolates (92%) with the H antigen were resistant to at least one out of the twelve resistant genes tested (Table 1). For example, 11 isolates were positive for *stx*2 and all were positive to genes for ampicillin resistance bla_{CMY}^{-2}, 8 were positive to genes for streptomycin resistance (*aadA*), and 9 were positive to *tet*A. Overall, 40.2% of the 72 isolates were resistant to bla_{TEM}, 44.4% to *aad*A, 53% to *tet*B, and 18% to *str*A/B.

Antimicrobial susceptibility profiles of *E. coli* isolates from wetland

Sixteen antimicrobials were used for susceptibility tests of the remaining 421 *E. coli* isolates. Most isolates were resistant to tetracycline (67.46%), followed by erythromycin (25.42%), ampicillin (22.33%), streptomycin (18.53%), sulfisoxazole (16.63%), and kanamycin (6.89%) (Fig. 3). The rest of the antibiotics had <5% resistance rates. *E. coli* isolates were subsequently characterized for MDR profiles, i.e., resistance to more than one antimicrobial (Table 2). The antimicrobials associated with most MDR were tetracycline with 195 isolates (46.3%), ampicillin with 88 isolates (20.9%), erythromycin with 85 isolates (20.2%), streptomycin with 77 isolates (18.3%), and sulfisoxazole with 69 isolates (16.4%). Isolates resistant to the rest of the antibiotics were <10%. Detailed examination of each section of the wetland and the number of *E. coli* isolates resistant to more than one antimicrobial showed that 42.1 % (24) of *E. coli* isolates showed resistance to tetracycline and other antimicrobials in the swine house effluent and the high resistant levels of isolates to tetracycline continued to the final effluent (11.8). Based on our data, tetracycline produced the highest percent resistant phenotype in this wetland in the final

effluent followed by erythromycin (8.8%), and streptomycin (5.9%). One isolate (2.9%) each were resistant to sulfisoxazole and nalidixic acid at the wetland effluent. The final effluent did not contain isolates carrying resistance to ampicillin and kanamycin as did many isolates with MDR at the swine house effluent.

Prevalence of antimicrobial resistance genes

Antimicrobial resistance genes were analyzed in 421 *E. coli* isolates collected from the wetland (Fig. 4). Multiplex PCR detected resistance genes for ampicillin (*bla*$_{TEM,}$ *bla*$_{SHV}$, bla$_{CMY}$-2), tetracycline (*tet*A, *tet*B, and *tet*C), streptomycin (*aadA*, StrA/B), erythromycin (*mph*A), and sulfisoxazole (*sul*1, *sul*2, and *sul*3) in some of the isolates (Fig.4). Antibiotic resistant *E. coli* isolates predominantly carried *tet*B (51%), and *tet*A (27%) genes. The second most predominant resistance genes were for streptomycin, with 33% of isolates carrying *aad*A gene and 28 %, StrA/B genes. About 27% of the isolates carried genes for ampicillin resistance (*bla*$_{TEM}$) and 12% for bla$_{CMY}$-2 while none had sequences for *bla*$_{SHV}$. Sulfisoxazole (*sul*2, *sul*3) genes were detected at lower levels than genes for tetracycline, streptomycin, and ampicillin (Fig 4).

Genetic diversity of *E. coli* isolates with **BOX AIR-PCR.**

E.coli isolates with fingerprint patterns similarity above 90% were considered clonal populations and were subtyped using BOX AIR DNA fingerprinting with Jaccard similarity coefficients and UPGMA (Fig. 5 with the dotted line representing the 90% cutoff point). All isolates (n = 421) were grouped into 176 unique genotypes with Shannon diversity index (*H'*) of 4.78 (Table 3). The distributions of 176 unique genotypes and their detection frequencies, i.e., number of

isolates per genotype is shown in Figure 6. Temporal variation of *E. coli* genotypes showed samples collected during March, August, and November from wetland effluent and analyzed by BOX AIR-PCR (Fig.7). The *E. coli* isolates were fingerprinted and clustered to identify unique genotypes and their diversity. During late winter (March) 244 isolates with 83 unique genotypes were obtained with genetic diversity (*H'*) of 3.96, followed by autumn sampling in November, with 111 isolates, 60 unique genotypes, and *H'* of 3.93. However, during the summer (August) fewer numbers of isolates (57) were obtained with 41 unique genotypes and a Shannon diversity index (*H'*) of 3.58 (Table 3). The frequencies of obtaining a unique *E. coli* genotype, as indicated by ratios of genotypes versus isolates, were 0.32, 0.67 and 0.54 for isolates collected in March, August and November, respectively.

