

IN VIVO TRANSFER OF ANTIBIOTIC RESISTANCE GENES BETWEEN THE RESIDENT
INTESTINAL MICROFLORA OF BROILER CHICKENS AND *SALMONELLA*
TYPHIMURIUM

by

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(Under the Direction of John Maurer)

ABSTRACT

The emergence of multi-drug resistance in *Salmonella typhimurium* and *Salmonella newport*, has sparked increased debate over the impact of veterinary usage of antibiotics on the development of resistance. For drug resistance to develop in *Salmonella*, there must be a reservoir of mobile, antibiotic resistance genes. Newly hatched commercial broiler chicks were orally inoculated with *S. typhimurium* nalidixic acid-rifampicin resistant isolates and subsequently administered intestinal microflora composed of poultry litter containing antibiotic resistant enteric bacteria collected from a commercial broiler chicken farm, which was propagated in specific-pathogen free (SPF) chickens. For six- weeks, the typical maturation period for commercial poultry, resistance transfer to *Salmonella* was monitored weekly by plating tetrathionate enrichments onto XLT4 selective media supplemented with antibiotics. At the end of the six-week study, resistant *Salmonella* were enumerated by a modified three-tube MPN procedure.

INDEX WORDS: Antibiotic Resistance, *Salmonella*, *In vivo* Transfer, Integrons, Broiler Chickens, Most Probable Number (MPN)

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DEDICATION

To my husband whose undying love, trust, and encouragement continues to inspire me in my journey to find my true purpose in life; this accomplishment is just as much yours' as it is mine.

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To my Lord and Savior, Jesus Christ for helping me stand still and not be weary, and for the numerous blessings in my life and those to come in the future! To my parents Donnie and Linda Lyles for their support and encouragement and for teaching that with hard work and perseverance anything can be accomplished. To my sister and brother whose friendship I have grown to cherish, my friends for helping me relax, vent and have fun.

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Chapter 1

INTRODUCTION

Antibiotics have been in clinical use for more than 60 years. These antimicrobials comprise a variety of substances with different mechanisms for antibacterial activity. Some of the earlier (first generation) antibiotics originated from natural sources secreted as by-products from bacteria and fungi. The discovery and use of antibiotics has been one of the greatest scientific accomplishments in history. Antibiotics have transformed our way of life. Diseases like the plague and cholera are no longer a problem thanks to antibiotics.

The development of antibiotic resistance among zoonotic organisms, like *Salmonella*, has been recognized as a global healthcare risk. It was only a decade ago that the health officials and the public began to take the issue of antibiotic resistance seriously. Prior to the current “resistance era”, antibiotics were oftentimes over prescribed and misused by veterinary and human physicians. Unfortunately, the agricultural industry has been targeted at the culprit. Low- level antibiotic usage in food animals is primarily blamed for the production of antibiotic resistant bacterial strains. Outbreaks of *Salmonella enterica* serotype Typhimurium DT104 in the early 1990s and most recently ceftriaxone resistant *Salmonella* and multiple drug resistant *Salmonella enterica* Newport strains, resistant to third generation cephalosporins (21, 53, 62, 70, 71) are causing the greatest concern to human and animal health, particularly because most of these resistant organisms have been traced back to food animals like cattle and poultry.

The incidence of antibiotic resistance in poultry and livestock pathogens and commensal organisms (15, 74) demonstrates the extent and the diversity of resistance among gram-negative enterics. It also strongly indicates that bacteria are truly remarkable and adaptable organisms. Secondary to antibiotic resistance, genetic elements such as plasmids, transposons, and

integrations, have greatly influenced the dissemination of resistance genes among different bacterial populations (174). These genetic elements, particularly plasmids and bacteriophages have been known to contribute to bacterial evolution by horizontal gene transfer. A large number of commensal organisms within the *Enterobacteriaceae* family have been characterized as natural reservoirs of resistance determinants. Consequently, they may act as potential donors of genetic elements and their associated antibiotic resistance genes to pathogenic organisms (110) like *Salmonella enterica*. Interestingly, most of these isolates have been associated with mobile genetic elements of some sort. Since the discovery of integrations, nearly 14 years ago, it has become evident that integrations represent a very important vehicle for spreading and acquiring multiple antibiotic resistance genes.

Objectives

While the genetic mobility of antibiotic resistance genes in medically significant bacteria, both nosocomial and food- related, is well documented (74) less attention has been given to the collectively understanding the role integrations play in acquiring antibiotic resistance given a large reservoir of various resistance genes when direct selective pressure is applied. The objective of this study was to determine if sensitive *Salmonella enterica* serovar Typhimurium strains could acquire resistance genes from the intestinal microflora of commercial poultry. The primary objective was to perform an *in vivo* conjugal mating experiment while also evaluating the emergence of resistance in *Salmonella*. The second objective was to quantitatively determine the rate at which resistance develops in *Salmonella* in response to therapeutic antibiotic treatment.

Chapter 2

LITERATURE REVIEW

“Antibiotics are arguably the single most important and widely used medical intervention of our era. Almost every medical specialty uses antibiotic therapy at some point. These drugs have prevented incalculable suffering and death and are perhaps still the closest medications we have to a ‘magic bullet’.”
-Steve Heilig 2002

Antibiotic Use in Agriculture

The use of antimicrobials in food animals has been an integral part of the agriculture industry. Just like humans, all food animals at some point during their lives, although short, will be treated with antibiotics. Antimicrobials are used primarily for two reasons. Therapeutically, to treat existing infectious diseases and sub-therapeutically, to enhance production yields and improve feed conversion (135). Antibiotics used therapeutically tend to be applied at the onset of clinical signs and only after diagnosis of a specific disease-causing agent. Such treatment is governed by licensed clinical veterinarians according to federally mandated Food and Drug Administration (FDA) guidelines. Subtherapeutic or prophylactic treatment of animals occurs when infectious organisms are overwhelmingly present within an environment or when animals are stressed (environmentally or immunocompromised) and consequently more susceptible to disease.

Nearly all farm animals receive antibiotics in their feed as growth promotants. Supplementing animal feed with antimicrobials has been a common practice since the late-1940s. Stokstad et al. were the first to demonstrate the positive effects of antibiotic use in poultry. They noticed the enhanced growth and weight gain of broiler chickens (178). Antibiotics are added to feed at varying concentrations, however the dosages tend to be low, at approximately <200 grams/ton of feed (135). Unfortunately, the use of growth-promoting antibiotics in food animal production has been met with much controversy. Fast food restaurants

like Wendy's, McDonald's and Popeyes have refused to purchase chickens treated with antibiotics (8, 10). While proponents of growth promoters attest to their numerous benefits such as an overall improvement in animal health and decrease feed cost, opponents argue that antibiotic residues in the water and feed increase risks associated with eating milk and meat products (135, 192).

Most antibiotic use in poultry is subtherapeutic. Using antibiotics therapeutically depends highly upon the cost of the drug and severity of disease or mortality (91). These antibiotics are broad-spectrum drugs effective against gram-positive and gram-negative bacteria. Currently, the U.S. poultry industry uses bacitracin, virginiamycin, bambarmycin and lincomycin for growth promotion (135). Two drugs, the cephalosporin ceftiofur and the aminoglycoside gentamicin, are used in day of hatch chicks after vaccination against viral pathogens to prevent secondary bacterial abscesses (135). Before the approval of enrofloxacin and sarafloxacin (two synthetic fluoroquinolones derived from nalidixic acid) in the mid-1990s, the tetracyclines were the only drug class allowed in poultry to treat *Escherichia coli* associated infections (15, 135, 202). Other drugs approved for use in chickens and turkeys include neomycin, novobiocin, penicillin, streptomycin, spectinomycin, the sulfonamides, the macrolides, and ionophores such as monensin and salinomycin (135).

There are different antimicrobial regimens within the food animal industry. It is estimated that out of all the antibiotics used in the United States (human and animal medicine) approximately half are used in agriculture (115) and growth promotants account for approximately 15% antibiotic usage (144). According to the Institute of Medicine (IOM), 60-80% of livestock and poultry will receive antibiotics at some point in their lifespan (135). Low levels of antibiotics are also used in the swine industry and include the antibiotics bacitracin,

chlortetracycline, penicillin and virginiamycin, which increase growth and feed conversion. Dairy and beef cattle may be given ampicillin, ceftiofur, streptomycin, sulfonamides, bacitracin or oxytetracycline to enhance growth and production levels or to treat any number of infections (135). Although antibiotics have been used to genetically modify crops, antibiotics used in plant agriculture account for less than 0.5%, with oxytetracycline and streptomycin as the mostly frequently used antibiotics (128).

Over the past few decades the food animal industry has evolved. It is projected, by 2004, that the annual revenue for livestock production (red meat and poultry) in the United States will be to equal approximately \$84,365 million (11). The economic consequences of a ban on antimicrobial usage in food animals would be devastating to the U.S. economy and ultimately international trading (28). The financial burden to U.S. consumers would be even greater, costing nearly \$1.2 billion to \$2.5 billion per year (135). Antimicrobial drug use in agriculture is like a double-edged sword, where maintaining the US agricultural economic vitality and global competitiveness conflicts with the issue of public health. This has spawned a contentious debate where presently no one really knows the true risks associated with agricultural use of antibiotics, the development of antibiotic resistance in opportunist or zoonotic pathogens, and the likely transmission to consumers.

Antibiotics and Associated Risks

The discovery of antimicrobial agents is one of the greatest scientific accomplishments of the 20th century. Antibiotics have had a tremendous effect on not only veterinary and human medicine but allowed the agricultural industry to flourish as well. Unfortunately, these innovative “cure alls” did not come without serious repercussions. Our copious use of antibiotics has made antimicrobial resistance a global human and animal health crisis. Soon after

the discovery and clinical use of the first antibiotic, penicillin, resistance emerged. A similar trend occurred with subsequent antibacterial compounds (43, 160).

Low- level antibiotic use in agriculture is a growing concern. Much debate is focused on the use of antibiotic growth promotants (AGP) in food animals; particularly the glycopeptides: avoparcin, streptogramin, virginiamycin, and the fluoroquinolones: enrofloxacin and sarafloxacin. The issue of avoparcin usage and selection for vancomycin resistant enterococci in the community is a growing concern in Europe. There is substantial evidence indicating the food chain as the primary source of vancomycin resistant enterococci in humans (1, 102, 191). The medical community believes drastic measures are necessary to control development of antibiotic resistance through the judicious use of antibiotics and banning those antibiotics in agriculture that are analogous to drugs in human medicine. As a precaution, Denmark which first banned the use of all therapeutic antibiotics per the recommendations of the Swann Committee Report (183) and the European Union has recently followed a ban on the use of growth promoting antibiotics (2, 135, 160, 191).

Reviews on the effectiveness of prohibiting antibiotic growth promotants use are mixed. On one side, it appears that despite the ban, drug resistance among *Enterococci* remains prevalent. A study by Gambarotto et al. (2001) detected 66% vancomycin- resistant *Enterococci* in swine and poultry meat products following the ban on growth promoting antibiotics (67). On the other side, Aarestrup et al. (2001) reports that in Denmark between 1995 and 2000, resistance to vancomycin and other AGPs among *E. faecium* isolates from broiler chickens decreased significantly and this decrease was associated with a decrease in antibiotic usage (2). Now we face the problem of *Enterococcus* species becoming resistant to the vancomycin's replacement

Synercid® and the new drug class linezolid (3, 75, 171). Might we lose effectiveness of Synercid® due to veterinary use of an analogous streptogramin, virginiamycin?

The emergence of antibiotic resistance among zoonotic organisms is on the rise (2, 126, 165, 171, 206). For example, several studies have illustrated the recent increase in fluoroquinolone resistance among *Campylobacter* and *Salmonella* species. Hakanen et al. (2003) observed a dramatic increase from 40% to 60% in ciprofloxacin resistance among *Campylobacter jejuni* strains isolated between 1995- 1997 and 1998- 2000 (83). A change in susceptibility to quinolones (fluoroquinolones) among *Salmonella enterica* serotypes isolated from food animals and humans has developed as a worldwide trend over the years (65, 84, 120). Likewise, penta-drug resistant *Salmonella enterica* serotype Typhimurium definitive type DT104 (187) has been associated with a large number outbreaks throughout the world (73). In addition to having a unique resistance pattern, *Salmonella* DT104 has acquired resistances to trimethoprim, aminoglycosides and the fluoroquinolone, ciprofloxacin (51, 63, 188). The cause for this boost in resistance is subject to debate as to whether the approval and subsequent use of veterinary fluoroquinolones in poultry is responsible or is foreign travel to countries where antibiotics are easily obtained without a prescription is to blame (44, 167, 169)?

Ceftriaxone resistant *Salmonella* has become the latest threat to the medical community (56, 60) particularly, for treatment of systemic *Salmonella* infections in children and the elderly. The number of ceftriaxone-resistant *Salmonella* isolates, reported by the CDC in 1997, was 0.07% (89) and by 1998, the incidence of resistance rose slightly to 0.5% (56); this was the first episode of ceftriaxone resistance within the United States. According to the 1999 National Antimicrobial Resistance Monitoring System (NARMS) Annual Report, the percentage of *Salmonella* isolated from food animals with resistance to ceftriaxone (MIC \geq 16) increased from

0.1% in 1996 to 2% in 1999 (34). By 2001, the NARMS survey reported 3% of *Salmonella* with decreased susceptibility to ceftriaxone while 2% were resistant to ceftriaxone (35). The increased prevalence of multi-drug resistant (MDR) *Salmonella enterica* Newport, resistant to third generation cephalosporins and as many as nine additional antibiotics from the other antibiotic classes. Some of these isolates displayed a decreased susceptibility to ceftriaxone and cross-resistance to ceftiofur, a cephalosporin currently approved for use in poultry and cattle (148, 206, 208). The question that comes to mind is... are the value of antibiotics like ceftriaxone being undermined as a direct consequence of veterinary use of analogous drugs that select for drug resistance in foodborne pathogens like *Salmonella*? Powerful drugs, such as the fluoroquinolones and cephalosporins, may become limited in its utility to treat gastroenteritis and subsequent systemic illnesses that develop and require medical intervention, if drug resistance is left unabated to develop in *Salmonella* and *Campylobacter*.

