INTEGRON AND TETRACYCLINE DETERMINANT CARRIAGE AMONG STRAINS OF COMMENSAL *ESHERICHIA COLI* ISOLATED FROM BROILER CHICKENS

by

JACINTA L. SMITH

(Under the Direction of Margie D. Lee)

ABSTRACT

Some authorities question the use of antimicrobials in food production based on data that suggests usage may lead to an increase in multiple drug resistance among food-borne pathogens. The purpose of this study was to investigate the influence of antibiotic administration on the ecology of antibiotic resistance. We detected a high prevalence of resistance to antibiotics that were not used on the chicken farms, suggesting that antibiotic usage patterns may not be predictive based on phenotypic data. Sarafloxacin administration did affect the *E. coli* strain population in a flock. However, we discovered that other factors such as physiological status and the environment might also affect the ecology of resistance. Overall, our data indicated that multiple drug resistance may occur at a high prevalence without an accompanying antibiotic administration selective pressure. This data also revealed the many factors that influence multiple drug resistance among commensal bacteria in the chicken intestine.

INDEX WORDS: Antibiotic resistance, *Escherichia coli*, Integron, Tetracycline determinant, Poultry, Agriculture

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DEDICATION

I dedicate this thesis to my mother, Cynthia Smith and my major professor, Dr. Margie D. Lee……two of the most influential mentors in my life.

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CHAPTER 1

LITERATURE REVIEW

Introduction

Over the past 50 years, antibiotics have been the classic "magic bullet" for bacterial infectious diseases, the discovery of which vastly changed the relationship between bacteria and people (36). However, the wide usage of antibiotics over the decades may have selected for a population of multiply drug resistant bacteria that have significant implications on public health and food safety. The concern over antibiotic resistance has influenced many entities of society, including government in legislation and public policy, fast-food industries in purchasing supplies of meat, pharmaceutical drug development, animal husbandry practices, and managed care practices. It is important for scientists to continue antibiotic resistance research in order to fully ascertain the processes underlying the acquisition, spread, ecology, and evolution of antibiotic resistance.

Antibiotics: General overview

The word "antibiotic" is used to describe any low-molecular weight compound, whether it be a microbial metabolite, or a synthetic compound, that at low concentrations will kill or inhibit growth of microorganisms (49). Antibiotics that stop bacteria from growing are bacteriostatic while others that cause bacterial cell death are bactericidal. Some antibiotics can display bacteriostatic activity in some circumstances and bactericidal activities in others. This can occur when sufficient damage to one or more cell pathway or structure yields a net bactericidal response (64). Most antibiotics are directed against a specific target molecule that is unique to the bacteria, interfering with structures or processes that are essential for growth or survival, while causing little to no harm to the host (6). Antibiotics are grouped into "classes"

based on either their mechanism of action or chemical structure (31). The major classes of antibiotics include: (1.) b-lactam antibiotics such as penicillin and glycopeptides that inhibit bacterial cell wall synthesis (2.) Aminoglycosides and tetracyclines which inhibit protein synthesis binding by the 30S ribosomal unit, (3.) macrolides and lincosamides which inhibit protein synthesis by binding to the 50S ribosomal unit, (4.) quinolones, which block DNA replication, (6.) sulfonomides and trimethoprim, which interfere with DNA synthesis by blocking the folate coenzyme biosynthetic pathway, and (7.) other miscellaneous drugs such as rifampin, which inhibits DNA-directed RNA polymerase (64, 31, 49, 20). These major classes of antibiotics have been used in both human and/or veterinary medicine.

Although antibiotics used in food animals belong to the same classes as those used in human therapy, there are relevant differences that exist within classes and usage of antibiotics in veterinary medicine (9, 55). Antibiotics in food producing animals are primarily used for the prevention and control of bacterial infections and growth promotion (55). The control or prevention of bacterial infections is achieved by either therapeutic (treatment of an infection), metaphylactic (rapid treatment of animals in a group to prevent further extension of the infection), or prophylactic (preventative) application of antimicrobials (55).

Thirty-two antimicrobial compounds, that do not require a veterinary prescription, are approved for use in broiler feed in the United States (15, 28). Fifteen of the 32 approved compounds are used in the treatment and prevention of coccidiosis. Some of these 32 antibiotics are also used in growth promotion of poultry. Eleven of the 32 approved antimicrobials licensed as growth promoters are given at low concentrations to improve weight gain during the growth period of the animals (15). Seven of these compounds are used in human medicine (bacitracin, chlortetracycline, erythromycin, lincomycin, novobiocin, oxytetracycline, and penicillin). Some

believe that the use of closely related antimicrobials in human and veterinary medicine creates a hazard because of the development of antimicrobial resistance among food-borne pathogens. The use of antimicrobials in the growth promotion of food animals has sparked a debate among public health and food safety experts. The debate centers around the risk associated with the use of antimicrobials in agriculture as a potential threat to human health and the influence of antibiotic usage on the ecology of resistance.

Antibiotic Resistance

A bacterial strain is considered "resistant" to an antibiotic when it is not inhibited by the minimal concentration (MIC) of the antibiotic that inhibits the growth of a typical strain of that species (20). Some of the nomenclature used in publications to describe bacteria as "resistant" is contingent upon *in vitro* antibiotic concentrations which can be greater than concentrations achievable *in vivo*; however, a bacterial strain is in fact resistant to an antibiotic when it can persist and grow in the presence of antibiotics at or above concentrations achievable in the serum or tissues (63). Antibiotic resistance occurs when a microorganism acquires the ability to destroy or change an antibiotic target molecule, or prevent the uptake of the antibiotic into the microorganism. The first antibiotic resistance mechanism was identified in 1940 when Abraham and Chain described the presence of a penicillinase in resistant *Escherichia coli* (1). Therefore, antibiotic resistance was recorded shortly after the discovery of the antibiotics themselves.

Mechanisms of Resistance

Bacterial resistance can be acquired through mutations, the acquisition of genetic elements, or it inherently occurs due to the absence or inaccessibility of the antibiotic target (57). An example of intrinsic antibiotic resistance includes the *Pseudomonas aeruginosa* Mex-AB-

OprM efflux pumps, that are able to reduce the intracellular concentrations of many compounds including antibiotics (52). In addition, the *Streptomycetes* sp produce antibiotics as a means of self-protection, thus requiring an intrinsic resistance to its antibiotics (6). Bacteria can acquire a variety of mechanisms to defeat antibiotics. These include: limiting the intracellular concentrations of the antibiotic by efflux of the drug; modification or neutralization of the antibiotic by enzymes that use the drug as a substrate; alteration of the target of the antibiotic so that the drug no longer binds to it; and elimination of the target by the acquisition of different metabolic pathways (29).

Genetic Basis of Resistance

Bacteria may acquire resistance determinants on genetic elements such as plasmids, transposons, and integrons. Horizontal transfer is a common way bacteria exchange genetic information through conjugation, transduction, or transformation. Plasmids are dispensable extra-chromosomal elements (61). Many plasmids are self-transmissible and one bacterium can simultaneously harbor several different plasmids (26,61). Plasmids may carry a range of different determinants such as virulence genes. These genes may encode for virulence factors that do damage to the host such as the virulence plasmids in *Agrobacterium tumefaciens* that incite crown gall tumors in susceptible plants (65,67). Virulence genes may also encode for adhesins, allowing the bacteria to adhere and colonize on cell surfaces and establish disease such as those found in Enteropathogenic *E. coli* (47). Plasmids may confer other various genetic traits to their bacterial host such as pathogencity factors, degradation of aromatic compounds, and resistance to antibiotics and heavy metals (62). Plasmids may carry genes that specify resistance to antibiotics, thus acting as vectors to a variety of antibiotic resistance genes. Akiba et al provided the first evidence of multiple resistance plasmids in pathogenic bacteria in the 1960's

(2). Later, Mitsuhashi et al unveiled how these resistance plasmids moved between different bacterial species by horizontal gene transfer (41). Since some plasmids have a broad-host range, these vectors may serve as the most active vehicles for promoting the rapid spread of antibiotic resistance genes to many bacteria (61).