In order to determine spatial variations of *E. coli* genotypes, distribution of genotypes among the eight sampling point and their detection frequencies (i.e., the number of isolates per genotype) at different point and their diversities were obtained (Table 4). A total of 135 isolates were analyzed from swine house effluent (S1) with 69 unique genotypes and *H'* of 3.96. The frequencies of obtaining a unique *E. coli* genotype, as indicated by ratios of genotypes versus isolates (Goto and Yan, 2011), were 0.51(*H'* 3.96) for isolates from S1, 0.63 for isolates from S2, 0.73 for isolates from S3), 0.73 for isolates from S4, 0.25 for isolates from S5, 0.37 for isolates from S6 0.47 for storage pond isolates (S7), and 0.21 for the final effluent isolates that was spread on land (S8). Genetic diversity of *E. coli* isolates decreased from the swine house to the final effluent (Table 4). As shown, genetic diversity was lower from continuous marsh influent (S5) to the final effluent (S8) than from the swine house to the storage tank. There was a two to three fold reduction in the number of unique genotypes and diversity between the storage tank and the final effluent in comparison to genotype ration obtained from the first four sampling

points of the wetland. The net effect of the wetland seems to suggest a more clonal population towards the final effluent with low diversity and low frequency ration (Table 4).

Discussion

Pathogenicity and antimicrobial characteristics

Constructed wetlands are known to reduce *E. coli* populations between the wetland influent and effluent significantly. Higher bacterial removal in constructed wetlands, therefore, means much lower antibiotic resistant bacteria loadings to the environment (Sidrach-Cardona and Bécares, 2013). This may result in significant reduction in the emergence of pathogenic MDR bacteria (Frigon et al., 2013; Call et al., 2003), contamination of ground and surface waters, soils, and crops by waste containing antimicrobials and resistant microorganisms. Many *E. coli* strains isolated from our wetland carried heat-stable enterotoxin a and b (STa and STb)-encoding genes which are characteristic of ETEC (Table 1). Other studies have shown high prevalence of STa and STb-encoding genes from *E. coli* samples isolated from swine (Chapman et al., 2006) and greater reduction of potentially pathogenic *E. coli* from waste water treatment (Frigon et al., 2013; Pereira et al., 2013).

Most fecal bacteria released by animals into the environment may carry antibiotic resistance genes (Durso et al., 2012). Their fate and the transfer of antibiotic resistances by gene transfer to other bacteria are of great concern to human health (Normark and Normark, 2002). A number of studies have measured antibiotic resistance in animal production environments (Haley at al., 2012; Brichta-Harhay et al., 2011), and the general public perception is that agricultural environments have more antibiotic resistance than natural and non-agricultural

environments. However, a close examination of current research data suggests that these may all be perceptions, and that in many watersheds non-agricultural environments may produce the same or higher antibiotic resistant bacteria (Ibekwe et al., 2011; Storteboom et al., 2010ab; Pruden et al., 2006; Pruden et al., 2012; McKinney et al., 2012; Berendonk et al., 2015) in the absence of antibiotic challenges. Pathogens with increased resistances may be transported from the animal via feces or other mechanisms into rivers and groundwater (Aarestrup et al., 2000) where the water is used as a source for domestic water supply. This was confirmed by our recent study with 600 isolates of generic *E. coli* from a southern California watershed (Ibekwe et al., 2011). Resistance genes are often associated with integrons or mobile DNA elements such as plasmids and transposons that facilitate the integration and spread of resistance genes (Jacoby. 1994; Murinda et al., 2005; Tenover and Rasheed, 1998). More often, there is a linkage between many of these resistance genes on mobile elements and the distribution of antibiotic resistant bacteria in the environment (Gow et al., 2008; Sharma et al., 2008; Smith et al., 2002). Therefore, pathogens with increased resistances may be transported from animals via feces into rivers and ground water (Baquero et al., 2008).