Foodborne Disease and *Salmonella*

Preventing foodborne infections has been major public health challenge. The Centers for Disease Control and Prevention (CDC) estimates that in 1997, food-borne infections caused approximately 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths (129). The surge in foodborne associated infections lead to the development of several federally funded surveillance initiatives. By late1997, FoodNet, a national population based surveillance program was established to investigate food related infections in the U.S.

Salmonella enterica is one of the leading causes of foodborne outbreaks in the U.S. *Salmonella* is estimated to cause nearly 1.4 million cases of gastroenteritis and over 600 deaths annually in the United States (129). In 2000, there were 4,330 laboratory-confirmed cases of foodborne illnesses attributed to *Salmonella* and 3,964 of these isolates were typed as either

serovars Typhimurium (830 cases), Enteritidis (585 cases), Newport (412 cases) or Heidelberg (252 cases). Surprisingly, out of eight different states participating in the FoodNet surveillance program, the state of Georgia had the highest rate of *Salmonella* infection reported (1491) (33).

A large number of these *Salmonella*- related infections go unreported for several reasons, primarily because diarrhea can be caused by any number of intestinal irritants or predisposing health conditions. In some cases, people seeking medical care may not always provide stool samples or provide them to late. FoodNet estimates that 97% of *Salmonella* illnesses went unreported during 1996- 1997. The economic losses attributed to food- borne *Salmonella* infections are tremendous. For fatal cases, the estimated average cost could possibly range from \$0.5 million to \$3.8 million per case (64). The emergence of drug- resistance in foodborne pathogens has only complicated the issue concerning food safety.

Approximately 95% of *Salmonella* infections result from the ingestion of foods of animal origin contaminated with this organism (16, 31, 132, 133). Other potential sources of *Salmonella* contamination include fresh fruits and vegetables such as cantaloupes, strawberries, and alfalfa sprouts in addition to cilantro, reptiles and dog treats (9, 29, 41). Animal manures have been implicated as a potential source for *Salmonella* contamination of produce through contaminated water or irrigation systems (136). Human waste has also been implicated as a vector for introducing *Salmonella* into the food chain (32, 157) mainly a result of poor hygiene practices.

Antimicrobial Resistance

Resistance to antibiotics may be intrinsic or acquired. Intrinsic resistance occurs when bacteria are naturally resistant to an antibiotic, without selecting modifications in the target gene or acquisition of extrinsic gene(s) that alters its susceptibility to the drug (139). Cell structure, drug permeability, and structural alterations in the drug's target (ex. point mutations in rRNA)

inherent to that microbial species, are important factors of intrinsic resistance. The lack of a peptidoglycan cell wall also makes *Mycoplasma* species naturally resistant to β -lactam antibiotics. *Pseudomonas aeruginosa* is naturally resistant to the macrolides as a result of multi-drug efflux pumps (87).

Bacteria acquire resistance to antibiotics as a result of chromosomal mutations or exchange of genetic material via conjugation, transduction, or transformation (17). The exchange of resistance genes typically occurs in susceptible bacteria undergoing environmental change: antibiotic selective pressure or population shifts in size and composition. Consequently, organisms may become resistant as a means of adaptation (113, 134, 190). In other words, the evolution of features (be it physiological or morphological) that make an organism better equipped to survive in a particular environment by increasing the fitness of a organism (170). Within each antibiotic class, there are numerous resistance mechanisms. The main mechanisms include decreased drug uptake, modification of antibiotic target or antibiotic, over expression of drug efflux, or any combination of these mechanisms (99, 160).

Antibiotic Resistance within the Enterobacteriaceae Family

The family *Enterobacteriaceae* is a diverse group of gram-negative organisms found throughout nature (20). The wide-spread occurrence of antibiotic resistance among gram-negative enterics, from diverse environments and hosts, suggests that these organisms readily acquire, maintain, and spread antibiotic resistance genes, and thus serve as a likely reservoir for disseminating genes to naïve bacterial populations. Enteric bacteria have been found in aquatic environments (run-off from groundwater, lakes and streams), soil, sewage, plants (rhizospheres) (38, 204). It also comprises many of the organisms found in the intestinal tract of humans and animals (59, 204). Within this group, nearly 30% of the known genera are potential human

pathogens. These pathogenic strains have been associated with nosocomial wound, urinary tract and respiratory infections, and diarrhea (69). Currently, the six major antimicrobial classes used to treat gram-negative infections are the quinolones, the β -lactams and cephalosporins, the aminoglycosides, the tetracyclines, sulfonamide and trimethoprim, and chloramphenicol. Unfortunately bacteria have developed different mechanisms to circumvent these exact antibacterials.

i) Quinolones

The quinolones represent the first generation of synthetic chemotherapeutic agents. The broad-spectrum, bactericidal activity of this class makes the quinolones effective against many gram-negative and gram-positive pathogens important in veterinary and human medicine (13, 198). They are most commonly used to treat infections in companion pets, cattle, swine, horses, and poultry (94). The fluoroquinolones, third generation quinolones, are currently being used worldwide to treat human infections (94). Of the fluoroquinolones, ciprofloxacin is the most prescribed antibiotic in human medicine. It is often the drug of choice for most foodborne illnesses because of its broad antibacterial spectrum (13). However, in the United States, fluoroquinolone use in veterinary medicine has been somewhat restrictive.

The predominant mechanism of resistance to the quinolones is created by mutations in DNA gyrase and topoisomerase IV enzymes, which are essential for the initiation of DNA replication (99). DNA gyrase is a type II topoisomerase composed of two subunits (*gyrA* and *gyrB*) responsible for negative supercoiling, nicking and re-joining bacterial DNA (194). Mutations have also been identified in the *parC* loci, a homologue of subunit A encoding topoisomerase IV (58, 88, 105). Quinolone resistance is largely due to spontaneous, single-step, point mutations in the subunit A of gyrase (*gyrA*) (93) and recently novel *gyrB* mutations (79)

have been discovered. These gene mutations tend to be chromosomal (77, 205) although recent plasmid encoded mutations have been characterized (93, 124, 189, 199). Wang et al showed that 8% of the high-level ciprofloxacin resistance among *E. coli* isolated from hospitals in China were carried on transferable plasmids ranging from 54kb to >180kb. These plasmids carried the In4 class 1 integron backbone with additional *resistance* genes, open reading frame (orf513), and a second 3' conserved region (195).

Alterations in porin channels and efflux pumps are additional mechanisms of quinolone resistance, both of which are chromosomal mutations (92, 93). Bacteria prevent the quinolones from penetrating the cell membrane by elongating O-antigen side chains, to increase the lipid layer (131). However, hydrophilic quinolones similar to ciprofloxacin are able to circumvent the thickened LPS (36). Another resistance mechanism utilizes AcrAB-TolC efflux pump systems to actively transport quinolones out of the cell. The production of excess efflux pumps down regulates the expression of outer membrane proteins and thereby reducing antibiotic permeation into the cell (93). Although these pumps tend to be associated with low-level, broad-spectrum, quinolone and fluoroquinolone resistance, when coupled with chromosomal mutations *gyrA* and *parC*, high levels of quinolone resistance can be observed (58, 93).

In recent years, fluoroquinolone resistant bacteria have been on the rise in both clinical and veterinary isolates of *E. coli* and *Campylobacter* species (169, 201). A similar trend has been observed with fluoroquinolone resistance in *Salmonella* (65, 78, 97, 145). Overwhelming attention has been given to fluoroquinolone resistance in *Salmonella* DT104 isolates because of the imminent danger of treatment failure.

ii) Tetracyclines

The tetracyclines are a collection of broad-spectrum antibiotics, developed in the late 1940s, most effective against actively dividing cells (146). Tetracyclines are weakly lipophilic molecules that are able to diffuse across the lipid bilayers of the cytoplasmic or inner membranes through porin channels (185). Transport across the membrane is regulated by an energy dependent proton motive force (159). These antibiotics work by reversibly binding the 30S bacterial ribosome to inhibit protein synthesis (40). Tetracyclines inhibit protein synthesis by obstructing the binding of aminoacyl-tRNA to the ribosomal A (acceptor) site, which blocks the tRNA-mRNA interface essential for translation (146). They are useful against a wide-range of microorganisms that differ in cellular composition. Gram-positive, gram-negative bacilli and cocci; aerobic and anaerobic organisms; cell-wall deficient organisms like *Mycoplasma*; as well as obligate intracellular parasites and protozoa; they are all inhibited by tetracyclines (40). The first generation in this drug class is a group of naturally occurring tetracyclines: chlortetracycline, oxytetracycline, and demethylchlortetracycline, all of which are derived from various *Streptomyces* species between the years of 1948-1957. The second-generation antibiotics are semi-synthetically produced. They include doxycycline, tetracycline and minocycline, which were discovered around the late 1960s and early 1970s. The third generation in the tetracycline family, the glycylines, is currently undergoing clinical trial testing (40).

Resistance to the tetracyclines is accomplished by one of three processes: active efflux, ribosomal protection, and enzymatic inactivation (39). It was once thought that decreased intracellular accumulation into susceptible organisms served as the basis for resistance to the tetracyclines. Later, we learned that active efflux systems were responsible for such resistance (109). The key tetracycline resistance mechanism entails an inducible active efflux system that

works by impairing or reducing intracellular uptake (109, 112, 185). Determinants encoding for tetracycline efflux system proteins are *tetA- tetE, tetF, tetG- tetH, tetI tetJ- tetK, tetP, tetV, tetY, tetZ, otrB, tcr3, tet30- 31* (40, 112, 152). These efflux genes are distributed widely among gram-negative and gram-positive bacteria (40, 152).

As the name suggests, ribosomal protection proteins shield the 30S ribosomal subunit from the bacteriostatic activity of the tetracyclines by changing the ribosome conformation while allowing protein synthesis to occur (40). At present, eight ribosome protection proteins have been identified: TetM, TetO, TetP, TetQ, TetS, TetT, TetW and OtrA (40, 112). Of the ribosomal protection determinants, TetM and TetO are most notable. TetM determinant was first characterized in *Streptococcus* species (26, 121) and commonly found associated with gram-positive conjugative transposons like Tn916 and Tn1545, which have also been found among gram-negatives like *Bacteroides* species (121, 151).

Enzymatic modification of tetracycline is rare among resistance mechanisms associated with this drug. The *tetX* gene is the only tetracycline determinant encoding for an enzyme that chemically inactivates tetracycline in the presence of NADPH and oxygen (152). It is found located on transposons carried by strict anaerobes such as *Bacteroides* species, although the gene appears to only work in aerobic bacteria (152, 175).

iii) Aminoglycosides

Aminoglycosides are bactericidal antibiotics first discovered nearly 60 years ago for the treatment of infections caused by both gram-negative and positive organisms. Aminoglycosides are effective against numerous pathogens, for example, *Staphylococcus* spp., *Enterococci* spp., *Pseudomonas* spp., *E. coli* and others within the *Enterobacteriaceae* family. Aminoglycosides such as gentamicin, neomycin, and amakacin are considered broad- spectrum antibiotics,

whereas kanamycin and streptomycin have a narrower antimicrobial spectrum. Semi-synthetic aminoglycosides, netilmicin and sisomicin, were developed to overcome problems associated with toxicity and bacterial resistance. Structurally, all aminoglycosides are polycationic amino sugars connected by glycosides linked to a central hexose (146).

Aminoglycosides are effective as bactericidal agents because they passively diffuse into the bacterial cell through the outer membrane and travel into the inner membrane via an energy-dependent pathway (25). Once inside the cell, aminoglycosides bind irreversibly to the 30S ribosomal subunit. However, this binding does not stop the formation of the initiation complex (small ribosome subunit- mRNA- fMet/tRNA). Consequently, the 50S subunit is unable to join the 30S in order to form a functional ribosome. Thus, preventing the movement of tRNA into the A site and truncates the elongating peptidyl chain causing a translational misreading and premature termination (146).

The most common and significant mechanism of aminoglycoside resistance is antibiotic inactivation. Aminoglycoside-modifying enzymes function by adding *N*-acetyltransferase (AAC), *O*-adenyltransferase (also referred to as *O*-nucleotidyltransferase (ANT)), and *O*-phosphoryltransferase (APH) onto antibiotic residues, which affect the ribosome binding ability of the antibiotic (104, 163). Each modifying enzyme can vary in substrate specificity and confer resistance to multiple aminoglycosides. There are more than 50 aminoglycoside-modifying enzymes to date. Resistance by aminoglycoside-inactivating enzymes can be encoded on either non-conjugative, conjugative plasmids or any other mobile element. Such resistance can be chromosomally encoded as evidence of point mutations leading to increased resistance levels (53). High-level streptomycin and spectinomycin resistance through rapid single step mutations in the chromosomally located *strA* (or *rpsL*) gene are created in this manner (146).

Aminoglycoside resistance can also develop by two additional mechanisms, 1.) reduced uptake/ decreased accumulation and 2.) target (ribosome) alteration. Altering transport of antibiotics across the inner membrane, active efflux, and changes in outer membrane permeability all contribute to a decrease in antibiotic uptake (4, 119). Ribosomal protein modifications or 16S rRNA methylation coded by *armA* (aminoglycoside resistance methylase) gene (66) are other ways in which bacteria may inherit aminoglycoside resistance.

iv) β - lactams and cephalosporins

β - lactam and cephalosporin antibiotics are the most widely used class of antimicrobial agents. The class is comprised of a diverse number of semisynthetic compounds (penicillin, cephalosporins, monobactam, and carbapenems), which act as cell wall inhibitors. All of these agents target peptidoglycan transpeptidases, cell-wall synthesizing enzymes present in the cytoplasmic membrane (70). By inhibiting peptide cross-linking of the peptidoglycan, β -lactams and cephalosporins weaken the cell wall, which eventually results in lysis of the bacterial cell. Although these antibiotics are generally bactericidal, growth of some bacterial species may only be inhibited (146). Different β -lactams and cephalosporins vary in their range of antibacterial activity. Some are effective against both gram-negative and gram-positive, whereas others are only effective for gram-negatives or gram-positives alone. For example third generation cephalosporins, cefotaxime and ceftriaxone are effective in treating nosocomial infections caused by multi-drug resistant gram-negative bacteria (82).