Transposons are DNA segments capable of inserting themselves at a new location in the plasmid, chromosome, or bacteriophage. Transposons are widespread in nature and have been identified in many organisms (7). Transposons contain insertion sequences (IS), which may flank antibiotic resistance genes or toxin genes. Some transposons, such as Tn*10*, encode resistance to tetraryclines (30). Others like Tn7 encode resistances to trimethoprim, streptomycin, and streptothrycin (25). These IS elements may insert themselves into the bacterial chromosome, enabling transposons to spread antibiotic resistance genes through vertical evolution as well as horizontal transfer.

Integrons are genetic elements that, although unable to move themselves, contain gene cassettes that can be mobilized to other integrons (17). Approximately 60 gene cassettes have been characterized that confer resistance to antibiotics, heavy metals, and disinfectants (16). Movement of the gene cassettes is catalyzed by a site-specific recombinase (*intI*) that recognizes the 59 base element in the cassette and the *attI* site in an integron (24, 50, 53). The site-specific recombination results in the integration of a circular gene cassette into an integron (11). Several gene cassettes containing different resistance genes may be found in a single integron. Up to five gene cassettes have been reported in a integron from a clinical *Pseudomonas aeruginosa* strain (33) and eight functional resistance gene cassettes have been characterized in a clinical *E. coli* strain isolated from a hospital patient in France (45). Integrons are classified into 8 different categories based upon differences in the integrase amino acid sequence. Class 1 integrons are

the most common class encoding resistance among gram-negative bacteria from clinical (5, 19, 59) and environmental sources (4, 34). Some of the resistances associated with class 1 integrons include B-lactam antibiotics, aminoglycosides, trimethoprim, chloramphenicol, rifampin, erythromycin, and quaternary ammonium compounds (17). Class 1 integrons are commonly found in gram-negative bacteria; however, recent studies have described the presence of a class 1 integrons in gram-positive bacteria and the potential of these organisms to serve as reservoirs of integron carriage among commensal bacteria (46, 48). Bass et al described the widespread dissemination of the class I integron associated with transposons Tn*21* in multiple drug resistant avian *E. coli* isolates (5). Tn*21* and other related transposons were first described in multiple drug resistant *Shigella* strains isolated in Japan in the 1950's (60) and since have been identified in many bacterial genera and families. Class 2 integrons are associated with transposon Tn*7* and other related transposons such as Tn*1825* and Tn*1826* (25). Only three antibiotic resistance gene cassettes have been characterized among class 2 integrons. These cassettes encode for trimethoprim (*dfr1*), streptomycin (*aadA1*), and streptothrycin (*sat*) resistance (25). A class 3 integron has been described in a transposon-like element with several gene re-arrangements (56). This class 3 integron carried the bla_{imp} gene, a cassette associated with broad-spectrum B-lactam antibiotic resistance. The class 3 integron has been detected only among *Serratia marcescens* (3) while the class 2 integron has been characterized primarily among the *Enterobacteriaceae* organisms (25). The ability of integrons to incorporate more than one antibiotic gene cassette, the association of integrons with transposons that my carry additional antibiotic resistance genes, and the association of multiple transposons with the same plasmid may explain the common occurrence of bacterial strains that are concurrently resistant to many different antibiotics (23).

Antibiotic Resistance: An Emerging Public Health Concern

"*Losing the Battle of the Bugs: Common bacteria are now so resistant to antibiotics that they can kill. We have no one to blame but ourselves"* (53).

Similar headlines have recently graced the covers of newspapers and periodicals throughout the nation. There has been a recent increase in the frequency of antimicrobial resistance conferences and symposiums as well as a multimillion dollar increase in the funding of antibiotic resistance research and surveillance. Why is there a resurgence in the debate over antibiotic resistance, especially since resistance has been characterized since the early 1940's? In the last 50 years, bacteria have remarkably demonstrated the ability to develop or share resistance to almost every antibiotic developed and the increasing development of resistance has been well documented in human and veterinary medicine. (14, 35, 54). Some believe that the frequent prescribing of drugs to humans have resulted in the emergence of resistant bacteria strains. Others believe that the widespread use of common antibiotics in veterinary medicine have aided in the emergence of resistance. Therefore, some propose prudent usage of antibiotics in both populations as a way to combat the growing prevalence of antibiotic resistant bacteria in human (58) and veterinary (16, 58) medicine. Public concerns and new research about antibiotic usage in food safety has prompted the federal government to take action and withdraw the approval of fluoroquinolone administration in poultry husbandry (16). Bayer, a drug company that manufactures enrofloxacin for use in poultry, recently lost a federal court case against the Federal Drug Administration (FDA) resulting in the pending revocation of the approval of enrofloxacin (14). Other countries have banned the use of growth promoting antibiotics altogether (13), resulting in a suggested decrease of resistance to some antibiotics and a moderate prevalence of resistance to others. However, many studies have not supported the "cause-and-

effect" relationship associated with antibiotic usage and prevalence rates. In fact, the data from these studies demonstrated a mutlifactorial relationship associated with the ecology of resistance (32, 43, 44).

Ecology of Resistance in Agriculture

In addition to antibiotic usage, there are other elements that may influence the epidemiology of resistance in agriculture. These factors include animal diet, farm management, the environment, and the natural evolution of bacteria strains and resistance genes. Some studies show that physiological stress may alter the intestinal bacteria community resulting in the shedding of resistant bacteria strains in the fecal contents of the animal (43, 44). Other studies demonstrate the evolution of bacteria strains over time that result in an improved fitness to carry antibiotic resistance gene loads (42). Due to the monitoring of clinical human and veterinary isolates, the prevalence of resistance is being commonly studied in pathogenic bacteria. Some studies have shown the widespread dissemination of clonal pathogenic strains that carry antibiotic resistance genes (for ex: emergence of *Salmonella* DT104) (18). Other studies have shown an increased prevalence of resistance to certain antibiotics among pathogenic bacteria strains due to linkage of antibiotic resistance genes on virulence plasmids (12). Current studies are investigating the role of commensal bacterial in the ecology of resistance (19, 22, 38, 39, 46). The data from these studies suggest that commensal bacteria serve as reservoirs for some antibiotic resistance genes, resulting in a possible exchange of genetic information to pathogenic bacteria and other non-related organisms in the intestine.

Environmental Impact of Genes in Manure, Litter, and Sewage

Environments such as manure, litter, and sewage, may influence the ecology of resistance. Some antibiotic resistance occurs naturally in the environment (21). However, farming and industry practices may impact the prevalence of resistance genes and resistant bacteria in the environment. Agricultural practices, such as integrated fish farming, combines the use of animal manure (mainly chickens and pigs) into fish ponds as fertilizer to support the growth of photosynthetic organisms. Recent studies showed an increase in the prevalence of resistance among aquatic bacteria in these environments (51). Based on this data, some would suggest that integrated fish farming favors antimicrobial-resistant bacteria in the pond environment due to the introduction of antimicrobial resistant bacteria from animal manure. However, other plausible explanations for an increase in resistance could be due to the increased density of bacteria among ponds sediments after many applications of organic material such as manure. Therefore, the normal bacterial ecology of the pond environment could be altered. Other factors such as a past history of antibiotic usage may have also affected the prevalence or resistance. Recent studies have demonstrated the dissemination of a newly characterized antibiotic resistance gene (*tet 36*) among gram-negative proteobacteria and gram-positive bacteria isolated from swine manure, indicating the occurrence of horizontal transfer between divergent polygenetic groups in the farm environment (66).