In a study to determine the impact of nontherapeutic use of antibiotics on swine manure-impacted water sources, surface water and groundwater situated up and down the gradient from a swine facility were assessed for antibiotic-resistant enterococci and other fecal indicators (Sapkota et al., 2007). The median concentrations of enterococci, fecal coliforms, and *Escherichia coli* were 4 to 33 fold higher in down-gradient versus up-gradient surface water and groundwater. Higher numbers of erythromycin- and tetracycline-resistant enterococci were detected in down-gradient surface waters. These findings demonstrated that water contaminated with swine manure could contribute to the spread of antibiotic resistance in the environment. We

also observe high levels of resistance to tetracycline and streptomycin in our study from the swine wetlands, suggesting the need for the treatment of swine waste before disposal. Other antibiotics that showed high resistance rates in our isolates were sulfisoxazole and ampicillin (Fig. 4). In general, swine exposed to antibiotic treatments seem to show higher percentages of *E. coli* isolates resistant to one or more antibiotics (Anderson et al., 2003). However, many efforts have been undertaken to control the spread of contaminants from animal waste to surface and ground waters, especially where concentrated animal feeding operations are located (Ibekwe et al., 2012).

Genetic diversity of *E. coli* population

E. coli were enumerated throughout the wetland during March, August, and November to capture the population densities and genetic structure during winter, summer, and autumn seasons. The data obtained showed both spatial and temporal behavior of *E. coli* throughout the three seasons in the wetland (Fig. 2). *E. coli* populations from influent were significantly higher than those from effluent of the wetland ranging from 5·5 $\log_{10}$ MPN 100 ml^{-1} for influent and 3.5 $\log_{10}$ MPN 100 ml^{-1} for the final effluent. Others have reported the typical enteric bacteria removal between 1 and 3 $\log_{10}$ from constructed wetlands (Vymazal and Kropfelova 2008; Graves and Weaver, 2010; Steer et al., 2002). Our results are in agreement with the above studies from the wetland described (Reddy et al., 2001; Dong and Reddy, 2010; Poach et al., 2004). Previous authors using this wetland associated the decreased bacterial counts with spatial nutrient content differences in the wetland where the concentrations of TN, NH^{+}_{4}, TP and PO^{3-}_{4} decreased from influent to effluent of the wetland. Our previous study with subsurface constructed wetland also showed a 99% decrease in total and fecal coliform and a 98% decrease in *E. coli,* and these

reductions were significantly correlated with decreases in TN, NH^{+}_{4}, TP and PO^{3-}_{4} from influent to effluent in the wetland (Ibekwe et al., 2003). The most significant reduction started occurring in continuous mash wetland cells (S5) where there are likely interactions of microbial activities with wetland plants where most of the nutrients like N and P are broken down through nitrification and other processes. The concentration of *E. coli* in the final effluent (S7) was significantly lower especially during March for final application to pasture or to recycle into the swine house.

E. coli isolates used in this study were subsequently characterized temporally and spatially to understand the effects of season and wetland cells on genetic diversity of the population. It has been reported that at the temporal scale, changes in *E. coli* composition in surface water could be a consequence of seasonal fecal population structure change with summer populations derived from numerous sources than winter populations (Whitman et al., 2008; Lyautey et al., 2010). More importantly, fecal *E. coli* communities are known to change during the lifetime of animals, or to be influenced by diet which may also vary with seasons. In this study higher diversity was observed in spring and autumn months (March and November) than during the summer month (March). However, in March higher numbers of isolates were found in each genotype than in August and November (Table 3). Changes in *E. coli* diversity associated with seasons had been well documented in water communities (Goto and Yan, 2011; Duriez and Top, 2007; Russell et al., 2000), but little has been done in constructed wetlands. In fact, most of the studies in constructed wetlands seem to focus on enteric bacterial populations at the effluent level since this is used for water quality measurements.

In summary, constructed wetlands may provide adequate means to minimize pathogens in waste water and to minimize their cell numbers in rivers and groundwater and to improve water

quality as a whole. In this study, the significant reduction of *E. coli* between influent and effluent water samples is a prime example, and this will also reduce the number of pathogens/bacteria with ARGs that may enter surface water from animal waste. Their fate and transfer of antibiotic resistances by gene transfer to other bacteria are of great concern to human health, and therefore, any strategy that reduces the transfer of antibiotics or ARGs from sources should be the first option in any mitigation program. Therefore, the use of low-cost technical solutions, such as constructed wetlands and other educational activities for water quality improvements must be encouraged at all levels in every society from developed to developing countries.