Bacteria have developed four mechanisms of β -lactam/cephalosporin resistance: 1.) enzymatic inactivation; β -lactamases, enzymes that cleave the β -lactam ring causing the antibiotic to become inactive; 2.) alteration of the target; mutations in penicillin-binding proteins (PBPs) modify the binding affinity so that these proteins are unable adhere to the antibiotic; 3.)

reduced drug uptake due to changes in cell wall/ membrane permeability or active efflux; and 4.) resistance to lysis by antibiotic tolerance. Tolerance is where the antibiotic impedes bacterial growth without killing the cell. Once β -lactam antibiotic levels decrease, bacteria are capable of resuming growth (146).

β -lactam and cephalosporin hydrolyzing, enzymes are ubiquitous in nature. Beta-lactamases account for the majority of resistance to the β -lactam antibiotics (143). These enzymes have been divided into four classes (A-D) based on their respective nucleotide and amino acid sequence homology; pI and molecular weight; and susceptibility to β -lactam inhibitors. Class A consists of plasmid-encoded TEM β -lactamases, including the most frequent β -lactamase gene *bla*_{TEM-1}, and SHV β -lactamases. Likewise, *SHV bla* genes identified in *Klebsiella pneumoniae* maps to chromosomal DNA. The extended-spectrum β -lactamases (ESBLs) are also considered class A, β -lactamases, where single and multiple amino acid substitutions alters the enzymes spectrum of activity. Class B enzymes are the chromosomal carbapenem hydrolyzing IMP-1 β -lactamases, (116). Class C enzymes represent both chromosomal and/or plasmid- mediated AmpC and CMY β -lactamases. These enzymes unlike class A, β -lactamases, are resistant to β -lactam inhibitors like clavulonic acid. Plasmid-mediated CMY-2 β -lactamases are responsible for the increasing emergence of ceftriaxone-resistant *Salmonella* isolated (56). These AmpC β -lactamases have also been characterized in farm and companion animals (158, 203). The last class, class D, includes Oxa- type enzymes which are plasmid-encoded as well (116).

v) **Trimethoprim and Sulfonamides**

Trimethoprim and the sulfonamides are synthetic antibacterial compounds that reduce the production of tetrahydrofolic acid, otherwise know as folic acid, an essential cofactor for nucleic

and protein synthesis. The sulfonamides generally have a broad-spectrum of antibacterial activity against gram-negative, gram-positive and protozoal organisms. Over the years, the sulfonamides have been used to treat infections in humans and animals. Sulfisoxazole, sulfamethizole and sulfamethoxazole are commonly coupled with trimethoprim in treatment regimens due to the synergistic effect of these two antibiotic classes (146). Sulfonamides are structural analogs to para-aminobenzoic acid (PABA) substrate. Resistance to these antibiotics develops when sulfonamides compete with PABA in a series of reactions catalyzed by the bacterial enzyme dihydropteroate synthetase (DHPS) (96, 166). Trimethoprim is likewise a synthetic antimicrobial agent that acts as a competitive inhibitor of dihydrofolate reductase (DHFR) (96).

There are several mechanisms of resistance to sulfonamides and trimethoprim. The most prevalent mechanisms are: 1.) mutational loss involving thymine synthesis, 2.) regulatory point mutations that generate an overproduction of the target enzymes, 3.) mutations and/or recombination changes in chromosomal *dfr* genes, and 4.) horizontal acquisition of resistance by drug-resistant DHFR variants (95).

Sulfa resistance is primarily plasmid mediated, however chromosomal resistance has been characterized in the *folP* gene encoding for DHPS among pathogens like *Escherichia coli*, *Streptococcus pyogenes*, *Haemophilis influenza*, *Campylobacter jejuni*, and *Neisseria meningitidis* (57, 72, 96, 184). Of the plasmid mediated resistance to sulfonamides there are two types commonly associated with Gram-negative enterics, *sul1* and *sul2*. Type I sulfonamide resistance gene, *sul1*, generally appears on conjugative plasmids, Tn21 related transposons and is located in the 3' conserved region of nearly all class 1 integrons (62, 147, 166, 177, 180). Type II sulfonamide resistance gene, *sul2* is primarily found on non-conjugative plasmids in junction with the streptomycin resistance genes *strA* and *strB* (57). High-level resistance is attributed to

the acquisition of *sul2* in *Haemophilus influenzae* isolated from Kenya and the United Kingdom (57).

Approximately 20 different trimethoprim resistance genes, *dfr*, have been characterized, versus only two sulfonamide resistance genes. Several types of plasmid encoded trimethoprim resistance genes have been characterized among gram-negative enterics (69). The most prevalent of these genes, *dfrI* and *dfrII* variants confer high-level resistance to trimethoprim and these antibiotic resistance genes are frequently located on transposons and integrons (95, 96) as antibiotic resistance gene cassettes. Recently, novel trimethoprim resistance gene cassettes, *dfrVIII* and *dfrXV*, have been found inserted into the class 1 integron of commensal *E. coli* (6, 7). These *dfr* gene cassettes are commonly observed among multi-drug resistant clinical isolates (5, 71, 140, 158).

vi) Chloramphenicol

Chloramphenicol belongs to a group of antibiotics commonly referred to as the phenicols. The phenicols consist of both chloramphenicol and florfenicol. Chloramphenicol is one of the first broad-spectrum antibiotics used to treat gram-positive and gram-negative bacterial infections, as well as parasitic related infections. Depending upon the nature of the organism, chloramphenicol can be either bacteriostatic or bactericidal. Chloramphenicol is an inhibitor of protein synthesis. The antimicrobial agent interferes with transpeptidation by binding to the 50S ribosomal subunit (146). Like with most antibiotics, bacteria have devised several mechanisms of resistance to the phenicols. These mechanisms include, but may not be limited to, 1.) reduced ribosomal binding, 2.) reduced membrane permeability, 3.) modifying enzymes, and 4.) efflux systems (127). The major resistance of mechanism to chloramphenicol is enzymatic inactivation by the plasmid/ transposon-mediated chloramphenicol acetyltransferase (CAT) enzyme. Type I

CAT (*catA* gene) is most often encoded on the transposon *Tn9* while type II CAT (*catB* gene) has been chromosomally located or as part transposable elements (52, 62). The *cmlA1* gene conferring nonenzymatic chloramphenicol resistance through efflux pumps, has been identified as the first integron gene cassette with its own promoter inducible by low levels of chloramphenicol (62, 68).

Chloramphenicol resistance determinants are widespread in nature. The emergence of chloramphenicol, florfenicol resistant *E. coli* isolated from poultry and bovine (42, 100, 200) not to mention, chloramphenicol resistant *Salmonella enterica* serovar *Newport* (130, 176) and *Salmonella enterica* serovar *Typhimurium* DT104 (22) suggests that a large number of gram-negatives carrying resistance determinants have amplified this drug resistance and passed it on to important human pathogens like *Salmonella*. As a precaution, chloramphenicol usage in food producing animals was banned in the United States due to potential human risks associated with antibiotic residues and the development of aplastic anemia (118, 161).

Acquisition of Resistance Genes by Horizontal Gene Transfer

Bacteria can exchange and/or acquire resistance genes by one of three mechanisms: transduction, transformation or conjugation (17). Transfer of foreign genes by transduction involves DNA exchange from one bacterial cell to another by bacteriophages. Bacteriophages are bacterial viruses that rely solely on the infected cell for replication and assembly. Briefly, phage viral DNA is injected into the bacterial cell. At which time, the phage DNA, for lyogenic phages, may incorporate itself into the bacterial host genome where it may undergo either a lysogenic or lytic cycle. In the lysogenic cycle, the phage DNA lies dormant as prophage replicating along with the host genome and remains so until environmental factors such as UV irradiation or chemical triggers cell lysis (19). During the lytic cycle, resistance plasmids or

chromosomally-borne drug resistance genes are incidentally packaged into a new virus particle, which subsequently infects a new bacterial host and passes on genetic information (19). DNA exchange by transduction, unlike transformation and conjugation, is more likely to occur because the DNA is protected from degradation within the phage particle (179). However, on a shorter evolutionary scale, there several factors that may limit frequency and breadth at which generalized transduction occurs in nature. Although phages have been detected in over 60 different species of bacteria throughout many environments (103), phages are highly host specific, recognizing specific cell surface receptors that are limited in their distribution to a species or sub-population. The bacterium's restriction/modification system may also play an important defensive mechanism against phages and genetic information transmitted by the virion.

Transformation involves the transfer of “naked” DNA (be it plasmid or chromosomal) into the genome of competent bacterial cells. Under *in vitro* conditions, transformation is the most efficient way of introducing plasmids into bacteria for gene cloning purposes. However, *in vivo*, transformation has a limited role in gene transfer (17, 54). Only a few bacteria are naturally transformable. *Campylobacter coli*, *C. jejuni*, and *Streptococcus mutans* are naturally competent and transformable with either homologous DNA or foreign DNA (141, 196, 197).

Another mechanism of gene transfer is conjugation. Conjugation differs from transduction and transformation in that it requires physical cell-to-cell contact between donor (F^+) and recipient (F^-) cells. Horizontal transfer via conjugation is usually carried out by self transmissible, conjugative plasmids and/or transposons (17). For conjugal transfer to occur, the genetic element, whether plasmid or transposon must have a complex of *tra* genes (regulates pilin synthesis), an *oriT* (origin of transfer) and *mob* (mobilization) genes (156). Transfer begins

at the *oriT* when the sex pilus forms a conjugative bridge from the donor bacteria containing the F plasmid (fertility) then linearized single-stranded DNA is transferred to the recipient bacterial cell. Replication of plasmid DNA, either bidirectionally or unidirectionally is initiated by one of three mechanisms: theta (θ), strand displacement, or rolling circle replication (55, 186). Transfer of chromosomal genes by conjugation occurs when the F plasmid integrates by homologous recombination into the host chromosome as an episome. These rare cells are known as Hfr (high-frequency recombination) cells. The process of incorporating plasmid into chromosome is reversible, yet when the Hfr cell mates with a F⁻ (recipient) this cell now becomes a F' (F prime) bacterial cell (27, 137).

Mobile Genetic Elements

The development and spread of antibiotic resistance genes often involves genetic elements that can effectively capture and disseminate these resistance determinants throughout a variety of gram-negative and gram-positive species (156). Plasmids, transposons, and integrons serve as vehicles in transporting antibiotic resistance genes throughout the environment. Plasmids and transposons are responsible for gene transfer in approximately 80- 90% of drug-resistant bacteria (18). Recent studies show that integron carriage also plays a tremendous role in the development of multi-drug resistance among gram negative enterics (106, 123).

Plasmids are double-stranded linear or circular, extra-chromosomal elements capable of replicating independently of the host chromosome (27, 160, 186). They are a diverse group of elements found in gram-negative and gram-positive organisms of veterinary and medical importance. Their sizes typically range from two kilobases (kb) to >100kb. A single bacterial cell is not limited in the number of plasmids it may contain as long as they are unrelated plasmids. When closely related plasmids coexist in a bacterial cell, they segregate during cell

division, which leads to the loss of one of the plasmids. To date, over thirty different incompatibility groups have been used to classify plasmid in gram-negative bacteria based on narrow-host-range plasmids such as IncB/O, -FIA, -FII, -FIB, -H11, H12, and I1 and broad-host-range plasmids such as IncQ, -P, and -N carrying a variety of antibiotic resistance markers (21).

In addition to an origin of replication, plasmids also contain accessory DNA regions encoding for virulence factors, multiple antibiotic resistance genes, and resistance to heavy metal compounds to mention a few. Some large plasmids encode genes important for their transfer (*tra* and *oriT* genes) and mobilization (*mob* genes) (160). Plasmids encoding their own replication and transfer/mobilization machinery are referred to as self-transmissible or conjugative. The IncF plasmid of *Escherichia coli* is the prototype conjugative plasmid. F plasmids are narrow-host-range low-copy number plasmids with 1-2 copies per cell. Most of these plasmids are greater than 100kb and tend to be associated with both virulence and genetic elements carrying multi-drug resistances as seen with the *spv* (*Salmonella* plasmid virulence) plasmid of *Salmonella enterica* serotype Typhimurium (80) proving that plasmids are tools for disseminating antibiotic resistance genes on transposons and integrons (160). Plasmids that have their own replication and mobilization genes but lack the ability to transfer themselves are non-conjugative, mobilizable plasmids. IncQ plasmids require the *tra* functions of self-transmissible (helper) plasmids such as the IncP, RP4, to transfer from one cell to another by conjugation. These small yet promiscuous plasmids have been detected in several gram-negatives including *Salmonella typhimurium*. The most common Q plasmid is RSF1010 carrying resistances to streptomycin and sulfonamides with 10- 12 copies per cell (21, 149).

Transposition is mechanism of gene rearrangement by which segments of DNA are transferred to new locations within the genome. Unlike plasmids, transposons do not direct their

replication, instead they must incorporate genes into mobile carriers such as conjugative plasmids for survival (27, 160). The smallest and simplest types of transposon are insertion sequences (IS). IS elements contain transposase flanked by two DNA sequences with transposase binding sites. Composite and conjugative transposons are transposable elements that can capture and transport resistance genes. Composite transposon elements contain two insertion sequences flanked by antibiotic resistance genes. The most commonly studied composite transposons are: Tn5 containing genes for kanamycin and streptomycin resistance (GenBank Accession No. U00004), Tn9 for chloramphenicol resistance gene (GenBank Accession No. V00622) and Tn10, responsible for the wide spread dissemination of tetracycline resistance (GeneBank Accession No. V00611) (27, 160).

Like conjugative plasmids, conjugative transposons can mobilize and replicate any necessary genes for transfer. Likewise, conjugative transposons are similar to lysogenic bacteriophage in their excision and integration into bacterial host genome (156). These complex yet simple entities show no preference for targets, they simply “hop” to new locations in the bacterial genome scattering resistance determinants. Transposon 916, conjugative transposon first discovered in *Enterococcus faecalis* is 18.5kb in size carrying a tetracycline resistance (Tc^R) gene *tetM*. A unique group of transposons unrelated to Tn916, have been found in gram-negative anaerobes termed *Bacteroides* conjugative transposons (156).