In addition to manure, sewage facilities may have an environmental impact on the ecology or resistance. Iverson *et al* studied the transmission of an ampicillin-and ciprofloxacin-resistant *Enterococcus faecium* from humans to the environment (27). The nosocomial *E. faecium* strain isolated from patients in a Swedish hospital was found in samples of hospital sewage (50%), surface water (35%), treated sewage (28%), and untreated sewage (17%),

suggesting a possible transmission route for nosocomial *E. faecium* to the environment. Many would conclude that the resistant strains in the environment are of hospital origin, but it may have been possible that the source of the nosocomial strain originated in the surveyed environment and entered the hospital through environmental contamination from sources such as surface water. Similar studies published data indicating waste effluent from a pharmaceutical plant was likely to cause a change in the distribution of antibiotic resistant *Acinetobacter* sp by selecting or introducing resistant strains into recipient sewers (21).

There is a microcosm of bacteria that exist within the litter environment. We have characterized the unique microbiota associated with chicken litter and we have also shown that the litter may contain a diverse load of antibiotic resistance genes (34, 38). Therefore, the litter environment may be an important reservoir for resistance genes that are available to the abundant members of the litter bacterial community. The fitness cost in carrying certain plasmids that contain antibiotic resistance genes can be reduced as generations of *E. coli* strains evolve in an environment. Therefore, the co-evolution of bacteria populations and resistance genes in the environment may influence the prevalence of antibiotic resistance also.

Although many studies have explored the influence of antimicrobials and resistance in natural, agricultural, and industrial environments, little is known about the epidemiology of resistance gene deposits in research facilities. How does this environment contribute to the dissemination and prevalence of resistance? There is also a gap in the knowledge associated with the stability of certain antimicrobials and their metabolites in the environment and its influence on the ecology of resistance. Little has been reported on the genetic exchange of antibiotic resistance genes among the intestinal bacteria of animals and humans. The purpose of this study was to investigate the ecology of resistance among commensal *E. coli* isolated from

the broiler chicken intestine by detecting specific antibiotic resistance genes among commensal strain types. The information from this study explores the factors that contribute to the dissemination and prevalence of resistance among commensal bacteria from animals.

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CHAPTER 2

INTEGRON AND TETRACYCLINE DETERMINANT CARRIAGE AMONG STRAINS OF COMMENSAL *ESHERICHIA COLI* ISOLATED FROM BROILER CHICKENS[1]

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Abstract

Some authorities question the use of antimicrobials in food production based on data that suggests usage may lead to an increase in multiple drug resistance in human pathogens. The purpose of this study was to investigate the influence of antibiotic administration on the ecology of antibiotic resistance among commensal *E. coli* isolated from the broiler chicken intestine. We detected a high prevalence of resistance to drugs such as tetracycline, sulfonamides, and streptomycin among treated and untreated flocks. We also found that the population of *E. coli* in the microflora of an untreated flock consisted of several dominant strain types, some of which persisted as the flock matured. Although some strain types persisted among flocks on the same farm, we did not observe the same strain of *E. coli* on multiple farms, suggesting that farm-specific resident strains may not be prevalent elsewhere. Sarafloxacin administration did affect the *E. coli* strain population in the flock. However, when examining the affect of physiological status on the ecology of resistance, we found that heat stress did not change the strain prevalence of *E. coli* nor did it significantly change the prevalence of tetracycline resistance. However, class I integron carriage significantly decreased ($p=0.0415$) and class II integron carriage significantly increased ($p=0.0285$), suggesting that health status of the birds may influence gene carriage. We were not able to correlate resistance with antibiotic usage because of the wide variation in prevalence rates and MICs among isolates from different flocks and different commercial farms.

Introduction

The poultry industry is a significant economic force across the United States, with the value of poultry production averaging over 50 billion dollars in 1997 (31). The demand for poultry has increased over the past 50 years from 1 million pounds of chicken per year in 1950 to

over 40 billion pounds in 2000, resulting increased production by over 20 fold (31). However, economic loses due to cellulitis and airsaculitis infections in broiler chickens were greater than $80 million in 2002 (32). *Escherichia coli* is the primary causative agent of cellulitis, septicemia, and airsacculitis in poultry; therefore, it is the most significant bacterial pathogen of broiler chickens (3,12). There are several antibiotics approved for treatment of *E. coli* infections in broiler chickens such as tetracycline and streptomycin (8, 36, 34). However, some are not cost effective while others are ineffective due to bacterial acquired resistance (1, 16, 17).

Due to increasing human food safety concerns, Abbott Laboratories withdrew sarafloxacin, a fluoroquinolone used to treat *E. coli* infections in poultry, from the market in 2001 (9). The use of antimicrobials in food production is controversial because of data that suggest that usage may lead to an increase in multiple drug resistance in human pathogens (8, 30). These human food safety concerns have been influential in triggering the European Union to ban the use of antibiotics as growth promotants in food production (5) and increase their surveillance for bacterial resistance among food-borne pathogens and indicator organisms (16).

Several antibiotic resistance studies suggest that the long term use of antibiotics for therapy and growth promotion selects for multiple drug resistant gram-negative pathogenic bacteria (13, 16). In farm environments, commensal and environmental bacteria may serve as a reservoir for the transfer of antibiotic resistance genes to pathogenic bacteria. We have reported on the gene load of resistance determinants among the bacterial community in chicken litter (18, 24). Since bacteria acquire resistance genes through horizontal transfer, many conjugative genetic elements such as plasmids and transposons are common vectors in the dissemination of antibiotic resistance genes to diverse microorganisms. The purpose of this study was to investigate the influence of antibiotic administration on the ecology of antibiotic resistance

among commensal *E. coli* isolated from the broiler chicken intestine by detecting specific antibiotic resistance genes among commensal strain types. The information gleaned from such a study should help elucidate the ecology of resistance among commensal bacteria in a farm environment.

Materials and Methods

Sample Collection. Fresh cecal droppings were collected from the top of the wood shavings of flocks raised on three commercial broiler chicken farms in Northeast Georgia. Samplings occurred at various ages from 3 wks of age to 7 wks of age and the history of antibiotic usage was known for these commercial farms.

In addition, experiment broiler chicken flocks were raised on wood shavings for bedding in bleach-disinfected concrete floored pens in a research facility (Poultry Diagnostic and Research Center). One hundred twenty research broiler chickens were split into 4 treatment groups at 4 weeks of age. The birds were administered therapeutic concentrations of antibiotics in their drinking water at 4 weeks of age. The antibiotic doses for the treatment groups were as follows: sarafloxacin: 20ppm for 5 days; enrofloxacin: 25ppm for 3 days, and oxytetracycline: 25mg/lb for 5 days. All treatment groups were fed a commercial corn/soy meal broiler diet. The broiler feed contained monensin (90 grams/ton) and bacitracin methylene disalicylate (50 grams/ton). Ten birds were euthanatized by carbon dioxide asphyxiation at 3 wks, 5 wks, and 7 wks of age and the cecal contents were collected for bacterial isolation.

Bacterial Isolation and Identification: Thirty to one hundred and twenty cecal droppings from each commercial farm sampling time were collected using sterile wood applicators and placed in 30 tubes containing 1mL of BHI broth. The 30 tubes were pooled together into 10 tubes, diluted in saline (10^{-3} and 10^{-5}), and plated on MacConkey agar (Difco).

The plates were incubated overnight at 38°C. Thirty isolated colonies were collected from the 10 plates and subcultured onto MacConkey plates. These were stored at- 80°C in freezer stock solution (15% glycerol and 1% peptone). Similarly the samples of cecal contents from the research birds were plated on MacConkey agar and 30 isolated colonies were collected and archived. *Escherichia coli* isolates were identified according to a panel of biochemical tests that noted gas production and sugar fermentation reaction of Triple Sugar Iron agar media, indole production, citrate fermentation, ornithine decarboxylase fermentation and oxidase reaction (10). Ten avian pathogenic *E. coli* (APEC) isolated from clinical cases of septicemia and colibacillosis were acquired from the Poultry Diagnostic Research Lab at the Poultry Diagnostic Research Center.