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Table 1. Virulence gene and antibiotic resistance genotypes of potentially pathogenic *E. coli* from swine constructed wetland

Sample names	Serotype		Virulence genes encoding								Antibiotic resistance genes											
	O type	H type	LT	STa	STb	Stx1	Stx2	EAE	CNF 1	CNF 2	Sul1	Sul2	Sul3	*bla*$_{TEM}$	bla$_{SHV}$	bla$_{CMY}$$^{-2}$	mphA	*aadA*	*StrA/B*	*tet*A	*tet*B	*tet*C
W1C	-	43	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
W1F	-	43	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
W1G	-	43	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
W1U	-	19	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-
W1W	-	4	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
W2C	-	11	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	+	-	+	-
W2D	-	11	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	+	-	+	-
W2E	-	11	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
W2K	-	43	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
W2P	88	38	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-
W2Y	-	4	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-
W2Z	-	4	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-
W2AA	-	4	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
W2AB	-	4	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
W2AC	-	4	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
W3W	-	4	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-
W3X	-	9	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	+	-
W3Y	-	4	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-
SM1A	-	36	-	+	+	-	+	-	-	-	-	-	+	-	-	+	-	+	-	+	-	-
SM1C	-	36	-	+	+	-	+	-	-	-	-	-	+	-	-	+	-	+	-	+	-	-
SM1T	-	36	-	+	+	-	+	-	-	-	-	-	+	-	-	+	-	+	-	+	-	-
SM1U	-	36	-	+	+	-	+	-	-	-	-	-	+	-	-	+	-	-	-	+	-	-
SM1V	-	36	-	+	+	-	+	-	-	-	-	-	+	-	-	+	-	+	-	+	-	-
SM2N	98	5	-	-	+	-	-	-	-	-	-	-	+	+	-	-	-	+	-	+	-	-
SM2U	-	36	-	+	+	-	+	-	-	-	-	-	+	-	-	+	-	+	-	+	-	-
SM2V	-	36	-	+	+	-	+	-	-	-	-	-	+	-	-	+	-	+	-	+	-	-
SM2W	-	11	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SM2X	-	36	-	+	+	-	+	-	-	-	-	-	+	+	-	+	-	+	-	+	-	-
SM2Y	-	11	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SM2Z	-	19	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-
PW1A	-	19	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-
PW1B	-	19	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-
PW1C	-	19	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-
PW1D	-	19	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-
PW2A	-	19	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-
PW2B	-	19	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-
PW2C	-	19	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-
PW2D	-	19	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-
PW2E	-	19	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-
PW3A	-	19	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-
PSM2E	-	19	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-
PSM2F	-	19	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-
PSM2G	-	19	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-
PSM2H	-	19	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-
PSM2I	-	19	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-
PSM2J	-	19	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-
PSM2K	-	19	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-
PSM2L	-	19	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-
PSM2M	-	19	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-
PSM2N	-	19	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-
PSM2O	-	19	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-
PSM2P	-	19	-	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-

W1C	-	11	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	+	-	+	-
W1H	-	11	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	+	-	+	-
W1J	-	11	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	+	-	+	-
W1K	-	11	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	+	-	+	-
W2B	-	32	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-
W3E	178	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
W1L	-	11	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	+	-	+	-
W2K	-	11	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	+	-	+	-
W2L	-	11	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	+	-	+	-
W3A	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
W3B	2	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
W3C	-	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
W3G	-	11	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	+	-	+	-
W3H	-	11	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	+	-	+	-
W3J	-	11	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	+	-	+	-
W3O	-	4	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-
W6K	-	11	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	+	-	+	-
SM1G	-	30	-	+	-	-	+	-	-	-	-	+	-	-	-	+	-	-	+	-	+	-
SM1H	-	30	-	+	-	-	+	-	-	-	-	+	-	-	-	+	-	-	+	-	+	-
SM1L	-	30	-	+	-	-	+	-	-	-	-	+	-	-	-	+	-	-	+	-	+	-