Integrations are small DNA elements that act as natural expression vectors for disseminating resistance genes among gram- negatives, particularly *Enterobacteriaceae* species (125). The current definition of an integron evolved through the work of Stokes and Hall in 1989. Integrons are mobile genetic units that contain a site- specific recombination system responsible for the recognition, capture, and insertion of antibiotic resistance genes (177). There

are at least nine known classes of integrons to date, each based on the type of integrase gene they possess (62). Despite the differences between each integron class, the overall structures are similar. Class 1 integrons represent the most intensely studied of the eight classes (62). A large number have been associated with gram- negative organisms, including *E. coli*, *Klebsiella*, *Citrobacter* and *Salmonella* (24, 30, 50, 74, 125, 142, 177).

The class 1 integron found associated with Tn21 contains the *aadA1* gene and has been designated *In2*. Many class 1 integrons are found on Tn21- like transposons, which encode mercury and tetracycline resistances (15, 114, 125). The general structure of an class 1 integron (Figure 1.) consists an integrase gene (*intI1*), a nearby recombination site (*aatI*) and a strong promoter, P_{ant} (62, 85, 153, 177). Two conserved segments located at the 5' and 3' ends flank a central variable region that may contain multiple gene cassettes encoding resistance to specific antimicrobial drugs. The 3' region is made up of a *qacΔE*, *sulI*, and an *attC* or 59 base element (BE), followed by an open reading frame (ORF5) of unknown function (47, 62, 177). The *qacΔE* and *sulI* determine resistance to quaternary ammonium compounds and sulfonamide, respectively. Located downstream of each gene cassette inserted into the variable region an integron is a region of short inverted repeats called the 59- base element (177).

Class 2 integrons are found in the Tn7 family of transposons and contain a dihydrofolate reductase gene cassette (61, 128, 182). Tn7 contains three integrated gene cassettes, *dhfrI-sat-aadA1* (181). Unlike class 1, class 2 integrons have an inactive integrase gene (*IntI2*) and lack a *sulI* gene (30, 86, 150). The first class 3 integron was reported by Arakawa et al, in *Serratia marcescens* contained the *bla_{IMP}* gene cassette conferring resistance to extended spectrum β-lactams and partial *aacA4* gene sequence. Another class 3 integron has been identified in *Klebsiella pneumoniae* with a extended-spectrum β-lactamase *bla_{GES-1}* gene cassette (49). The

integrase gene, *intI3*, has 60% amino acid homology to class 1 *intI1* gene (12). The fourth class of integrons, called “super integrons”, has the *intI4* integrase gene of *Vibrio cholerae* within its chromosome and hundreds of gene cassettes. These super structures have been identified in nine diverse genera throughout γ -proteobacteria (138, 153, 154). Class 5 integrons is associated with *Vibrio mimicus* and has 74% homology to *intI4* (138). Integron classes six, seven, and eight were discovered by Nield et al, from environmental samples. These classes vary not only in their integrase sequences, but also have a promoter located at different locations outside of the integrase gene as observed in class 1 integrons (138).

Different classes of integrons have been reported to contain numerous gene cassettes. Gene cassettes are mobile genetic elements that, unlike other known mobile elements, do not include all the functions required for their mobility (86). Within an integron there are two promoters (P_{ant} and P_2) which influence the level of resistance expressed by gene cassettes (46). In order for the gene(s) to be expressed, a cassette must be inserted in the correct orientation relative to these promoters. The feature responsible for orientation and integration resides in the 59 BE (base element) (45, 46). Cassettes can exist in a free circularized form or integrated at the *attI* site, however only when it is integrated, at the *attC* site, is it formally part of an integron (86).

More than sixty distinct gene cassettes have been identified among integrons, most of which have been described for class 1 integrons (62). The gene cassettes identified determine resistance to a range of antibiotics including, the aminoglycosides, trimethoprim, chloramphenicol, the penicillins and the cephalosporins (86) all confer resistance by several mechanisms, for example, antibiotic modification (aminoglycosides, chloramphenicol, β -lactams and extended- spectrum β -lactams), metabolic by-pass (trimethoprim) antibiotic efflux

(chloramphenicol), and cell wall synthesis (the penicillins) (62, 86, 160). The most common integron gene cassettes are those that encode for aminoglycosides and trimethoprim resistance. At least 14 different cassettes have been described for the aminoglycosides and at least 12 different gene cassettes encoding for trimethoprim resistance (125). Levesque et al, found that approximately 75% of aminoglycoside resistant clinical isolates carried an integron (107). Next to aminoglycoside resistance, the *sulI* gene (conferring resistance to the sulphonamides) is the most common resistance associated with integrons, partly because it is a structural component of the class 1 integron located adjacent to the variable region (86). Surprisingly, not all integrons have gene cassettes. Integrons missing gene cassettes have been characterized among environmental isolates. Isolates with this cassette free configuration have 5' and 3' conserved regions that are contiguous (48, 86).

Insertion of antibiotic resistance genes by site-specific recombination plays an important role in the acquisition and dissemination of resistance genes (45, 86). The integrase gene, a member of the λ DNA integrase family codes for site-specific recombinase and insertion of gene cassettes (30, 45, 47). DNA integrase is also responsible for the deletion and rearrangement of cassettes (48). It has been experimentally demonstrated that DNA integrase drives site-specific recombination by the formation of co-integrates (122). The recombination cross-over has been shown to occur on either side of a conserved GTT triplet region which is part of a seven-base core site located at the end of the inserted region and flanking all inserted cassettes (48). A core site is found at either the 5' conserve segment with the first gene cassette or may constitute the last seven bases of the 59BE (177). The integrase also catalyzes excision and recombination that can lead to loss of cassettes from an integron and generate free circular cassettes (86).

Antibiotic Resistance: Consequences of DNA Transfer in Nature

The spread of resistance genes involves the integration of mobile genetic elements such as plasmids, transposons, and integrons into the chromosome of other bacteria. Numerous studies have reported the incidence of plasmids, specifically R plasmids (resistance plasmids) and how easily they can move from one bacterial population to another (37, 81, 168, 193). Oftentimes, these plasmids confer resistance to multiple antibiotics and in some cases, virulence factors (14, 23, 50, 76, 173). Significant research has been conducted to identify factors that may be conducive to gene transfer such as the environment, antibiotic selection, and microbial population shifts (98, 108, 172). For years, it has been known that selective pressure plays a role in the dissemination of antibiotic resistance. Unfortunately, this evidence is conflicting.

When bacteria are placed under environmental stresses by either antibiotics and/ or disinfectants, they ultimately have two choices: adaptation or death. Somehow, the mere fact that bacteria have existed for billions of years is a signal that they are extremely adaptable. Consequently, when exposed to toxic chemicals (i.e- antibiotics) their innate response is to become resistant (162). Bacteria may become hypermutable or swap resistance genes with neighboring bacterial cells to acquire a competitive advantage (117, 207). The ability to change one's genome is a highly dependent upon the organism's fitness (155).

The dissemination of antibiotic resistance genes among animal and human pathogens between indigenous microbiota is the prototype of horizontal gene transfer. Gene transfer systems, such as conjugative plasmids and transposons, act as vectors to allow for rapid exchange and acquisition of resistance genes between commensal populations commonly found in the respiratory, intestinal, and urinary tracts of humans and animals (19, 160).

Since many of the drugs used to treat animal infections are the same antimicrobials or a derivative of an antibiotic previously used in human medicine, there is much concern over the source of resistance. There are numerous studies, which document the movement of resistant microbes from human to animal, and vice versa. In 1969, Smith et al showed that antibiotic resistance transferred to resident *E. coli* resistant to nalidixic acid in the alimentary tract of a man to *E. coli* of both animal and human origin (171). Likewise, in 1976, Levy observed transfer of tetracycline resistance between chicken *E. coli* strains from chicken to chicken and from chicken to humans (111).

Hinnebusch et al. (2002) demonstrated, within the midgut of a flea, that *Yersinia pestis* could acquire a conjugative R plasmid from *Escherichia coli* (90). Similarly, a study by Khan et al. (2000) showed that resistance in *Staphylococcus aureus* strains taken from different ecosystems (poultry and human) could transferred among each other (101). Shoemaker et al. (2001) demonstrated that horizontal transfer mediated by the conjugative transposon of *Bacteroides* was responsible for the spread resistance to other *Bacteroides* species and other human colonic organisms (164).

“We cannot be sure that the searchers for new drugs and antibiotics will always win the race. However hard and successfully we may work in the search for new drugs we shall therefore continue to labour under discouragement so long as we are faced with the bugbear of drug resistance. The problem is one of microbial biochemistry, physiology and genetics, and can only be solved by work in these fields. Until we understand the problem we shall have no hope of overcoming it, and until we overcome it we shall have no real sense of security in our chemotherapy. The subject is not only of the greatest scientific interest and importance; it has also a background of practical medical urgency.”

-Sir Charles Harrington 1957

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Chapter 3

***IN VIVO* TRANSFER OF ANTIBIOTIC RESISTANCE GENES BETWEEN RESIDENT
INTESTINAL MICROFLORA OF BROILER CHICKENS AND *SALMONELLA*
*TYPHIMURIUM*¹**

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ABSTRACT

With the emergence of multi-drug resistance in *Salmonella typhimurium* and *Salmonella newport*, there has been increased debate over the impact of veterinary usage of antibiotics on the development of resistance. For drug resistance to develop in *Salmonella*, there must be a reservoir of mobile, antibiotic resistance genes. Newly hatched specific-pathogen free (SPF) broiler chicks were orally inoculated with *S. typhimurium* nalidixic acid-rifampicin resistant isolates (10^8 cfu/ml). Chicks were subsequently administered litter microflora containing antibiotic resistant enteric bacteria from a commercial broiler chicken farm. At 1 week of age, the birds were administered chlortetracycline (25mg/body weight/lb) or streptomycin (15mg/body weight/lb) *per os*. Over 6 weeks, we observed resistance transfer to *Salmonella* by plating weekly tetrathionate enrichments of drag swabs onto XLT4 selective media containing antibiotics: ampicillin, chloramphenicol, gentamicin, kanamycin, streptomycin, or tetracycline. We also monitored the level of resistance to these same antibiotics among gram-negative enterics present in poultry litter by plate counts on MacConkey agar with and without antibiotics. At the end of the six-week period, ceca were harvested and drug resistant *Salmonella* were enumerated using a modified Most Probable Number (MPN) procedure. Drug-resistant *Salmonella* were screened by PCR for several, common antibiotic resistance genes. The highest prevalence of resistance transferred was to ampicillin, followed by tetracycline, gentamicin, and kanamycin. There was no transfer of chloramphenicol resistance detected. The integron associated and non-integron associated recipient *Salmonella* isolates acquired several antibiotic resistance genes during the course of the experiment regardless of antibiotic treatment. Transfer of antibiotic resistance genes from the gut flora of chickens to *Salmonella* readily occurred.

INTRODUCTION

The transfer of antimicrobial resistance among *Enterobacteriaceae* and their increasing role in nosocomial and zoonotic infections is a worldwide concern. Foodborne pathogens cause nearly 76 million illnesses each year in the United States. Of these pathogens, *Salmonella* is one of the leading causes of foodborne gastroenteritis with 1.4 million cases reported annually (46). Between 1973 and 1987, contaminated poultry products were linked to 19% of *Salmonella* foodborne outbreaks in the United States (6). Antibiotic resistance among these pathogens has caused unease in both the human and animal health communities. The alarm transpired in the early 1990s, when a multi-drug resistant strain of *Salmonella enterica* serotype Typhimurium phage type DT104, R-ACSSuT, became a threat to humans and farm animals throughout the U.K. and the United States (21, 63). Since that time, clonal descendants of *Salmonella* DT104 have acquired resistance to trimethoprim and other antibiotics within the aminoglycosides and fluoroquinolone families (13, 18, 64). Multi-drug resistant (MDR) *Salmonella enterica* serotype Newport, the third most common *Salmonella* serotype, (54, 71, 72) is now at the forefront of this issue for several reasons. *S.* Newport is resistant to third generation cephalosporins and as many as nine additional antibiotics from the other antibiotic classes. These isolates have decreased susceptibility to ceftriaxone and cross-resistance to ceftiofur, a cephalosporin currently approved for use in poultry and cattle (54, 71, 72). Multiply resistant organisms and a potential for antibiotic treatment failure in humans are some of the driving forces behind the campaign to restrict antibiotic usage in agriculture.

Resistance to all antibiotic classes has been documented among the gram-negative enterics of human and animal origin. In most cases, these antibiotic resistances are encoded by genes contained on discrete mobile elements known as gene cassettes, located in integrons (28).

Class 1 integrons encode a site-specific recombinase (IntI1) that belongs to a distinct family of the tyrosine recombinase superfamily (11), responsible for the insertion of gene cassettes at *attC*, and also provides the promoter responsible for expression of the cassette-encoded genes (8). The 3' region contains the genes *qacAE* and *sulI* encoding resistance to quaternary ammonium and sulfonamide, in addition to an open reading frame (ORF5) of unknown function (10, 16, 60). Located downstream of each gene cassette inserted into the variable region is a segment of short inverted repeats called the 59- base element that play a part in recombination (60). Conjugative plasmids and transposons facilitate the movement of these gene cassettes (23, 24) among different enterobacteria populations (5, 22, 35, 67). Goldstein et al. (2001) found 46% *Enterobacteriaceae* isolates carried a class 1 integron and 79% of *Salmonella* isolated from poultry possessed this gene (22).

Antibiotics are dispensed to food animals, including poultry, for disease prevention and to improve feed conversion and increase production (31). Consequently, many commensal organisms are continually exposed to low levels of antibiotics. There is an extensive collection of epidemiological evidence which links antibiotic selective pressure to the emergence of resistance among commensal bacterial populations (39, 40). The large reservoir of antibiotic resistance genes in nature makes the potential for transfer inevitable. This study was an opportunity to use poultry farm environment as an ecosystem and investigate the role commensal organisms, bearing high level of resistances, play in disseminating antibiotic resistance genes. On a larger scale, this study attempts to understand the transferability of antibiotic resistance genes with a complex microbial community using the commercial poultry industry as a model. Since integrons are “natural vectors” for capturing gene cassettes and spreading antibiotic resistance genes, we wanted to test the ability of a class 1 integron in *S. enterica* Typhimurium to

gain resistance genes from the resident intestinal microflora of a chicken in response to antibiotic selective pressure. Although there are numerous *in vivo* studies documenting conjugative transfer of antibiotic resistance genes in cows, pigs, mice, turkeys and chickens (20, 25, 26, 30, 33, 44) none of these studies have attempted to assess integron resistance gene acquisition and quantitatively enumerate the level of resistance transfer.