Antibiotic Susceptibility Profiles: The minimum inhibitory concentrations (MIC) were determined using the Sensititre automated antimicrobial susceptibility system (Trek Diagnostic Sys; Westlake, Ohio). The antibiotics tested were those present on Sensititre avian antibiotic plates: amoxicillin, ceftiofur, gentamicin, neomycin, tetracycline, oxytetracycline, spectinomycin, streptomycin, sulfadimethoxine, sulfathiazole, sarafloxacin, and enrofloxacin. The nalidixic acid antibiotic was tested from a customized Sensititre fluoroquinolone plate (Trek Diagnostic Sys; Westlake, Ohio). Results were interpreted according to National Committee for Clinical Laboratory Standards guidelines for microbroth dilution methods for veterinary *E. coli,* (25). *E. coli* ATTC 25922 was used as a control strain for susceptibility.

Strain typing by Enterobacterial Repetitive Intergenic Consensus (ERIC) PCR. ERIC-PCR template was prepared from whole cell bacteria. A loop full of bacterial growth from a fresh blood agar plate was placed in 1ml of 70% ethanol in a sterile microcentrifuge tube and centrifuged at 12,000 rpm for 6 minutes. The ethanol from the tube was discarded into a

biohazard waste container and the remaining pellet was washed with 1 ml of deionized distilled water. The pellet was resuspended in deionized distilled water and centrifuged at 12, 000 rpm for 6 minutes. The supernatant was discarded into a biohazard waste container. The remaining pellet was resuspended in 50ul of deionized distilled water and a working template was prepared from a 1:100 dilution of the resuspended pellet. The ERIC-PCR was performed in a Rapidcycler (Idaho Technologies: Idaho Falls, ID) under the following program parameters: denaturation at 94°C for 1 second, annealing at 52°C for 10 seconds and extension at 72°C for 35 seconds for 30 cycles; ending in a final extension cycle of 72°C for 4 minutes. The 10ul PCR reaction mix consisted of 1mM deoxynucleotides, 3mM of $MgCl_2$, PCR buffer (50mM Tris), 50 pmol of each primer (38) and 0.5 units of Taq DNA polymerase (Roche Molecular Biochemicals, Indianapolis, Ind.). DNA products were separated on a 1.5% agarose gel containing ethhidium bromide (5ug/ml) at 65V for 2.3 hours. Strains were considered similar if they shared 3 or more bands.

Detection of Resistance Genes. The DNA probes for determinant analysis were generated by PCR using dig-labeled nucleotides with primers specific for tetracycline determinants *tet* (A), (B), (C), (D), (E) (26) and *int*I1 and *int*I2 determinants (11). DNA-DNA hybridization for the detection of resistance determinants were performed as described by Sambrook et al (29) with hybridization washes at 60°C for *int*I2 detection and 68°C for the other genes. Fragments were detected with anti-digoxigenin alkaline phosphatase conjugate (Boehringer Manheim, Indianapolis, Ind.). The NBT/BCIP substrate tablets were used for the detection of alkaline phosphatase and the development of the colormetric assay as described by the manufacturer (Roche Molecular Biochemicals). The class 1- associated antibiotic resistance

gene cassettes were detected in a PCR- enzyme-linked immunosorbent assay (ELISA) as previously described by Lu et al (18).

Fluoroquinolone Mutation Analysis. The quinolone resistance determining region (QRDR) was amplified as previously described by Weigel et al. (39). PCR was performed in a Rapidcylcer (Idaho Tehnologies: Idaho Falls, ID) under the following program parameter: an initial denaturation at 94°°C for 2 min, denaturation at 94°C for 1 min, annealing at 57°C for 1 min., extension at 72°C for 1 min., and a final extension at 72°C for 5 min. The 50ul total volume PCR mixture consisted of following components: 250uM deoxynucleotides, 1.5 mM $MgCl_2$, PCR buffer (50mM Tris), 100pM of each primer (39), and 2.5 U Taq DNA polymerase (Roche Molecular Biochemicals, Indianapolis, Ind.). DNA products were separated on a 1.5% agarose gel containing ethidium bromide (5ug/ml). In order to detect the *gyrA* mutations at Ser-83 and Asp-87 in the PCR amplicon, a restriction fragment length polymorphism assay was performed as described by Ozeki et al (28).

Statistical Analysis. In order to detect differences in antibiotic resistance in *E. coli* isolated from different farms and flocks and among birds of differing ages or treatment groups, Fisher's exact method (for comparison of two groups) and the Cochran-Mantel-Haenszel method (for comparison among three groups) were used to determine whether isolates from one group were significantly more resistant than others. For detecting the differences in the mean MIC among the treatment and age groups in the research flock, a Kruskal-Wallis test was used to determine whether at least one group had significantly higher mean MICs than others. The Wilcoxon-Mann-Whitney test was used to identify the group that had significantly higher mean MICs. To analyze antibiotic resistance gene and integron carriage rates, we fitted logistic models to determine whether one group (by age, farm, flock, heat stress, or antibiotic usage) was

more likely to have an antibiotic resistance gene or integron than another. For comparison in the logistic model, the p-value was determined by the Wald-Chi squared method. Fisher's exact method was used to test whether there was a significant difference in the prevalence of carriage of antibiotic resistance genes between two groups when it was appropriate for the data.

Results

Prevalence of Resistance. Table 1 shows the antibiotic susceptibility of *E. coli* isolated from three flocks of healthy chickens on a farm that had not used therapeutic antibiotics in the flock house for over a year. We detected a high prevalence of tetracycline (36-97%), sulfa (50-100%), and streptomycin (53-100%) resistance at all sampling times. This suggests that there may be a high background of resistance to some antibiotics irrespective of past usage on this farm. However, we detected a low prevalence of resistance to fluoroquinolone and B-lactam antibiotics that may be associated with the lack of usage of these antimicrobials on this farm. Significantly higher gentamicin MICs ($p=0.0064$) were detected among isolates collected from young birds. The company that supplied these birds used gentamicin *in ovo*. Gentamicin is approved for use *in ovo* and in day-of-hatch birds (33) as is ceftiofur (35). Higher neomycin and oxytetracycline MICs were also detected in isolates cultured from 3 week old birds suggesting that the microflora of young birds is likely to consist of resistant bacteria perhaps due to the ecology of population succession of normal flora *E. coli*.

Two of the farms in our study had used therapeutic antibiotics in several flocks of chickens prior to our sampling and in two flocks that were included in our study. Table 2 shows the susceptibility of the isolates collected from the treated flocks. We detected a high prevalence of tetracycline (58-90%), sulfa (77-100%), and streptomycin (74-100%) resistance among the isolates. Both of these farms had a history of enrofloxacin and sarafloxacin usage. Isolates

cultured from a flock that was administered sarafloxacin had higher MICs (P=0.001) to sarafloxacin than isolates from the farm with no recent history of usage. However, we did not observe a significant increase in the prevalence of tetracycline resistance associated with oxytetracycline administration in the treated commercial broiler flock. One of our difficulties in demonstrating a statistically significant change in tetracycline resistance was related to the high levels of background resistance among isolates from untreated flocks and the variation in the prevalence of resistance due to differences in birds' age and farm. We detected significant age-related differences in the gentamicin (P=0.0064), oxytetracycline (P<0.0001), and neomycin (P=0.0035) MICs of the isolates. The pattern of increasing or decreasing resistance related to age and varied by farm and flock.