Table 2. Multiple antimicrobial resistant *E. coli* isolates from swine wetland

Antimicrobial	Total isolates* 421	swine house effluent S1- 135	primary lagoon 1 (S2)-57	secondary lagoon 2 (S3)- 57	storage tank (S4)-48	continuous wetland influent (S5)-24	continuous wetland effluent (S6)-30	storage pond (S7)-36	land application (S8) -34
Azithromycin	1.2 (5)	0 (0)	1.8 (1)	0 (0)	6.3 (3)	4.2 (1)	0 (0)	0 (0)	0 (0)
Nalidixic Acid	1 (4)	1.5 (2)	1.8 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2.9 (1)
Cefoxitin	0.2 (1)	0 (0)	1.8 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Ceftiofur	0.2 (1)	0 (0)	1.8 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Ceftriaxone	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Kanamycin	6.9 (29)	2.2 (3)	14 (8)	15.8 (9)	14.6 (7)	4.2 (1)	0 (0)	2.8 (1)	0 (0)
Trimethoprim/ sulfamethoxazole	0.5 (2)	0 (0)	1.8 (1)	1.8 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Sulfisoxazole	16.4 (69)	19.3 (26)	15.8 (9)	17.5 (10)	25 (12)	4.2 (1)	16.7 (5)	13.9 (5)	2.9 (1)
Amoxicillin/ Clavulanic Acid	1.7 (7)	0 (0)	1.8 (1)	0 (0)	2.1 (1)	0 (0)	0 (0)	13.9 (5)	0 (0)
Ampicillin	20.9 (88)	20 (27)	24.6 (14)	28.1 (16)	18.8 (9)	4.2 (1)	20 (6)	41.7 (15)	0 (0)
Chloramphenicol	5 (21)	10.4 (14)	1.8 (1)	1.8 (1)	4.2 (2)	0 (0)	0 (0)	8.3 (3)	0 (0)
Ciprofloxacin	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Erythromycin	20.2 (85)	14.8 (20)	17.5 (10)	29.8 (17)	33.3 (16)	4.2 (1)	16.7 (5)	36.1 (13)	8.8 (3)
Gentamicin	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Streptomycin	18.3 (77)	19.3 (26)	12.3 (7)	22.8 (13)	18.8 (9)	4.2 (1)	50 (15)	11.1 (4)	5.9 (2)
Tetracycline	46.3 (195)	49.6 (67)	42.1 (24)	63.2 (36)	56.3 (27)	8.3 (2)	50 (15)	55.6 (20)	11.8(4)

*Represents number of isolate

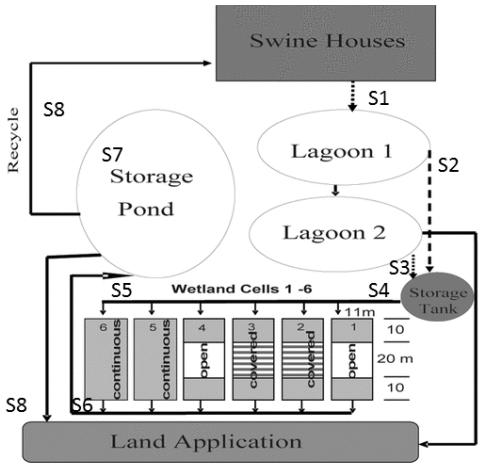
Table 3. Temporal variations of Shannon diversity indices (*H*') of *E. coli* isolate based of BOX PCR

Season	No. of isolates	No. of Unique genotypes	*H'* index	Frequency ratio
March	244	83	3.96	0.34
August	57	41	3.58	0.72
November	111	60	3.93	0.54
Total	421	176	4.78	0.42

Table 4. Shannon diversity indices (*H*') of *E. coli* isolates based on BOX PCR from different locations within the wetland

Sampling locations	No. of isolates	No. of Unique genotypes	H index	Frequency ratio
swine house effluent (S1)	133	69	3.96	0.51
primary lagoon 1 (S2)	57	39	3.55	0.63
secondary lagoon 2 (S3)	44	23	2.97	0.73
storage tank (S4)	47	35	3.48	0.73
continuous wetland influent (S5)	24	6	1.06	0.25
continuous wetland effluent (S6)	30	11	2.05	0.37
storage pond (S7)	35	17	2.55	0.47
land application (S8)	34	7	1.11	0.21

FIGURES



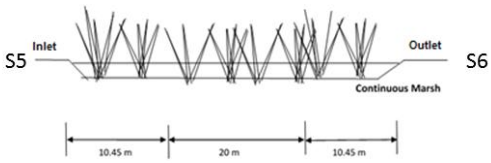


Figure 1B. Schematic diagram showing continuous marsh (CM) constructed wetland.