MATERIALS AND METHODS

Salmonella strains

Salmonella enterica Typhimurium isolates, designated 3147 and 934, were used as recipient strains in an *in vivo* mating experiment. The *Salmonella* isolates were obtained from a commercial poultry farm in northeast Georgia and were the same genetic type, as determined by pulsed-field gel electrophoresis (PFGE). *S. enterica* Typhimurium isolate 3147 is pan-susceptible, while isolate 934 is resistant to a single antibiotic, streptomycin. *Salmonella* isolate 3147 was found to naturally contain a class 1 integron (*intI1*) with an empty integration site, as determined by PCR (37). *Salmonella* isolates were cultured on MacConkey agar and submitted to the National Veterinary Services Laboratory (Ames, Iowa) for phage typing based on bacteriophage typing designations outlined by Anderson et al. (1977) (3). The *Salmonella* recipients were made rifampicin (RIF) and naladixic acid (NAL) resistance through selection, using increasing concentrations of each antibiotic to obtain resistance to 64 µg/ml (34).

One day prior to colonizing one-day old broiler chickens with *Salmonella*, frozen glycerol stocks of each *Salmonella* strain were streaked onto XLT4 (Xylose Lysine Tergitol 4) selective agar (Becton- Dickinson and Co., Sparks, MD) containing nalidixic acid (NAL) (64ug/ml) and rifampicin (RIF) (64ug/ml) agar. Each strain was inoculated into 500ml BHI broth (Becton- Dickinson and Co., Sparks, MD) and incubated at 37°C, statically for 4 H, prior

to administration to broiler chicks. At 4 H, the culture was serially diluted 10-fold in 1X PBS, the dilution series between 10^4 - 10^9 was plated onto MacConkey agar and incubated overnight at 37°C to determine final *Salmonella* titer given to birds. *Salmonella* recipient, 934 and 3147 administered to chickens, was grown to a final concentration of 6×10^7 and 1.5×10^8 colony forming units (CFU)/ml, respectively.

***Salmonella* enrichment, isolation and identification**

We used tetrathionate brilliant green broth (TBG) (Becton Dickinson and Co., Sparks, MD) as our selective enrichment medium for *Salmonella*. After incubation at 41.5 °C for 18-24 H, a loopful of the overnight, TBG enrichment was streaked, for isolation, onto XLT4 (Xylose Lysine Tergitol 4) selective agar (Becton Dickinson and Co., Sparks, MD) and incubated overnight at 37°C. Identity of H₂S producing colonies as *S. enterica* Typhimurium was confirmed by whole-cell agglutination test using polyvalent *Salmonella* cell wall “O” and Group B antiserum (Becton-Dickinson and Co., Sparks, MD) (27, 47, 53, 65).

Preparation of donor, chicken microflora

Eighteen day-old, embryonated chicken eggs from specific pathogen free (SPF), white leghorn chickens (SPAFAS, Inc., North Franklin, CT) were cleaned and disinfected by immersing in pre-warmed (100°F) 10% commercial bleach solution (5.95% sodium hypochlorite, 0.15% sodium hydroxide, 94.60% water) (James Austin Co., Mars, PA) for approximately three minutes as previously described (66). Prior to placing chicks, Horsfall isolator units were sterilized by washing with bleach solution followed by fumigation with formaldehyde for approximately 24 H (69). SPF chicks were allowed to hatch in electrically heated isolator units under positive pressure biosafety level-two facilities at the Poultry Diagnostic and Research Center (PDRC) at the University of Georgia.

Approximately fifty-eight, day of hatch, SPF chickens were used to propagate and multiply litter flora into chicken intestinal microflora. SPF chickens were separated into four groups containing 14 chickens each. Each bird was orally inoculated with 0.5ml of pooled, poultry litter microflora harvested from commercial broiler farms in the Northeast Georgia area (42). Litter microflora harbored a variety of antimicrobial resistance genes (42) and they were thus chosen as donors to reconstitute the chicken's intestinal microflora for the *in vivo* mating experiment. At three weeks, post- inoculation, chicks were sacrificed by cervical dislocation. The cecal contents from chicken intestines were pooled, homogenized for 5 min at maximum speed using a stomacher (Tekmar Company, Cincinnati, OH) with 1ml of 1X phosphate buffered saline (PBS). Homogenates were filtered through gauze and administered to experimental broiler chicks during the mating experiment. A sample of donor homogenate was screened by culture for the presence of *Salmonella* as described above.

***In vivo* mating experiment between *Salmonella* and chicken intestinal microflora**

Experiments were conducted with day of hatch, broiler chicks housed in colony blockhouses to simulate husbandry conditions as practiced on integrated commercial poultry farms. Eighteen-day old, embryonated chicken eggs, from Cobb/ Cobb (Siloam Springs, AR) line broiler chickens, were obtained from a commercial hatcher. Broiler eggs, hatching incubators and floor pen house at the Poultry Diagnostic and Research Center (PDRC) (University of Georgia, Athens, GA) were disinfected and fumigated according to the methods described in the previous section. Five days prior to placing broiler chicks, the floor pens, walls and floors were randomly sampled with drag swabs (9) and tested for *Salmonella* according to procedures described above.

Commercial broiler eggs were collected from incubators at day of hatch and transferred

to an adjacent floor pen house containing 18- 10 ft² x 10ft² pens lined with fresh softwood shavings. Chicks were divided into six groups of approximately 50 birds per pen, as listed in Table1: control; donor- chicken intestinal microflora; recipient-*Salmonella* strain 934^{NAL,RIF^R}; recipient *Salmonella* strain 3147^{NAL,RIF^R}; donor x *Salmonella* 934 or *Salmonella* 3147. To prevent competitive exclusion of salmonellae from the birds' intestinal flora, day of hatch broiler chickens were first colonized with NAL/RIF^R (64µg/ml) recipient *Salmonella* strain 934 or 3147 by orally inoculating each chick with 0.1ml of 6 x 10⁷ and 1.5 x 10⁸ CFU/ml salmonellae, respectively. One day after receiving the *Salmonella* recipient strains, a 1 ml suspension of donor intestinal microflora cultured in SPF birds, as mentioned previously, was added to the chicks' drinking water. All birds were given clean drinking water daily *ad libitum* and fed commercial feed supplemented with monensin sodium (Elanco Animal Health, Indianapolis, IN) (110g/ton) and bacitracin methylene disalicylate (Alpharma, Inc., Fort Lee, NJ) (5g/ ton). To determine if therapeutic treatment selects for resistant bacteria, at two weeks of age the birds were administered antibiotics: chlortetracycline or streptomycin via their water drinkers at concentrations of 25mg/body weight/ lb and 15mg/body weight/lb, respectively. Control groups, birds which received no antibiotic treatment, were also included in this study.

Sampling, collection and isolation of gram-negative enterics including *Salmonella*

Drag swab and chicken litter from each pen (n=16) were collected weekly, for the six-week duration of the study. Water and feed were also collected at each sampling period. Sterile drag swabs (1' x 1' cotton gauze pads) soaked in 2X skim milk (Becton- Dickinson and Co., Sparks, MD) were dragged across the poultry litter (9). Each drag swab was placed in 100ml of TBG and incubated at 41.5°C for 18-24 H (7). Ten microliters of each TBG drag swab enrichment were streaked onto XLT4 selective agar (Becton-Dickinson and Co., Sparks, MD)

(27) with no antibiotics and XLT4 containing NAL (64µg/ml) and RIF (64µg/ml). Additionally, samples were streaked onto XLT4, NAL (64µg/ml), RIF (64µg/ml) combined with each of the following antibiotics: ampicillin (AMP) (10µg/ml), chloramphenicol (CHL) (25µg/ml), and gentamicin (GEN) (16µg/ml), kanamycin (KAN) (25µg/ml), streptomycin (STR) (25µg/ml), and tetracycline (TET) (10µg/ml). Antibiotic stocks were prepared according to manufacturer's specifications (Sigma Aldrich, St. Louis, MO). All selective agar plates were incubated for 24 H at 37°C. Isolates were stocked in freezer stock medium (1% peptone, 15% glycerol) and stored at -70°C.

Chicken litter samples were randomly collected from five general areas within a pen then pooled. One sample was collected per pen (n= 18). Five grams of litter was resuspended in approximately 30ml of 1X phosphate buffered saline (PBS) and vigorously shaken for 10min with a "wrist-action" shaker set at maximum speed (Burrell Scientific, Pittsburgh, PA). Samples were then filtered through sterile gauze and centrifuged at low speed (50 X g for 15min at 4°C) to remove litter debris. The bacteria were pelleted by high-speed centrifugation (3,650 X g for 15min at 4°C), supernatant discarded and the bacterial pellet resuspended in 1X PBS. A 0.5ml aliquot was again pelleted by high-speed centrifugation, resuspended in Superbroth media (52) with 20% sterile glycerol and subsequently stored at -70°C.

Water and feed samples were also tested for presence of *Salmonella*. For water testing, 100ml of a sample was added to 100ml of double- strength TBG. Feed samples (approximately 25g) were mixed with 225ml of TBG. All enrichments were plated onto XLT4 agar at 37°C for 18-24 H.

Enumeration of total *Salmonella* and antibiotic-resistant *Salmonella* using a modified most probable number (MPN) technique

The total number of salmonellae and antibiotic resistant *Salmonella* were enumerated using a modified version of the most probable number (MPN) technique (52, 68) in a 96- well format. Briefly, at the end of six-week mating period, nine birds from each pen were sacrificed by cervical dislocation the ceca were then aseptically removed. Those nine caecae were divided into triplicate, placed in sterile Whirl-Paks (Fisher Scientific, Inc., Pittsburg, PA), and immediately placed on ice. The samples were then homogenized with a stomacher (Tekmar Company, Cincinnati, OH) in 1ml of 1X PBS for 5 min at maximum speed. The homogenized samples were diluted in 100ml TBG then diluted 10-fold to final dilution of 1/1000 in 9ml of 0.9% saline. From these tubes, 100 μ l was transferred into 96-well Deepwell plates (Eppendorf Scientific Inc., Westbury, NY) containing 1ml of TBG. The first row contained TBG alone, while subsequent rows contained TBG with each of the test antibiotics: AMP, CHL, GEN, KAN, STR, TET, plus an additional antibiotic, ceftiofur (CEF) at 16 μ g/ml. The 96- well blocks were incubated at 41.5°C for 18 H. Using a multi-pen inoculator (PGC Scientific, Gaithersburg, MD), approximately 6 μ l of the enriched cultures was spotted onto XLT4 agar, XLT4 agar with NAL + RIF, and XLT4 with each of the antibiotics listed above. The plates were incubated at 37°C for 24 H before recording MPN results as positive if H₂S producing colonies were present and negative if there was no growth and at which dilution range growth is present. The MPN values were determined from Standard MPN Tables and Thomas' approximation at 95% confidence levels according to Peeler et al. (51, 62).

Enumeration of antibiotic resistant gram-negative enterics, including *Salmonella* in chicken litter

Cell density of antibiotic resistant bacteria present in poultry litter was determined by serially diluting frozen-glycerol stocks of litter microbiota 10-fold in 1X PBS (dilution range:

10⁻¹ to 10⁻⁵), spread-plating 100 µl of cell suspensions onto MacConkey (MAC) agar, MAC supplemented with NAL/RIF to enumerate *Salmonella*, and MAC with other antibiotics (AMP, CHL, GEN, KAN, STR, or TET) to quantify antibiotic resistant coliforms and non-coliforms present in poultry litter. Plates were incubated for 18- 24 H at 37°C before determining plate counts (CFU/ml).

PCR detection of class 1 integrase integron gene cassettes and antibiotic resistance genes

DNA for PCR was prepared using whole cells as template as described by Bass et al. (5). All PCR was performed on RapidcyclerTM, hot-air thermocyclers (Idaho Technologies, Idaho Falls, ID) (70). All isolates were screened by PCR for integron associated genes *intI1*, *qacAE* and *sull* (36) (Table 2). For integron-positive isolates, presence of gene cassette(s) within the *attC* integration site (59 base element) was assessed by PCR (36). *Escherichia coli* K12 strain SK1592 (Tn21:: pDU202) and *S. enterica* Typhimurium strain SR11 served as positive and negative controls, respectively. PCR products were visualized on 1.5% agarose, 1X TAE, ethidium bromide (0.2µg/ml) gel at 100V for 1 H (57). Integron gene cassettes amplified by PCR were purified using a Qiagen Gel Extraction Kit (Valencia, California) and sequenced on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) at PDRC (Athens, GA). The identity of the sequenced product(s) was determined by using NCBI nucleotide BLAST Search (2).

Forward and reverse transposon Tn21 primers were used to amplify a 595-bp PCR product (Table 2). PCR reaction mixture contained 2mM MgCl₂, 0.1mM primers, 0.2mM nucleotides, and 1.0 U Taq polymerase per 10µl reactions (Roche). The PCR program parameters for the RapidcyclerTM (70) for all reactions was: 94°C for 0 sec (denaturing), 55°C for 0sec (annealing), and 72°C for 15 sec (elongation) for 30 cycles and a slope of 2. All DNA

products were visualized by gel electrophoresis with 1.5% molecular grade agarose, 1X TAE, ethidium bromide (0.2µg/ml) gel at 80V for 1 H (57). Resistant *Salmonella* isolates were also screen by PCR for genes encoding for streptomycin/ spectinomycin resistance (*aadA1*), gentamicin resistance (*aadB*), tetracycline resistance (*tetA*) (50), and *bla*_{TEM} (43), encoding for resistance to ampicillin (Table 2).