***E. coli* Strain Ecology**: Some of the variation in resistance may be due to changes in the microbial ecology and population biology of the chickens' intestine. Certain prevalent resistant strains of *E. coli* could dominate the gram-negative microflora and may persist on some farms. Thirty to fifty percent of the isolates from each flock were fingerprinted by ERIC-PCR in order to investigate whether *E. coli* strain carriage correlated with resistance. We considered the strains to be related if the fingerprint patterns shared three bands. We found that the normal succession of *E. coli* in the microflora of the chickens' intestine consisted of several dominant strain types that persist as the bird matures (Fig. 1). Although there were other *E. coli* strain types that were present in the microflora, two dominant strain types persisted in three flocks on the farm that did not use antibiotics, suggesting that a resident strain tends to colonize new flocks that enter the chicken house on the same farm. It is commonly believed that antibiotic usage selects for a dominant resistant strain. If the population of *E. coli* in an untreated flock is primarily composed of a dominant strain, how will usage affect the normal succession of strain

prevalence? We had flocks on different farms that were treated with tetracycline and sarafloxacin. There was a dominant strain that was present in the microflora of the maturing birds on Farm C and this dominant strain type persisted throughout the flock. Therefore, it was difficult to correlate usage with strain prevalence since the normal succession of *E. coli* in the microflora favored a dominant resistant strain type.

Although dominant strain types persisted among flocks on the same farm, we did not observe the same strain of *E. coli* from one farm to another, suggesting that the resident strain was farm specific and may not be prevalent elsewhere. (This was evident because Farm B and Farm C were managed by the same farmer.) There were different dominant strains on these farms although they were located across the road from each other. Although there was a dominant strain type, the prevalence of resistance varied with flock and with farm. Over 75% of the commercial isolates were resistant to 3 or more antibiotics. There were different strains that exhibited the same multiple drug resistance patterns and similar strains that displayed different patterns (Fig. 2). Therefore, multiple drug resistance patterns could not be explained based solely on strain prevalence, suggesting that there were other factors in addition to strain types that contributed to the prevalence of resistance.

The litter in the commercial chicken houses may serve as a reservoir for *E. coli* that colonize subsequent chicken flocks that reside on the bedding. Since statistical analysis showed variables such as the age, flocks, and different farms were confounding factors in the prevalence of antibiotic resistance; a group of research broiler chickens were raised under controlled conditions at our research facility. The research birds consisted of a flock that was placed onto disinfected concrete floors covered with fresh woodchips as bedding. These birds were administered therapeutic concentrations of an assigned antibiotic in their drinking water. Table 3

shows the antibiotic susceptibility of *E. coli* isolated from the research birds. Like the commercial farms there was a low prevalence of susceptibility to antibiotics such as tetracycline (17-57%), streptomycin (20-43%), and sulfathiazole (23-43%) among the untreated group. These data suggest that a high background of resistance was present in the intestinal microflora of young chickens despite the use of fresh bedding which may reduce the resistant strains picked up in the environment. This data also suggests that even under controlled settings (same management, farm, and flock), there was a high prevalence of resistance to certain antibiotics not used commonly in broiler chicken husbandry such as sulfonamides and streptomycin. It was difficult to correlate usage of antibiotics with prevalence of resistance among the commercial flocks due to inherent variation; however, many of those variables were controlled in the research setting. We detected significant differences in MIC levels between treatment groups. The *E. coli* isolates had higher MICs to neomycin, tetracycline, streptomycin, nalidixic acid, and sulfathiazole at 3 wks of age than at 5 wks of age ($p=0.05$), which suggests that age remained a confounding factor when interpreting resistance prevalence data. When comparing the treatment groups together, isolates from the enrofloxacin group at 5 and 7 wks of age were more likely to have higher fluoroquinolone MICs than the other treatment groups. Since enrofloxacin resistance in *E. coli* is characterized by two point mutations in the *gyrA* gene, one of which also confers resistance to sarafloxacin and nalidixic acid, this explains the linkage of sarafloxacin, nalidixic acid, and enrofloxacin resistance. We also found that isolates from the enrofloxacin group were less likely to have higher MICs to streptomycin than the other groups at 5 wks of age. This may be caused by the plasmid curing properties of fluoroquinolones that was first described by Weisser et al. (40). These data suggest that the presence of a quinolone could affect the carriage of resistance determinants.

Since there was variation in the prevalence of resistance among the research birds and commercial birds, that could not be explained by treatment status alone, we investigated whether there are specific strain types that would explain the fluoroquinolone resistance prevalence among the isolates. On Farm C, we detected 7 nalidixic acid resistant isolates from 3 wks old birds and 13 fluoroquinolone resistant isolates after sarafloxacin treatment at 6wks of age. There were 3 strain types among the 13 isolates that accounted for the resistance prevalence among the *E. coli* isolated after sarafloxain treatment. Only one of these strains was observed before treatment. These *E. coli* strains could have persisted in the litter between flocks; therefore, the data from the research flock could eliminate this confounding factor. Unlike the commercial flock where there were multiple resistant strains, we detected two dominant strains in the untreated research flocks. There was a prevalence of 33% of sarafloxacin resistance in the control group of the research flock. This may be representative of a normal background of resistance without resistant isolates contaminating the litter. In the research flock, there were 2 strains among the fluoroquinolone resistant isolates from the untreated flocks at all ages. These strains possessed the *gyrA* (ser83) mutation. The resistant strains among the sarafloxacin treated group also possessed the *gyrA* (ser83) mutation. The susceptible strain types from the research sarafloxacin group were different from the dominant resistant strain, but the sarafloxacin-treated commercial flock did not have a dominant resistant strain type. There may have been a variety of resistant strain types in the environment that may prevent one strain becoming dominant in the microflora.

Prevalence of Antibiotic Resistance Genes: Tetracycline resistance can be mediated by 36 different genetic elements (4). *E. coli* isolates have been found to carry *tet A, B, C, D, E, I,* and *Y*; however, *tet A-E* have been found to be the most prevalent (4). We wanted to determine

whether particular tetracycline determinants correlate with the presence of a particular strain. In untreated research flocks, related strain types carried the same tetracycline determinant. However, on the commercial farms, some similar strains carried different tetracycline determinants. This data suggests that there are many factors (litter, management, flock) that contribute to the ecology of tetracycline allele carriage on commercial broiler chicken farms.

In order to determine whether administration of tetracycline would affect or select for a particular determinant, we screened isolates cultured after oxytetracycline administration. Table 4 shows the prevalence of tetracycline determinant carriage in the commercial farm and research farm. *tet* A and *tet* B accounted for most of the tetracycline determinant carriage among the flocks irrespective of treatment. On farm C, there was no significant change of *tet A*, *tet* B, or *tet* C ($p>0.1$)) determinant carriage after sarafloxacin treatment. However, on farm B, there was a significant decrease in *tet B* carriage ($p=0.0003$) after tetracycline treatment with a significant increase in *tet A* carriage ($p<0.0001$). Also, *tet A* was more likely to be detected among isolates from farm B than farm A ($p=0.0002$) or farm C ($p=0.0005$), suggesting that the tetracycline administration on Farm B may have affected tetracycline allele carriage. Past usage of tetracycline on a farm may also affect the persistence of a particular tetracycline determinant to essentially reduce the cost of resistance; however, there were significant age ($p<0.0338$) and farm ($p=0.0041$) interactions that may confound the interpretation of the data. In some cases we found a higher prevalence of *tet* determinant carriage than tetracycline resistance, suggesting that silent carriage was not usual. We also detected a higher prevalence of tetracycline resistance than carriage of *tet* A-E alleles. This was particularly true on the commercial farms indicating that there may be a high diversity of *tet* determinants among the microflora or environmental bacteria.

We screened a group of avian pathogenic *E. coli* (APEC) for the presence of *tet A, B, C, D,* and *E* in order to determine the prevalence of tetracycline determinant carriage among clinical *E. coli* strains. All of the APEC strains were resistant to tetracycline. 45% of the isolates carried *tetA* , 56% carried *tet B*. Therefore, the prevalence of tetracycline determinant carriage in commensal and avian pathogenic *E .coli* is similar.