Figure 1. Constructed wetland located at a swine research facility at North Carolina Agricultural and Technical State University farm in Greensboro, NC, USA. (A) S1 indicates effluent from the from the swine house into a two-stage anaerobic lagoon system consisting of a primary lagoon 1

(S2) with overflow into a secondary lagoon 2 (S3) that flows to the storage tank (S4). Wastewater flows from the 8000 L storage tank by gravity to each continuous wetland cell influent for sampling point S5. The final effluent samples (S6) from the continuous marsh cells are discharged into a storage pond (S7) and recycled for flushing of the swine production facility and for land application (S8) (B) continuous mash flow section used for this study.

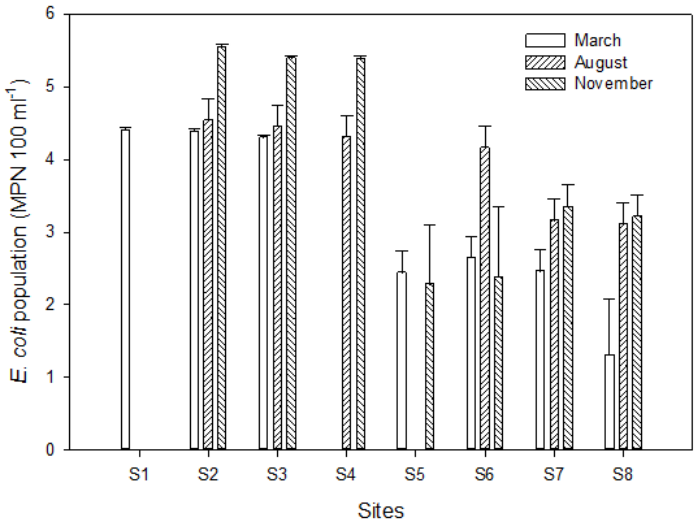


Fig. 2

Figure 2. *E. coli* population in November, March, and August in wetlands. Symbols on the X-axis are effluent from swine house (S1), two-stage anaerobic lagoon system consisting of a primary lagoon 1 (S2) with overflow into a secondary lagoon 2 (S3) that flows to the storage tank (S4), continuous wetland cell influent (S5), continuous wetland cell effluent (S6), storage pond (S7), final effluent samples (S8) where it was recycled for flushing of the swine production facility and for land application). All samples were collected in duplicate.

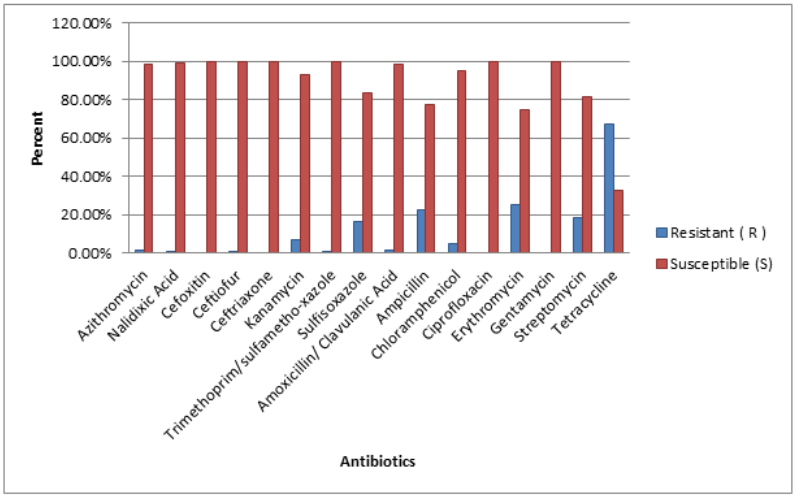


Fig.3

Figure 3. Percent susceptibility of 421 *E. coli* isolates against 16 antibiotics.