Molecular typing of bacterial isolates by PFGE and RAPD

Salmonella isolates were genetically typed using PGFE (4) by digesting agarose embedded bacterial genomic DNA with 2.5U of restriction endonuclease, *Xba*I (Roche Molecular Biochemicals; Indianapolis, IN) overnight at 37°C. DNA fragments were separated with a CHEF DR-II electrophoresis apparatus (Bio-Rad; Hercules, CA) at 6 V for 25h and pulse time of 2 to 40s (4). PFGE patterns were visualized with 1.2% low melting agarose (Bio-Rad) stained in ethidium bromide for 1 H and destained in 1X TAE (Tris-acetate EDTA) buffer overnight at 4°C. Yeast strain *Saccharomyces cerevisiae* YPH80 served as a chromosomal DNA standard (220- 1100 kb) BioWhittaker Molecular Applications; Rockland, ME). For isolates with indistinguishable band patterns and high background levels, 50µM thiourea (Sigma Aldrich) was added to the 0.5X TBE (Tris-borate-EDTA) running buffer to increase typeability (12, 55).

Random amplification of polymorphic DNA (RAPD) analysis (32) was performed on antibiotic-resistant *Salmonella*, recovered from chickens experimentally colonized with *S. enterica* Typhimurium 934 or 3147, to confirm PFGE results as to parental lineage of isolates. RAPDs were performed using the RapidcyclerTM (Idaho Technologies), hot-air thermocycler. RAPD conditions were used as described by Maurer et al. (45) using typing primer 1290 (5'-GTGGATGCGA- 3') (1, 29). Briefly, the PCR reaction was prepared by mixing 1µl of DNA template with 9µl of reaction mix, which consisted of 3.5mM MgCl₂, 0.1mM of primer, 0.2mM

deoxynucleoside triphosphate (Roche), 0.5 units of Taq polymerase (Roche). RAPD primer 1290 was chosen due to its discriminatory power for typing *Salmonella* (14, 32). DNA fragments were visualized on 1.5% agarose, 1X TAE, ethidium bromide (0.2µg/ml) gel at 100V for 1 H. DNA banding patterns for PFGE were interpreted based on Tenover criteria (61).

Antibiotic Susceptibility Profile

Antibiotic susceptibilities to a panel of nine antibiotics was first determined by the Kirby-Bauer disk diffusion method (48, 49) for the eight antibiotics corresponding to the panel of antibiotics screened for during the *in vivo* mating experiment: ampicillin(10µg), chloramphenicol (30µg), gentamicin (10µg), kanamycin (30µg), nalidixic acid (30µg), rifampicin (5µg), streptomycin (10µg), and tetracycline (30µg), with the exception of sulfisoxazole (250µg) (Becton-Dickinson and Co.), which was included in the initial screen. Acquired resistances were inferred based on wild type's antibiotic susceptibility profile. The Sensititre® susceptibility system (Trek Diagnostics Systems, Ltd.) was used to determine the minimum inhibitory concentration (MIC) for *Salmonella* isolates by the broth microdilution method (48, 49), in order to confirm and expand the antibiotic susceptibility profiles for these isolates generated by disk diffusion. The antibiotics contained in each Sensititre plate were enrofloxacin, gentamicin, ceftiofur, neomycin, oxytetracycline, tetracycline, amoxicillin, spectinomycin, sulphadimethoxine, trimethoprim/sulphamethoxazole, sarafloxacin, sulphathiazole, and streptomycin. Briefly, 3-5 isolated colonies picked from non- selective agar were inoculated into deionized water to a 0.5 MacFarland standard turbidity (approximately 10^8 CFU/ml). The bacterial suspensions were transferred to Mueller Hinton broth (Difco) aliquots then serial two-fold dilutions of approximately 10^5 CFU/ml were made in 96-well Sensititre plates containing different concentrations of antibiotics. After 18-24 H incubation at 37°C susceptibilities were

interpreted according to guidelines established by the National Committee for Clinical Laboratory Standards (NCCLS) (48, 49).

Statistical Analysis

The mean and standard deviations were computed for each experimental group and therapeutic treatment. Paired and unpaired student t-test analyses were used to evaluate the effects of therapeutic treatments over time on the degree of resistance to each antibiotic tested.

RESULTS AND DISCUSSION

Mortality and persistence of *Salmonella* in poultry environment following experimental challenge of day of hatch, commercial broiler chickens with salmonellae.

In our study, we orally inoculated broiler chicks with approximately equal numbers of *Salmonella* strain 934 and 3147. Significant mortality was observed 3 days following administration of *Salmonella* to one day-old chickens. Mortality rates varied from 25% to 40% (Figure 2). The high percentage of mortality could be attributed to the age of chicks, as well as the pathogenicity, invasiveness, and bacteremia of the *Salmonella* isolates (19). As expected mortality rates (2%) within the control group were significantly less than groups given salmonellae. Comparable mortality rates (4%) were observed in groups given donor microflora alone. The incidence of deaths caused by *Salmonella* infections tends to be age related. Consequently, we observed a decline in the number of deaths from 151 to zero, as the experiment progressed over the six-week period. Mature birds are significantly less susceptible to salmonellae. In most cases, older birds may not exhibit clinical signs of infection despite intestinal colonization, when experimentally challenged with salmonellae (19). Similar mortality rates were described by Fagerberg et al. (1976) wherein a 50% mortality rate was observed after orally challenging day of age broiler chickens with 1×10^9 cfu of *S. typhimurium*. The same oral

dose was lethal for 20% of three-day-old chicks, but no deaths were reported for 7-day-old chicks (15).

Both donor microflora and *Salmonella* easily colonized the chicks and persisted throughout the entire six weeks with varying population sizes (Figure 3). By the fourth week, we observed an increase in *Salmonella* titers that rose to approximately 10^5 cfu/g in litter. Although *Salmonella* cultures were given to the bird at high doses prior to the donor microflora, titers never reached the level at which they were first introduced into the broiler chicks. Gram-negative microflora remained fairly level, ranging from 10^6 - 10^7 cfu/g in litter (Figure 3). Even though *Salmonella* titers were low, we easily recovered resistant *Salmonella* isolates (transconjugants) throughout the experiment from tetrathionate enrichments of drag swabs and from litter collected from the pens. We observed the *Salmonella* titers peak at week 3 (log 4.56 CFU/g), followed by a steady decline of approximately 2 logs when birds reached 6 weeks of age. A similar pattern was observed for total gram-negative bacterial population in litter in which this population reached a steady-state level of log 7.70 CFU/g.

Impact of antibiotic usage in broiler chickens on the level of drug resistance in gram-negative bacteria present in poultry litter

To assess if antibiotic usage selects for drug resistant, gram-negative bacteria including *Salmonella*, antibiotics chlortetracycline and streptomycin were administered to broiler chicks for one week. For control groups (no chlortetracycline or streptomycin administration), resistance to the six antibiotics varied. Resistances ranged from <5 to <50% of the total gram-negative population (Figure 4A). This suggests that a reservoir, of resistance genes, was present despite antibiotic usage. We observed a high prevalence of ampicillin resistance (32%) and streptomycin resistance (27%) in this bacterial community over time. The level of resistance to

streptomycin gradually increased to more than 6-fold, from 4% to 27% by week 6. Tetracycline resistance was 9%, on average, never surpassing 15% of the total gram-negative population in litter, for this control group of birds.

In our analysis of the tetracycline treatment group, we found that the average ampicillin resistance increased by approximately 8.5% compared to control birds receiving no antibiotic treatment (Figure 4B). Based on student t-test analysis, tetracycline treatment had no significant effect on the emergence of ampicillin resistance, ($p= 0.1$). The average percentage of streptomycin resistance increased overall by 50%, when tetracycline was administered to birds as compared to 12%, in groups receiving neither this antibiotic nor streptomycin ($p= 0.2$). Tetracycline usage had no statistically significant effect on the level of streptomycin resistance observed, at least by student t-test. Kanamycin resistance rose from 2% at week 2 to 44% at week 4. We presumed that tetracycline resistance would select for the emergence of tetracycline resistance, but in comparison to other resistances, the incidence of tetracycline resistance was much lower (19% vs. 24% streptomycin and 41% ampicillin). Overall, tetracycline resistance slightly increased by about 2% in the tetracycline treated groups compared to non-treated groups.

Resistance to ampicillin among gram-negative bacteria, in litter, continued to be higher for the streptomycin treatment group, at approximately 34% compared to 32% for the control group ($p= 0.8$) (Figure 4C). There was no significant difference observed for ampicillin resistance between streptomycin treated and non-treated groups. We observed increases in resistances of 4-fold and 5-fold in litter gram-negatives for the antibiotics: tetracycline and kanamycin, respectively for week 3 through week 5. Streptomycin treatment, unlike tetracycline, increased the level of streptomycin resistance among gram-negatives in litter by five-fold. After streptomycin administration, between the weeks 3 and 5, resistance increased

from 16% to 81% at week 5. The resistances, for this treatment group, were considerably higher for the antibiotics: ampicillin (61%), kanamycin (59%), streptomycin (81%) and tetracycline (65%) compared to the other groups. A statistically significant difference was found for the four antibiotic resistances at week 5 when streptomycin treatment was administered as compared to tetracycline treatment ($p= 0.005$), at least by student t-test. The increased resistances to ampicillin, kanamycin, tetracycline, and streptomycin seen at week 5 were statistically significant between groups receiving streptomycin treatment compared to groups given no therapeutics ($p= 0.023$). In ranking their frequency of resistance in regards to therapy, streptomycin resistance followed, in order by ampicillin (34%), kanamycin (23%), and tetracycline (19%) were highest among the gram-negative, bacterial population isolated from the environment, in which birds received the antibiotic streptomycin. Tetracycline treatment resulted in a higher level of resistance among the litter gram-negatives to ampicillin (41%) followed by streptomycin (24%), kanamycin (16%) then tetracycline (19%). We observed no statistically significant differences ($p= 0.23$) among treatment and control groups for low level resistance of litter, gram-negative bacteria to the antibiotics gentamicin and chloramphenicol. Generally, we found that regardless of treatment, the level of ampicillin resistance fluctuated over time as the bird matured, and accounted for the majority of antibiotic resistance present within our simulated poultry farm environment. Here, as expected (38), antibiotic usage does appear to amplify resistance among commensal bacteria to certain antibiotics in poultry, but in several instances the outcomes were unexpected. For example, antibiotic usage appeared to amplify resistance to unrelated antibiotics (streptomycin treatment selecting for kanamycin resistance and tetracycline treatment selecting for streptomycin). A study by Gast et al. (1986) found when kanamycin added to the drinking water aided in the transfer of kanamycin

resistance. In this study, 40% of *Salmonella* (resistant to nalidixic acid, streptomycin) were recovered from turkey poultts given no kanamycin but in poultts given kanamycin, 87% of *Salmonella* acquired resistance to this antibiotic from *Escherichia coli* (resistant to tetracycline, ampicillin, and kanamycin) (20). Marshall et al. (1990) found that when heifer and bulls were administered chlortetracycline for five days, the frequency of tetracycline native microflora increased from $\leq 15\%$ to more 63% of the total *Enterobacteriaceae* population, however there was little to no effect on recovery of tetracycline resistant *Escherichia coli* (44). These findings conflict a study by Smith et al. (1970) where either tetracycline use reduced the number of *Salmonella* (58) or, as with our study had minimal effect on tetracycline resistance in *Salmonella*. This effect may have been the consequence of accidental, physical linkage of two different, antibiotic resistance genes onto the same genetic element, in which antibiotic selection for one resistance gene results in the co-selection of the other (56).

Emergence of antibiotic resistance in *Salmonella* experimentally colonizing broiler chickens

From our initial characterization of the recipient strains, we identified *S. enterica* Typhimurium 934, as negative for class 1 integron and its associated genes, *qac Δ E1*, *sull* and the spectinomycin/streptomycin resistance gene, *aadA1*. However, this strain was resistant to streptomycin and sulfisoxazole as determined by disk diffusion test. *Salmonella enterica* Typhimurium recipient 3147 contained a class 1 integron with an empty integration site as determined by PCR (35). This strain had an integrase gene (*intI1*), along with *qac Δ E1*, *sull* and *aadA1* genes. Antibiotic susceptibility testing of this strain revealed it susceptible to streptomycin. When evaluating the effect of antibiotics on *Salmonella* levels in the poultry environment, we found that titers for salmonellae in groups treated with streptomycin increased dramatically, from low levels, 9.90×10^2 cfu/g, to 7×10^4 cfu/g, following administration of the

antibiotic (Figure 5). During week 4, the level of *Salmonella* declined with respect to total gram-negative bacteria in litter. However, at week 5 the number of *Salmonella* increased by four log₁₀. Tetracycline treatment had less of an influence on *Salmonella* levels than streptomycin treatment. Following tetracycline treatment, the number of *Salmonella* reached its highest at 1×10⁴ cfu/g. Given the recipients' background, we expected that therapeutic streptomycin would amplify this population of bacteria, particularly since both recipients carried either the streptomycin resistance phenotype or the *aadA1* gene encoding for streptomycin resistance. There are conflicting reports concerning the effects of antibiotic selective pressure on *Salmonella* excretion in chickens. In a study examining the effects of feed supplemented with antibiotics and *Salmonella* excretion rates, feed containing bacitracin (10 or 100mg/kg) was shown to either have no effect or only increase the amount of *S. enterica* Typhimurium excreted slightly (59). Ford et al. (1981) examined the incidence of *Salmonella typhimurium* shedding and resistance when broiler chicks are fed salinomycin. They found salinomycin to have no effect on length of shedding time, number of salmonellae shed after treatment, nor the total number of resistance patterns. They observed salinomycin treated *Salmonella* group to be more susceptible to antibiotics like, gentamicin, tetracycline, and amikacin than non- treated *Salmonella*. The control birds exhibited more salmonellae resistant to eight or nine drugs while 82% of the salinomycin salmonellae maintained the streptomycin, sulfadiazine, and nalidixic acid which were the original phenotypes of the dosing *S. typhimurium* (17).