Tetracycline resistance can be chromosomally mediated; however, many are located on transmissible plasmids which often contain other antibiotic resistance genes. Our multiple drug resistance data indicated that these isolates may contain similar transmissible elements because of similar multiple drug resistance profiles. Since antibiotic resistance genes can be carried on mobile elements, the transfer of common genetic elements that carry antibiotic resistance genes such as integrons, plasmids, and transposons may contribute to variation in the prevalence of resistance even among similar strains. Integrons are common mobile genetic elements that encode for multiple drug resistance in gram-negative bacteria including *E. coli* (17). We have previously shown that avian *E. coli* commonly contain integrons as well as other antibiotic resistance genes (11) and that Tn*21* associated integrons are frequently possessed by avian *E. coli* (1). Therefore, we sought to investigate the prevalence of integrons among treated and untreated birds. The most common integron detected among the *E. coli* isolates was the class 1 (20%-100%) which most commonly contained the streptomycin resistance *aadA1* cassette (23.3%-100%) and the trimethoprim resistance *dfr* cassette (0-17.6%) (Table 5). The class 2 integrons detected (0-20%) among the flocks contained a *dfr* cassette (67%) and an *aadA1* cassette (83%). The presence of these cassettes among the class 2 integron is similar to the integron of the *Tn*7- associated class 2 integron. Antibiotic administration significantly affected the prevalence of integron carriage. On Farm B, there was a significant decrease in *intI1* carriage

after tetracycline treatment (p=.0077) and on Farm C, there was a significant decrease in *intI2* carriage (p=0.0053) after sarafloxacin treatment. These data suggest that antibiotic administration can affect integron carriage. However, significant differences between the age (p=0.0036), farm (p=0.0008), and flock (p=0.0001) suggest that significant variation exists in integron carriage.

Previous studies have shown that environmental stress can affect the physiological status of birds (22, 23). Could physiological status also affect the prevalence of antibiotic resistance? There were two flocks in our study that demonstrated increased mortality due to high summer temperatures and poor ventilation in the chicken houses. During this time, the birds were administered electrolytes and citric acid in their drinking water. Isolates collected before and after the heat stress were strain typed to determine the effect of physiological status on the population of *E. coli* in the birds. During this episode, strain prevalence of *E. coli* for Farm C did not change nor did it significantly change the prevalence of tetracycline determinants carriage (p>0.2402). However, class I integron carriage significantly decreased (p=0.0415) and class II integron carriage significantly increased (p=0.0285) after the heat shock episode on Farm B, suggesting that health status of the birds may influence gene carriage.

Discussion

Many questions have been raised over the usage of antibiotics in food animals. Are phenotypic resistance profiles predictive of antibiotic usage patterns? Does the administration of one antibiotic increase resistance to other antibiotics due to linkage of genetic antibiotic resistant genes? Do resistant strains persist to colonize new flocks that enter the farm? The purpose of this study was to investigate the influence of antibiotic administration on the prevalence of resistance among commensal *E. coli* from commercial broiler chickens in order to address some

of these questions. Furthermore, we evaluated the strain type and antibiotic resistance gene carriage in order to determine whether the prevalence of resistance correlated with colonization by certain *E. coli* strain types. Our results suggested that antibiotic usage patterns may not be predictive based on phenotypic data. We found a high prevalence of resistance to tetracycline, sulfonamides, and streptomycin among all flocks although these drugs were not used in most flocks (Table 1, 2). This data is similar to findings published in previous resistance studies where usage may not reflect resistance (13, 15, 37). Although there were higher MICs in the commercial flock that was treated with sarafloxacin compared to farms with no history of usage; there was also a high prevalence of multiple drug resistance among flocks that were not administered antibiotics. Therefore, it was difficult to correlate usage alone with increased resistance to other antibiotics.

Previous studies have shown the persistence of clonal pathogenic *E. coli* strains in some poultry houses and a wide diversity of genotypes among non-pathogenic *E. coli* (6). We demonstrated several dominant strain types among commensal *E .coli* suggesting a propensity for resistant strains to survive in the litter environment and colonize new flocks. Another plausible explanation for strain prevalence could be the parental source which may contribute to the *E. coli* strain prevalence. Although fluoroquinolone usage in the commercial flocks did not select for a particular *E. coli* strain, the research flock experiment showed that fluoroquinolone administration did affect strain prevalence. These data suggest that under controlled environmental conditions, antibiotic administration may select for a particular resistant strain type and this finding has important implications for poultry flock house management. Although there are certain practices that increase the efficiency of production on the farm (ex: air ventilation, rodent control, growth promotants, etc.), litter management varies as a practice

among poultry companies. Some poultry companies may remove litter from the house with every new flock while others may only replace the litter a few times a year. Past studies (18, 7, 20) have shown the microbial diversity and activity of organisms that survive in the litter; therefore, it is important to understand the implications of litter management on the persistent colonization of *E .coli* and other organisms. The fitness cost of carrying plasmids that contain antibiotic resistance genes has been shown to be reduced as generations of *E. coli* strains evolve (21). Therefore, the co-evolution of *E. coli* populations and the resistance gene load in litter may influence the prevalence of antibiotic resistance. We have also shown that litter may contain a diversity of resistance genes (18, 24), suggesting that the litter environment may serve as a reservoir for gene carriage and exchange among abundant members of the litter bacterial community.

Integron mediated resistance has been shown to be common in avian *E. coli* and other veterinary pathogens (1, 11). Our study also showed that class I integrons are very common among commensal *E. coli*. In some cases, antibiotic administration did significantly decrease the carriage of integrons and tetracycline determinants, suggesting the influence of antibiotic usage on genetic carriage. These findings are similar to those of Blake et al (2) in which tetracycline administration changed the carriage of particular tetracycline determinants among commensal *E. coli*. Our changes in carriage were not due to changes in *E. coli* community structure. Therefore, the antibiotic administration appeared to have more of an affect on gene distribution than on *E. coli* resistance prevalence.

We also examined how physiological status affects the prevalence of resistance. Previous studies have shown that transport stress, overcrowding in holding pens, and rough handling of pigs before slaughter resulted in increased fecal shedding of antibiotic-resistant

enteric bacteria (22). In addition, Moro et al (23) reported a significantly higher prevalence of resistance to ampicillin, neomycin, and tetracycline in *E. coli* isolated from heat stressed pigs. In our study, there were no significant differences in the prevalence of antibiotic resistance in heat stressed chickens. However, the heat stress did influence tetracycline determinant carriage, suggesting that physiological status may affect gene carriage among resident *E. coli* strains.

We encountered difficulty correlating resistance with antibiotic usage because of the wide variation in prevalence rates among the commercial farms. Therefore, the research flock was used to control some of the confounding factors present on the commercial farm such as differences in flocks and farms, management practices, and different environments. Langlois et al (14) found that the age of pigs and the location of housing had an effect on the antibiotic resistance of the fecal coliforms from pigs in an untreated herd. These findings are similar to our study in that younger birds were more likely to contain *E. coli* that were resistant to certain antibiotics such as neomycin and oxytetracycline and resistance varied among flocks and farms. *E. coli* is most abundant among the intestinal bacterial community when the birds are young and wanes as the birds mature (19). Therefore, poultry production practices that affect the density of resistant genes and affect the abundance of pathogens such as *E. coli* may be very important in influencing the prevalence of resistance.

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Tables

Table 1: Antibiotic Susceptibility (%) of *E. coli* isolated from cecal droppings of healthy commercial broiler chickens [a,b]

Antibiotics	FARM A					
	Flock 1		Flock 2		Flock 3	
	3wks	6wks [d]	3wks	6wks	3wks	6wks
Amoxicillin	92	96	90	77	100	90
Ceftiofur	100	100	100	100	100	100
Gentamicin [c]	8	27	13	32	20	70
Neomycin [c]	100	97	97	71	97	100
Oxytetracycline [c]	27	3	58	36	64	44
Streptomycin	0	0	0	0	17	47
Sulfathiazole	0	0	0	0	0	50
Enrofloxacin	100	100	100	100	100	96
Nalidixic acid	96	96	94	97	93	62
Sarafloxacin	96	97	94	97	97	75

[a] MICs were performed using the Sensititre microbroth dilution method with avian plates. Susceptibility was determined using NCCLS criteria (25).