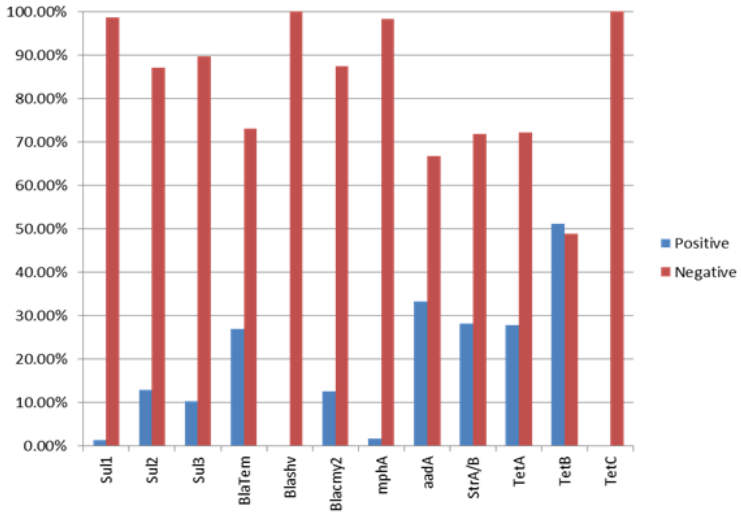


Fig. 4

Figure 4. Percent of bacterial isolates carrying selected antimicrobial resistant genes.

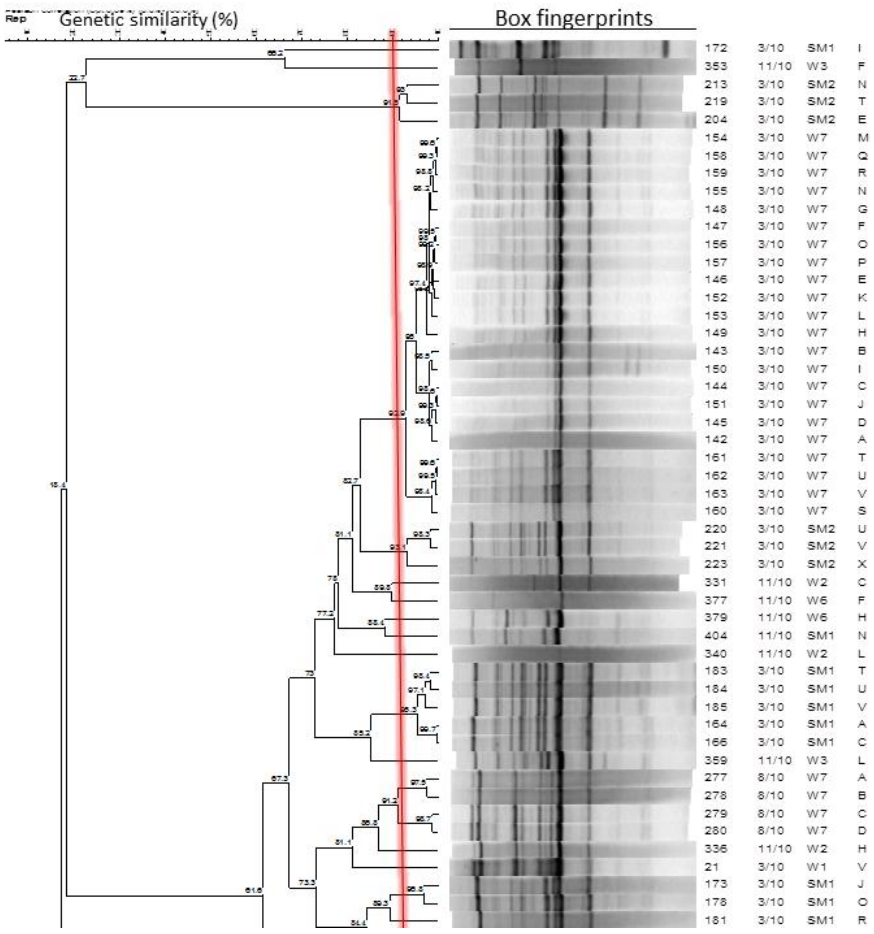


Fig.5

Figure 5. Representative dendrogram showing the genetic relatedness of *E. coli* from wetland based on their BOX-PCR DNA fingerprints. The red vertical line indicates the cutoff value of 90% for identifying unique genotypes.