Antimicrobial phenotypic analysis on both recipients demonstrated resistance to all nine antibiotics examined (Tables 3 and 4). A variety of resistance patterns were present within and between the two *Salmonella* strains recovered from the poultry environment. Resistant isolates from integron negative recipient strain 934 contained seven different antibiotic resistance

patterns (Table 3). These phenotypic resistance patterns had at least four antibiotics in their resistance profile. Sixty-seven percent were resistant to five antibiotics, 46% of which were resistant to nalidixic acid, rifampicin, sulfisoxazole, amoxicillin, and ampicillin (SuAmA). The majority of these *Salmonella* isolates had acquired the β -lactamase gene, *bla*_{TEM}. However, one isolate with the phenotypic pattern SSu, carried the streptomycin gene *aadA1*. All of these resistance patterns were a combination of all the antibiotics screened with the exception of gentamicin. Resistance to β -lactam antibiotics ampicillin and/or amoxicillin were prominent among *Salmonella* strain 934 recovered with >50% resistance (Figure 6). A number of these antibiotic resistances are often associated with mobile genetic elements. For instance, the TEM β -lactamases, can be either plasmid mediated or chromosomally encoded (41) and the aminoglycoside resistance genes are commonly found as mobile gene cassettes in integrons (16).

Salmonella strain 3147 isolates displayed resistances to eight different antibiotic combinations, some of which included resistances to all the antibiotics tested. Twenty percent of 3147 isolates were resistant to six or more antibiotics and 8% carried the SSuAmAGKT resistance pattern (Table 4). Resistance to three or four antibiotics (80%) was common among *Salmonella* 3147 isolates. The most prevalent resistance pattern, for 48% of the isolates, was the SSu antibiotic combination. Gentamicin resistance (100%) was observed only in *Salmonella* strain 3147 recovered from the environment of experimentally challenged birds (Figure 6). Despite the low level of gentamicin resistance among the litter gram-negative, population *Salmonella* recipient 3147 was able to acquire the gentamicin gene, *aadB* linked to integron gene cassette, from the poultry microflora. An overwhelming number of *Salmonella* 3147 isolates recovered were resistant to both kanamycin (83%) and streptomycin (78%) compared to *Salmonella* 934 isolates. We observed nearly equal percentages of resistance in both *Salmonella*

strains for tetracycline resistance (43% for 934 vs. 57% for 3147) and for sulfisoxazole resistance (47% for 934 vs. 53% for 3147) (Figure 6). While, all *Salmonella* isolates recovered from experimentally inoculated chicks were resistant to nalidixic acid and rifampicin, most of these isolates were from *Salmonella* strain 3147 (63%) while 37% represented isolates from *Salmonella* 934.

Twenty-eight percent of *Salmonella* 3147, the integron-associated strain, isolates recovered still possessed the class 1 integron, whereas 72% were characterized by PCR as integron negative. We observed that isolates with resistance to ≥ 8 antibiotics tended to possess class 1 integrons. *Salmonella* with multi-drug resistance patterns had multiple antibiotic resistance genes and produced a 1.7kb amplicon with primers flanking the integron's *attC* integration site (Figure 7). Of the integron-associated isolates, 57% generated a PCR amplicon. Sequencing these 1.7 kb amplicons revealed two gene cassettes; *aadB* and *aadA2*. *Salmonella* 3147 had also acquired antibiotic resistance genes: *tetA*, *aadB*, and *bla*_{TEM}. Forty-three percent of *Salmonella* recovered from the poultry environment possessed at least one of the antibiotic resistance genes. Most *Salmonella* isolates carried the *bla*_{TEM} gene that encodes for ampicillin resistance. Twenty-five percent carried the integrase gene of class 1 integron, all of which possessed antibiotic resistance genes: *aadA1*, *aadB*, *bla*_{TEM}, and *tetA*. Genetic fingerprinting by PFGE (Figure 8A) and RAPD (Figure 8B) confirmed the parentage of *Salmonella* recovered as strains 934 and 3147, thus, supporting our claim that resistant *Salmonella* recovered was the same strain administered to the chickens and that these salmonellae had acquired resistance from their poultry environment.

Enumeration using a modified three-tube MPN technique was an attempt to quantitatively determine the level of resistant and susceptible salmonellae still colonizing the

chicken. *Salmonella* resistant to tetracycline, ampicillin, and gentamicin were detected at levels ranging from 10^4 - 10^5 cfu/ml. We observed a high degree of resistance (86%) to gentamicin for *Salmonella* 3147 recovered from cecum of birds sacrificed at 6 weeks of age (Figure 9). There were differences regarding the level of tetracycline and ampicillin resistance. For *Salmonella* strain 934, MPN results showed a lower incidence of ampicillin resistance (4%) as opposed to tetracycline resistance (80%). As for *Salmonella* 3147, resistances to ampicillin (14%) and tetracycline (20%) were comparable. Using the MPN technique to enumerate, relies on several theoretical assumptions, primarily that if at least one organism (colony- forming unit) is present, then visible growth should be observed which is then used to estimate the number of bacteria present in a sample. In this case, the detection limit of the MPN was 3.6×10^4 cfu/ml (Table 5). Antibiotic susceptible *Salmonella* isolates were recovered from each group and were detected between 3.6×10^4 and 9.6×10^5 cfu/ml. We did not observe resistances among *Salmonella* colonizing chickens to the antibiotics: chloramphenicol, kanamycin, or ceftiofur.

CONCLUSIONS

Food safety is primarily centered on the epidemiological surveillance and identification of food related pathogens. As foodborne pathogens, like *Salmonella*, have emerged with multiple drug resistances, we are now aware of the public health risks associated with antibiotic resistance as it relates to food safety. Those most at risk are the elderly, the immunocompromised, and children. Popular opinion is that antibiotic use in food animals is the leading cause of multiple antibiotic resistant organisms. Our study has demonstrated that therapeutic antibiotic usage is capable of causing rapid and radical, yet sporadic changes within bacterial community with regards resistance frequency. However, without the selection pressure of antibiotics, bacteria, including *Salmonella* can readily acquire resistance from a reservoir of

commensal bacteria inhabiting the food production system. We showed that when there is a large reservoir of antibiotic resistance among the gram-negative bacteria they readily inhabit the poultry environment. Other studies have reported on the feasibility of antibiotic resistance transfer both *in vitro* and *in vivo*, but none have exploited the “natural cloning and expression” capability of an integron to acquire antibiotic resistance genes and to demonstrate the fluidity at which antibiotic resistances are disseminated in the intestine of a chicken.

There was a strong correlation between resistances acquired *in vivo* by our *Salmonella* recipient strains and the level of resistance in the gram-negative bacteria in litter. For instance, ampicillin resistance among the gram-negative enterics represented approximately 28- 32% resistance and *Salmonella* readily acquired this resistance through conjugal transfer *in vivo*. In addition to isolating ampicillin resistant *Salmonella*, we consistently observed resistances to tetracycline and streptomycin among salmonellae isolated from our poultry environment. These were the same common antibiotic resistances observed for commensal, gram-negatives isolated from the same environment. Likewise, we observed the same resistances for gram-negative bacteria isolated from the commercial poultry farm, from which we originally obtained our donor microflora (unpublished data). Hence, the acquisition of antibiotic resistance among *Salmonella* recipients 934 and 3147 actually followed with the level of resistance to these individual antibiotics among the commensal bacteria which inhabit the commercial farm environment.

Horizontal gene transfer has undeniably played a significant role in the development of drug resistance. The emergence of antibiotic resistance is complex, confounded by several factors relating to fitness, environment, selection pressure(s) and the ecology of genetic elements responsible for disseminating resistance. We showed that antibiotic selective pressure is not needed for resistant organisms to arise. We also showed that overall, integrons and mobile gene

cassettes contributed to the genetic diversity of *Salmonella* recovered during the mating experiment, as witnessed for integron-associated *Salmonella* strain 3147. However, there is no direct explanation for most of these isolates losing their class 1 integron. Several factors may have influenced this “jumping ship” episode. We observed a rapid loss in phenotypic antibiotic resistance when antibiotics were not included in the media. Plasmid instability may be a way bacteria cope with the lack of antibiotic selection pressure and consequently it may be an explanation of how and why we see resistance even when antibiotics are not used. We were not able to isolate enough plasmid DNA to perform restriction enzyme analysis to determine diversity as well as determine the location and linkage of the resistance genes acquired by our *Salmonella* strains. In addition to the stability of resistance genes and the elements which carry them, more focus needs to be on gene expression to understand the true impact of these mobile gene elements in nature and an explanation as to why more *Salmonella* isolates were not resistant even though there was a large pool of resistance genes within the environment.

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Table 1. *In vivo* mating of recipient *Salmonella* Nal, Rif^R strains with donor, chicken intestinal microflora: experimental groups \pm antibiotic administration.

GROUP	# BIRDS/PEN	TREATMENT
A	50	Control, Untreated*
B	50	Donor <i>Litter Flora</i>
C	50	Recipient, <i>Salmonella</i> 934
D	50	Donor + Recipient 934
E	50	Recipient, <i>Salmonella</i> 3147
F	50	Donor + Recipient 3147

\pm Experimental groups were done in triplicate. At week 2, birds were given chlorotetracycline, streptomycin, or no antibiotics for 1 week. Antibiotics were administered to the birds via their drinking water. * Untreated group received sterile phosphate buffered saline (PBS) as placebo.

Table 2. PCR primers.

Target Gene	Primer Sequence	PCR Conditions		Expected Size (bp)	Reference
		MgCl ₂	Annealing T _m (°C)		
5'CS- 3'CS	F:GGCATCCAAGCAGCAAG R:AAGCAGACTTGACCTGA	2mM	52	—	(37)
<i>int11</i>	F:CCTCCCGCACGATGATC R:TCCACGCATCGTCAGGC	2mM	55	280	(5)
<i>QacΔE1</i>	F:AAGTAATCGCAACATCCG R:AAAGGCAGCAATTATGAG	2mM	60	250	(5)
<i>sul1</i>	F:GGGTTTCCGAGAAGGTGATTGC R:TTGGGCTTCCGCTATTGGTCT	2mM	60	187	(37)
Tn21	F:GATAGCACTCCAGCCCGCAGAA R:AGGATCTGCTCGGCCATTCC	2mM	55	595	This Study

<i>bla</i> _{TEM}	F:ATAAAATTCCTTGAAGACGAAA R:GACAGTTACCAATGCTTAATCA	2mM	55	1079	(43)
<i>tetA</i>	F:GCTACATCCTGCTTGCCTTC R:CATAGATCGCCGTGAAGAGG	3mM	55	210	(50)
<i>aadA1</i>	F:GCACGACGACATCATTCCG R:ACCAAATGCGGGACAACG	2mM	60	307	This Study
<i>aadB</i>	F:ACGCAGGTCACATTGATAC R:CGGCATAGTAAGAGTAATCC	2mM	60	400	This Study

Table 3. Antibiotic resistance phenotype and genotype *Salmonella* 934 isolates recovered from poultry environment

Isolate	Time of Isolation (weeks)	Antibiotic Resistance Profile ^a	Class 1 Integron		Antibiotic Resistance Genes ^b
			<i>intI1</i>	<i>sulI</i>	
934 Recipient		S Su	-	-	-
8756	2	S Su T	-	-	-
8757	2	S Su	-	-	<i>aadA1</i>
8758	2	S Su K T	-	-	-
8759	2	S Su Am A	-	-	<i>bla</i> _{TEM}
8783	2	S Su T	-	-	-
8765	3	S Su A	-	-	<i>bla</i> _{TEM}
8784	3	S Su Am A	-	-	<i>bla</i> _{TEM}
8785	3	Su Am A	-	-	<i>bla</i> _{TEM}
8786	3	Su Am A	-	-	<i>bla</i> _{TEM}
8787	3	Su Am A	-	-	<i>bla</i> _{TEM}
8792	5	Su Am A	-	-	<i>bla</i> _{TEM}
8793	5	Su Am A	-	-	<i>bla</i> _{TEM}
8794	5	Su Am	-	-	<i>bla</i> _{TEM}
8768	5	Su Am A	-	-	<i>bla</i> _{TEM}
8769	5	Su Am A	-	-	<i>bla</i> _{TEM}

^a Antibiotic abbreviations: N, naladixic acid; R, rifampicin, S, streptomycin; Su, sulfamethoxazole; A, ampicillin; Am, amoxicillin; G, gentamicin; K, kanamycin; T, tetracycline.

^b Relevant resistance phenotype: *bla*_{TEM} = β -lactamase; *aadA1* = spectinomycin/streptomycin.

Table 4. Antibiotic resistance phenotype and genotype *Salmonella* 3147 isolates recovered from poultry environment

Isolate	Time of Isolation (weeks)	Antibiotic Resistance Profile ^a	Class 1 integron			Antibiotic Resistance Genes ^b
			<i>intI1</i>	<i>sul1</i>	Cassette Size (kb)	
3147 Recipient		Su	+	+	none	<i>aadA1</i>
8755	1	S Su	-	-	—	-
8760	2	S Su A G K T	+	+	1.7	<i>aadA1, tetA, aadB, bla_{TEM}</i>
8761	2	S Su	-	-	—	-
8762	2	S	-	-	—	-
8763	2	S Su	-	-	—	-
8764	2	S Su	-	-	—	-
8776	2	S	-	-	—	-
8777	2	S	-	-	—	-
8778	2	S Su	-	-	—	-
8779	2	S Su	-	-	—	-
8780	2	S Su G K	-	-	—	-
8781	2	S Su	-	-	—	-
8782	2	S Su	-	-	—	-
8766	3	Am A	-	-	—	<i>bla_{TEM}</i>
8788	4	S Su	-	-	—	-
8789	4	S Su	-	-	—	-
8790	4	S Su	-	-	—	-
8791	4	S	-	-	—	-
8767	4	S Su	-	-	—	-

8770	5	S Su Am G K T	+	+	1.7	<i>aadA1, tetA, aadB, bla_{TEM}</i>
8771	5	S Su Am A G K T	+	+	1.7	<i>aadA1, tetA, aadB, bla_{TEM}</i>
8772	5	S Su Am A G K T	+	+	1.7	<i>aadA1, tetA, aadB, bla_{TEM}</i>
8773	5	S Am	+	+	—	<i>aadA1, tetA, bla_{TEM}</i>
8774	5	S Am	+	+	—	<i>aadA1, tetA, bla_{TEM}</i>
8775	5	S Am	+	+	—	<i>aadA1, tetA, aadB, bla_{TEM}</i>

^a Antibiotic abbreviations: N, naladixic acid; R, rifampicin, S, streptomycin; Su, sulfisoxazole; A, ampicillin; Am, amoxicillin; G, gentamicin; K, kanamycin; T, tetracycline.