[b] Fresh cecal droppings were collected from the litter of broilers when they were 3 wks and 6 wks of age. Flock houses contained approx. 22,000 birds. Thirty *E. coli* isolates were collected after the cecal droppings had been plated on MacConkey agar plates. No antibiotics had been used in this flock house for greater than one year.

[c] *E. coli* isolated from young birds (3 wks of age) were significantly more resistant to gentamicin ($p=.0064$) neomycin ($p=.0035$) and oxytetracycline ($p<.0001$).

[d] Isolates from flock 1 at 6wks of age were more resistant to neomycin ($p=0.0035$) and oxytetracycline ($p=0.0175$) than those from flock 2 at 6 weeks of age.

Table 2: Antibiotic Susceptibility (%) of *E. coli* isolated from the cecal droppings of commercial broiler chickens that were administered antibiotics [a].

	FARM A [b]		FARM B[c]			FARM C[d]	
	3 WKS[e]	6 WKS	3 WKS	6 WKS [f]	7 WKS	3 WKS	6 WKS
Amoxicillin	92	96	76	100	90	93	100
Ceftiofur	100	100	100	100	100	100	100
Gentamicin	8	27	0	3	23	16	16
Neomycin	100	97	100	100	100	100	100
Oxytetracycline	27	3 [g]	10	42	23	33	26
Streptomycin	0	0	16	0	0	3	26
Sulfathiazole	0	0	0	0	23	6	16
Enrofloxacin	100	100	100	100	100	100	96
Nalidixic Acid	96	96	16	28	53	33	20
Sarafloxacin [h]	96	97	23	3	53	16	0

[a] MICs were performed using the Sensititre microbroth dilution method with avian and fluoroquinolone plates. Susceptibility was determined using NCCLS criteria (25).

[b] Fresh cecal droppings were collected from the litter of broilers when they were 3 wks and 6 wks of age. Flock houses contained approx. 22,000 birds. Thirty *E. coli* isolates were collected after the cecal droppings had been plated on MacConkey agar plates. No antibiotics had been used in this flock house for greater than one year.

[c] Oxytetracycline was administered in the birds' water when they were 6 1/2 weeks of age. Farm B had a past history of enrofloxacin and sarafloxacin usage.

[d] Sarafloxacin was administered in the birds' water when they were 6 weeks of age. Isolates were collected the day after the administration of Sarafloxacin. The flock house had a past history of sarafloxacin and enrofloxacin usage.

[e] Isolates from Farm A were significantly more resistant to amoxicillin than farm B ($p=0.021$) at 3 weeks of age

[f] Isolates from Farm B had significantly higher MICs to sarafloxacin ($p<0.0001$) and gentamicin ($p=0.0399$) at 6 weeks of age than the other ages.

[g] Isolates from Farm A were significantly more resistant to oxytetracycline ($p=0.0002$) at 6 weeks of age than those from Farm B.

[h] Isolates from Farm A were significantly more susceptible to Sarafloxacin ($p<0.0001$) at 3 weeks of age and 6 weeks of age than on Farm B and Farm C

Table 3: Antibiotic Susceptibility (%) of *E. coli* isolated from the cecal contents of research broiler chickens that were administered antibiotics [a, b]

	Grouped flock	Group 1 No treatment[f]		Group 2[c] Sarafloxacin		Group 3[d] Enrofloxacin		Group 4[e] Tetracycline	
	3wks[g]	5wks	7wks	5wks	7wks	5wks	7wks	5wks	7wks
Amoxicillin	100	100	93	100	100	100	97	93	93
Ceftiofur	100	100	100	100	100	100	100	100	100
Gentamicin	23	90	63	87	100	100	93	97	97
Neomycin	50	93	100	97	97	100	97	77	100
Oxytetracycline	16	53	57	53	60	70	60	0	3
Streptomycin	20	43	20	43	60	87	80	50	60
Sulfathiazole	23	43	27	43	80	50	83	30	97
Enrofloxacin	100	100	100	100	100	60	70	100	100
Nalidixic Acid	73	90	53	60	70	46	50	70	96
Sarafloxacin	93	97	67	83	67	50	53	100	100

[a] MICs were performed using the Sensititre microbroth dilution method with avian plates. Susceptibility was detected using NCCLS criteria (25).

[b] Fresh cecal contents were collected when the birds were 3 wks, 5 wks, and 7wks of age. The research flock contained 120 birds that were separated into 4 treatment groups at 4 weeks of age. Thirty *E. coli* isolates were collected after the cecal contents had been plated on MacConkey agar.

[c] Sarafloxacin (20ppm) was administered in the birds' water for 5 days when they were 4 weeks of age.

[d] Enrofloxacin (25ppm) was administered in the birds' water for 3 days when they were 4 weeks of age. The enrofloxacin group was more likely to have higher MICs to sarafloxacin ($p=0.0156$), enrofloxacin ($P=0.0125$), and nalidixic acid ($p<0.0342$) compared to other treatment groups at 5 wks and 7 wks of age. The isolates from the enrofloxacin group were more susceptible to streptomycin than the other groups at 5 wks of age ($p<0.001$).

[e] Oxytetracycline (25ppm) was administered in the birds' water for 5 days when they were 4 weeks of age. The isolates from the oxytetracycline group were more likely to be resistant to oxytetracycline than the untreated group and the fluoroquinolone treated groups at 5 wks and 7 wks of age ($p<0.001$) and less likely to be resistant to sarafloxacin and enrofloxacin at 7 wks of age compared to other groups ($p<0.0002$)

[f] The untreated group was more likely to be resistant to streptomycin ($p=0.001$) and sulfathiazole ($p<0.003$) at 7wks of age compared to other groups.

[g] *E. coli* isolated from 3 wk old birds were more likely to be resistant than those isolated from 5 wk old birds ($p<0.05$).

Table 4: Distribution of tetracycline resistant determinants from *E. coli* isolated from l broiler chicken flocks. [a]

	Farm	Age of bird	Tetracycline resistance (%)	tet (A)	tet (B)	tet (C)	tet (D)	tet (E)
Commercial Flock	Farm A	3wks	42	16	23	0	0	0
		6wks	90	33	36	0	0	0
	Farm B[b]	3wks	90	60	37	10	0	0
		6wks	55	10	50	0	0	0
		7wks	77	27	10	0	0	0
	Farm C[c]	3wks	65	13	36	10	0	0
		6wks	71	46	40	0	0	0
Research Flock[d]	Grouped	3wks	84	33	50	0	0	0
	Group 1; No treatment	5wks	47	16	60	0	0	0
		7wks	43	36	16	0	0	0
	Group 2; Sarafloxacin [e]	5wks	47	20	31	0	0	0
		7wks	40	28	12	0	0	0
	Group 3; Enrofloxacin [f]	5wks	30	0	13	0	0	0
		7wks	40	26	13	0	0	0
	Group 4; Tetracycline [g]	5wks	100	53	46	0	0	0
		7wks	97	30	66	0	0	0

[a] Tetracycline determinants were screened using DNA:DNA hybridization (26, 29). Each isolate that tested positive contained only one of the *tet* determinants screened.

[b] Oxytetracycline was administered in the birds' water when they were 6 1/2 weeks of age

[c] Sarafloxacin was administered in the birds' water when they were 6 weeks of age

[d] Antibiotics were administered in the research birds' water when they were 4 weeks of age.