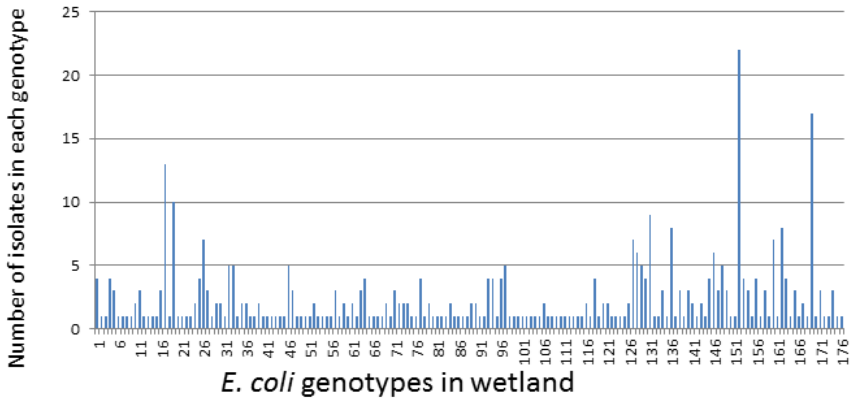


Fig.6

Figure 6. A total of 415 *E. coli* isolates from swine wetland were analyzed by BOX-PCR DNA fingerprinting and then grouped into 176 unique genotypes based on cluster analysis.

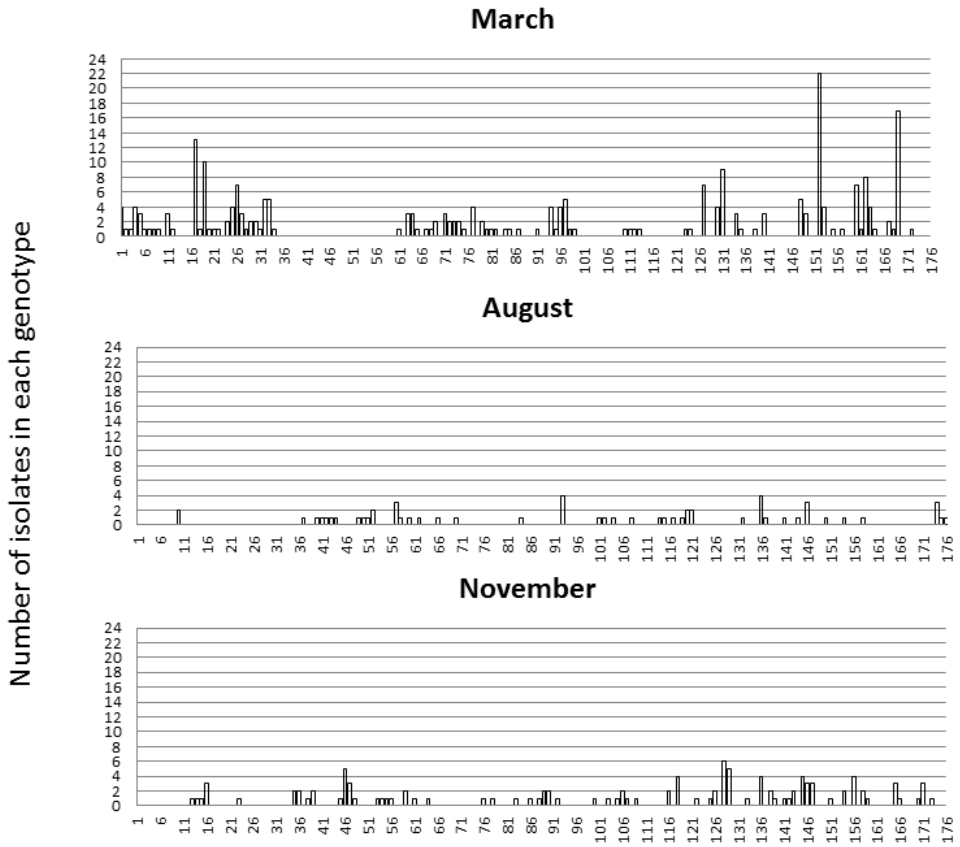


Fig.7

Figure 7. Temporal variations of *E. coli* isolates based on Box-PCR in wetland for March, August, and November.