Table 5: Distribution of integron carriage (% positive) and class I integron- associated cassettes among *E. coli* isolated from commercial broiler chicken flocks [a]

FARM	FLOCK	AGE	*intI*1	*intI*2	*aadA1,2*[b]	*BLA* [b]	*AG* [b]	*dfr*[b]
FARM A	Flock 1	3wks	96	0	94.1	0	0	17.6
		6wks	100	3	86.7	3.3	0	3.3
	Flock 2	3wks	40	6	90	0	10	0
		6wks	50	10	70	20	0	0
	Flock 3	3wks	51	3	90	3.3	10	6.7
		6wks	20	0	55.2	3.4	0	3.4
FARM B	Flock 1	3wks	97	6	100	0	0	6.7
		6wks	100	0	78.6	0	0	0
		7wks	93	6	76.7	0	0	0
	Flock 2	3wks	60	0	90	0	0	0
		6wks	66	6	83.3	0	0	6.7
FARM C	Flock 1	3wks	93	10	92.9	0	0	14.3
		6wks	83	0	86.7	0	3.3	16.7
	Flock 2	3wks	46	0	48.3	24.1	0	3.4
		6wks	23	13	23.3	0	0	0

[a] Class I and class II integrase genes were detected using DNA:DNA hybridization (11) Class I associated gene cassettes were screened using PCR ELISA as previously described (18).

[b] The probes to detect b-lactamase (BLA) dihydrofolate reductase (dfr) and aminoglycoside resistance (AG) cassettes were pooled to screen for the presence of these genes by PCR-ELISA. The AG pool did not contain the aadA1,2 probe. These gene cassettes were screened among *int*I1

positive isolates. Therefore, the gene cassette data represents the prevalence (% positive) of gene carriage among the *int*I1 positive isolates.

Figures

FIG 1. Strain types of *E. coli* isolated from a treated commercial chicken flock and an untreated flock. Lane L contains a molecular weight marker. Lanes 1 through 10 contain *E. coli* strain types from commercial birds before tetracycline administration. Lanes 11 through 20 include *E. coli* strains from the birds after tetracycline treatment. Lanes 21 through 28 contain *E. coli* strains isolated at 3 weeks of age from an untreated flock and Lanes 29 through 34 contain *E. coli* strain types isolated at 6 weeks of age from the same untreated flock.

L 1 2 3 4 5 6 7 8 9 10 L L 11 12 13 14 15 16 17 18 19 20 L L 21 22 23 24 25 26 27 28 L 29 30 31 32 33 34

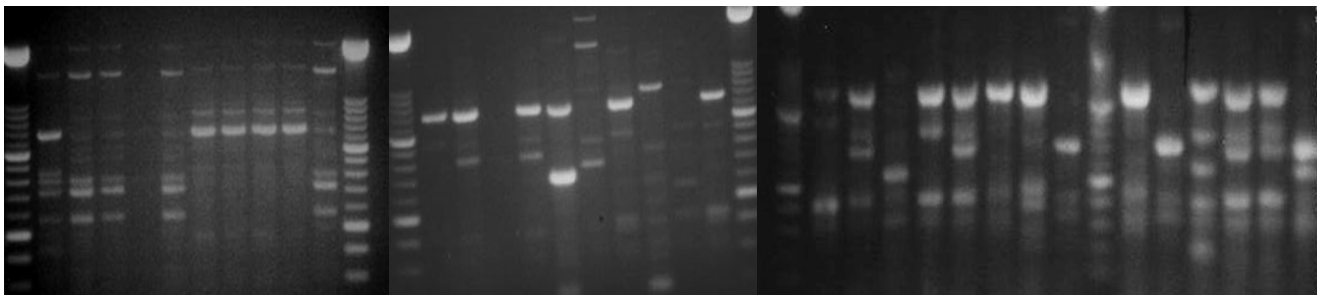
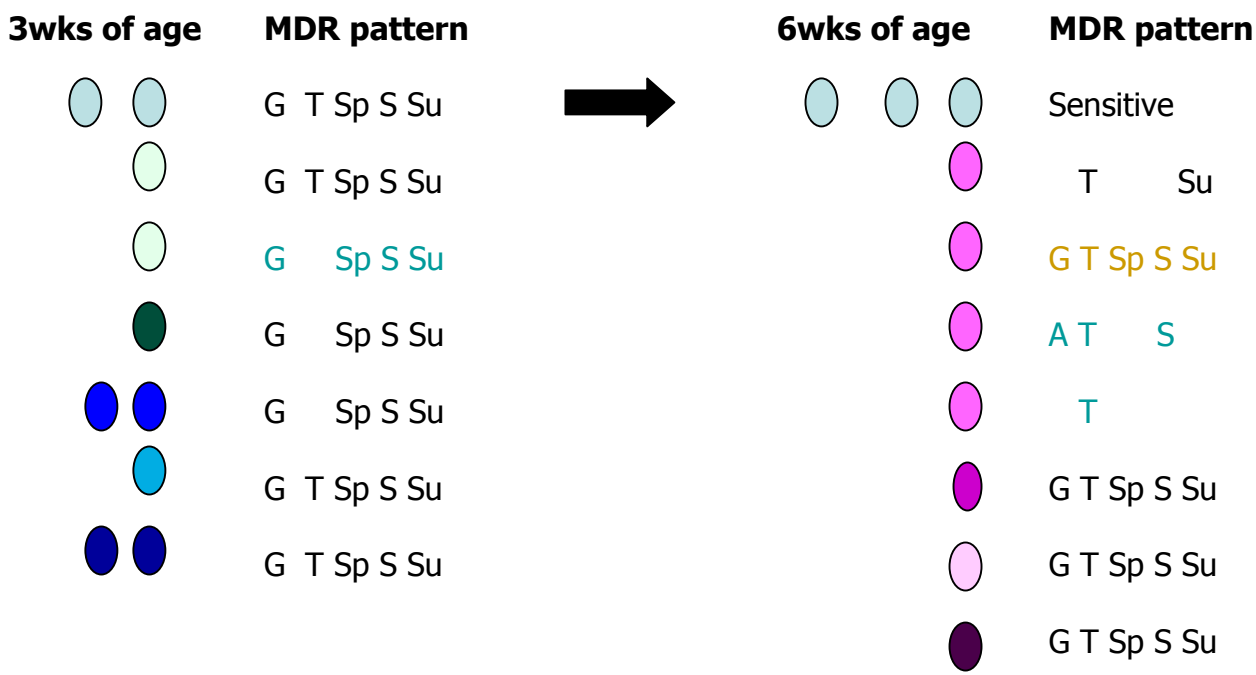


FIG 2. Schematic diagram illustrating the succession of resistant *E. coli* strain types isolated from an untreated commercial flock. The abbreviations for the antibiotics included in the multiple drug resistance (MDR) profiles are as follows: A=amoxicillin, G=gentamicin, T=tetracycline, Sp=spectinomycin, S=streptomycin, and Su=sulfathiazole


3wks of age
MDR pattern
G T Sp S Su
G T Sp S Su
G Sp S Su
G Sp S Su
G Sp S Su
G T Sp S Su
G T Sp S Su
6wks of age
MDR pattern
Sensitive
T Su
G T Sp S Su
A T S
T
G T Sp S Su
G T Sp S Su
G T Sp S Su

Concluding Remarks

In conclusion, the data from this research indicates that there are many factors that contribute to multiple drug resistance among commensal flora in animals. Currently, we are conducting microbial ecology studies to examine the activity of the microflora of the chicken intestine in response to selective pressures. Our data implies that there are many bacterial species that reside in the animal litter and intestines. Therefore, inferences on the ecology of antibiotic resistance may be limited based on the assessments made from one particular bacterial genera. Once we understand the bionetwork of bacteria in these environments, we can make inquiries on how much *E. coli* contributes to the overall ecology of antibiotic resistance with respect to genetic exchange and interaction with other organisms. The information gathered from these types of studies will help us manage the evolution of antibiotic resistance in the future.