^b Relevant resistance phenotype: *bla_{TEM}* = β -lactamase; *aadA1* = spectinomycin/streptomycin, *tetA* = tetracycline, and *aadB* = gentamicin.

Table 5. Enumeration of antibiotic resistant *Salmonella* recovered from birds as determined using a modified most probable number (MPN) technique.*MPN values and range are an estimation based on Thomas' approximation. †Average cfu/ml values were calculated using the 3-tube MPN method.

Antibiotic Resistance	Group	Avg. MPN/ml*	MPN range*
Susceptible	Recipient 934	0.36	0.0-0.36
	934+ Donor	9.74	0.36-43.0
	Recipient 3147	7.68	0.36-15.0
	3147 + Donor	0.14	0.0-0.43
Tetracycline	Recipient 934	0.30	0.0-0.30
	934 + Donor	8.25	1.5-15.0
	Recipient 3147	1.54	0.62-2.40
Ampicillin	934 + Donor	0.36	0.0-0.36
	Recipient 3147	1.10	0.0-1.10
Gentamicin	3147 + Donor	0.36	0.0-0.36

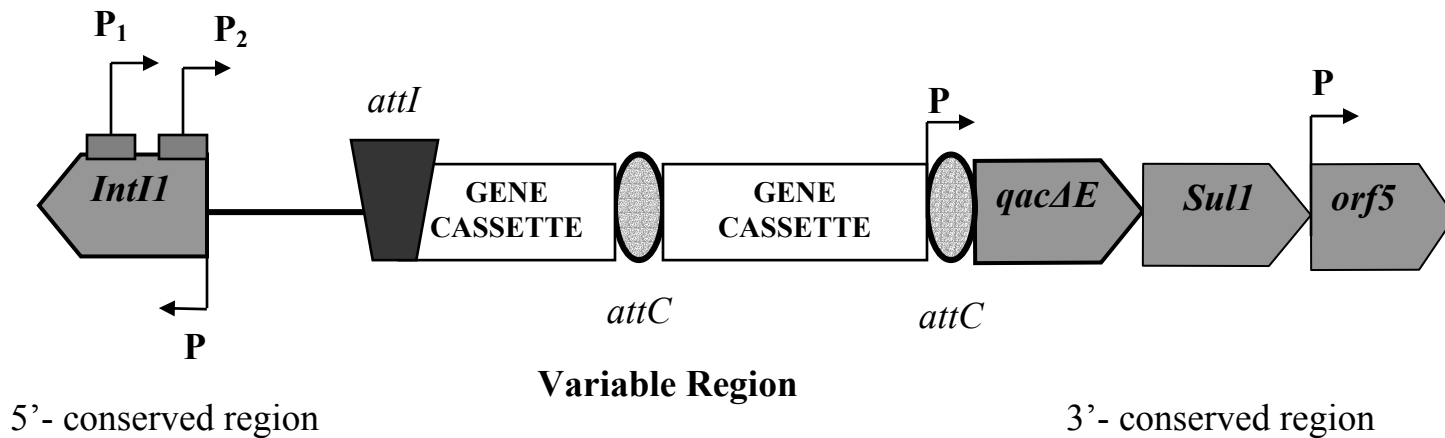


Figure 1. General structure of class 1 integrons. Cassettes are integrated into the variable region by the enzyme integrase (*IntI1*) using a site-specific recombination mechanism. The *attI* and *attC* (59 base element) sites are shown as black and grey-shaded ovals, respectively. The promoters are denoted by the letter P. The characteristic genes of a Class 1 integron are as follows: *intI1*, integrase; *qacΔE1*, which encodes for quaternary ammonium resistance; *sulI*, encodes for sulfonamide resistance; and *orf5*, a gene of unknown function.

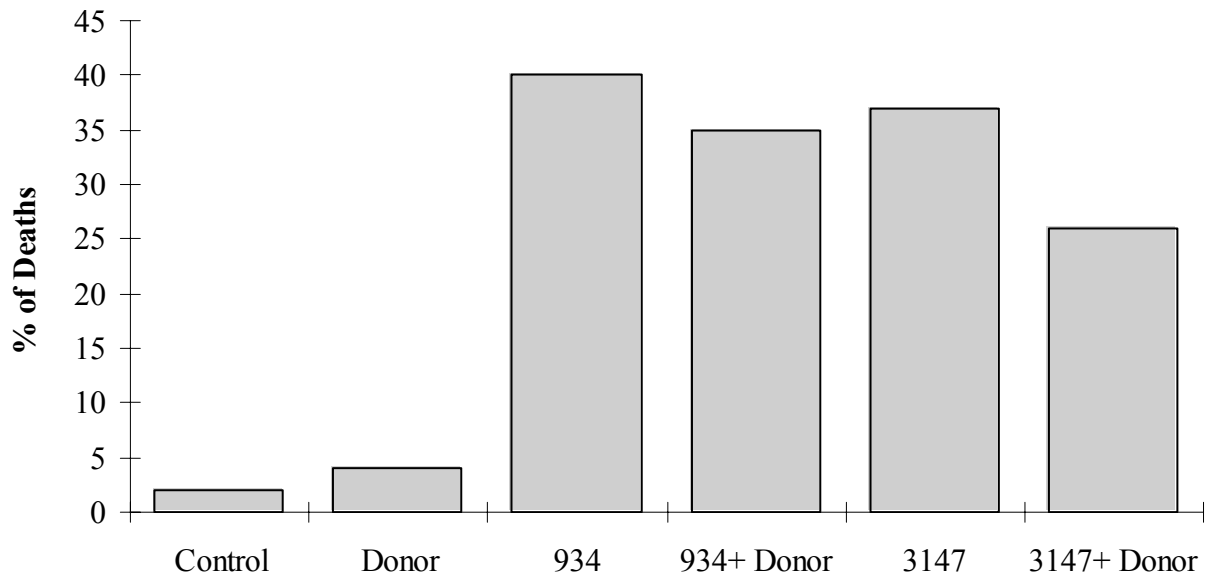


Figure 2. The percentage mortality observed and the number of deaths over six weeks for broiler chickens in the following treatment groups: sham – inoculated control group, each *Salmonella* strain alone, and donor microflora mated with each *Salmonella* strains.

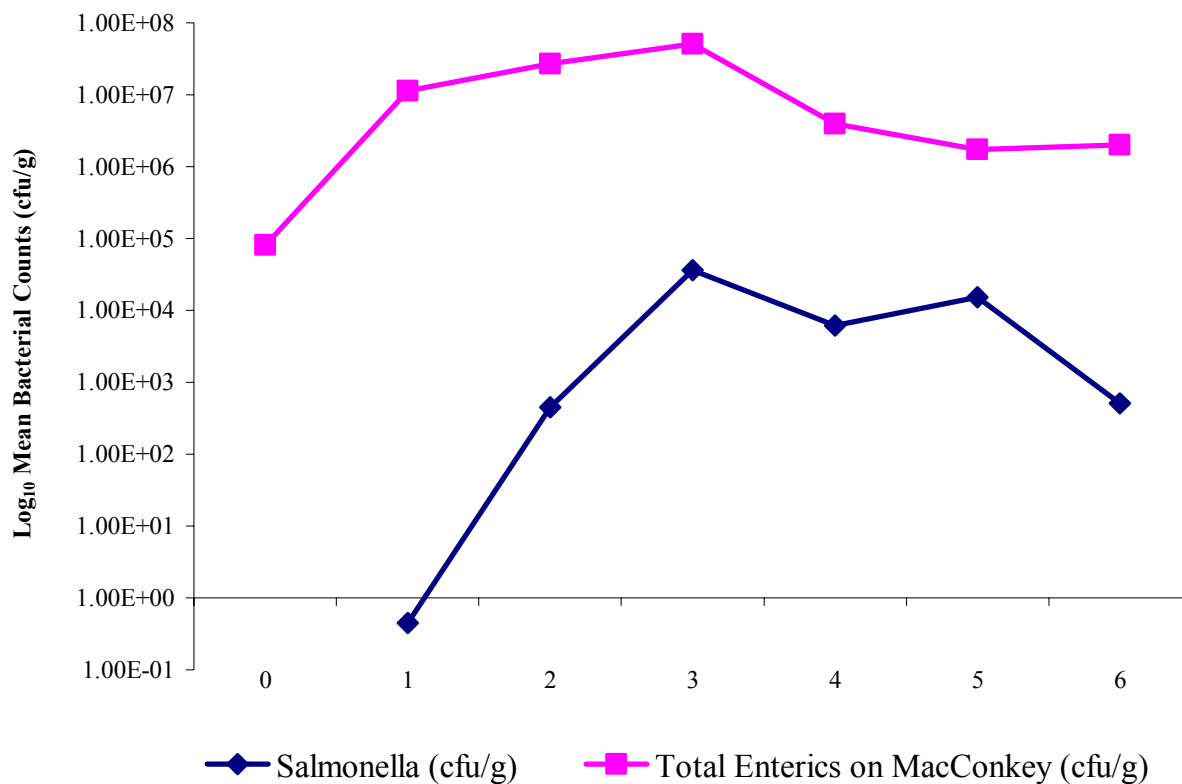


Figure 3. Level and persistence of *Salmonella* and other gram-negative enterics in broiler chicken environment. Values are represented as the mean number of *Salmonella* isolated for all groups, as defined as non-lactose fermenting colonies on MacConkey agar with nalidixic acid and rifampicin, compared to the number of gram-negative organisms isolated when grown on MacConkey agar without any antibiotics. Identity of *Salmonella* was confirmed by whole cell agglutination with *Salmonella* group B- specific antisera. Broiler chickens were administered, orally, with 10^8 CFU salmonellae at day of hatch.

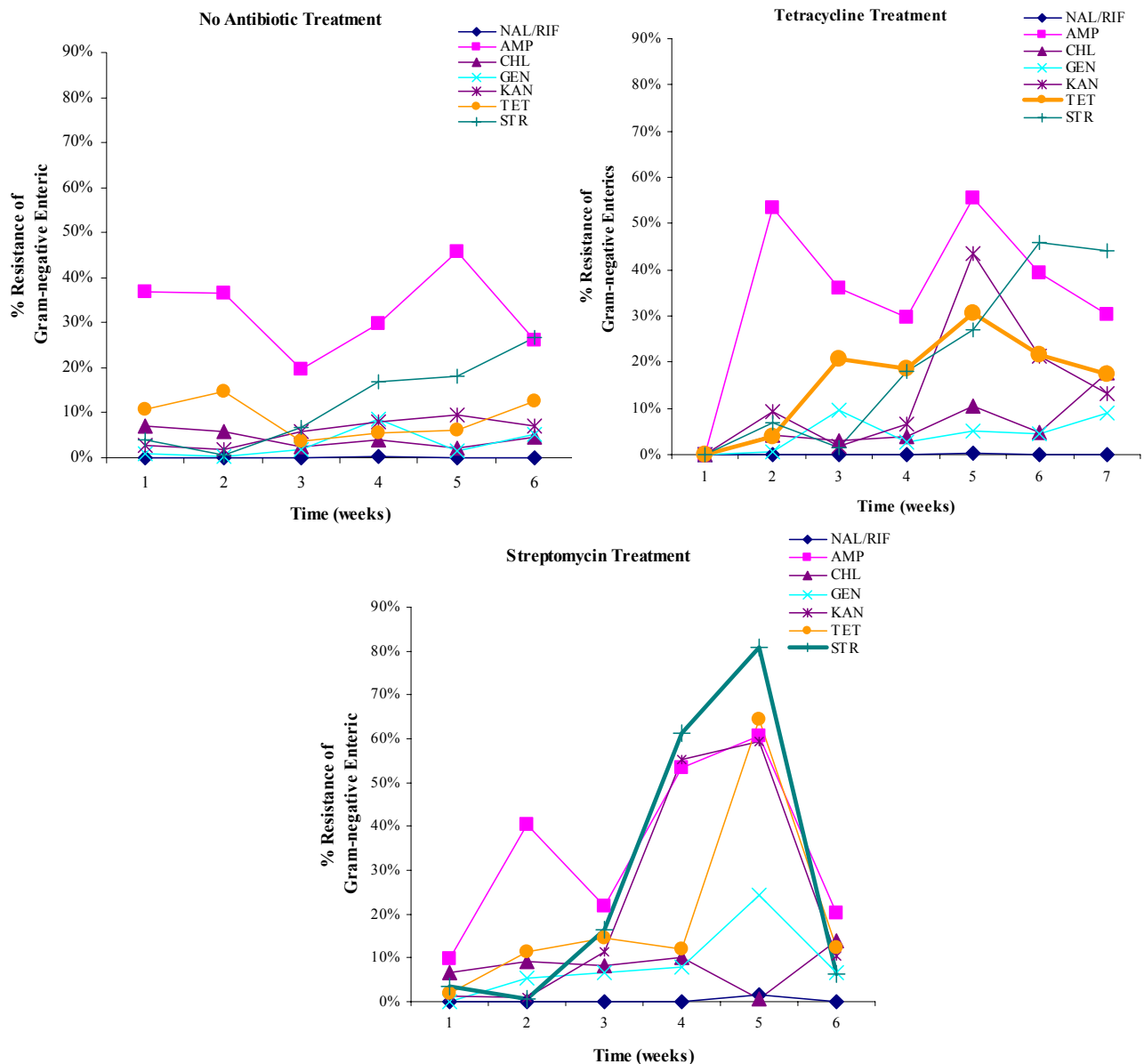


Figure 4. Incidence of antibiotic resistance among the total gram-negative bacterial population in poultry litter when antibiotic selective pressure is used. Untreated birds, control group (A); birds administered chlortetracycline (B); or streptomycin (C) at the second week for seven days. Percent resistance was calculated from average bacterial counts on MacConkey agar plates supplemented with antibiotics divided by the average count on MacConkey plates without antibiotics.

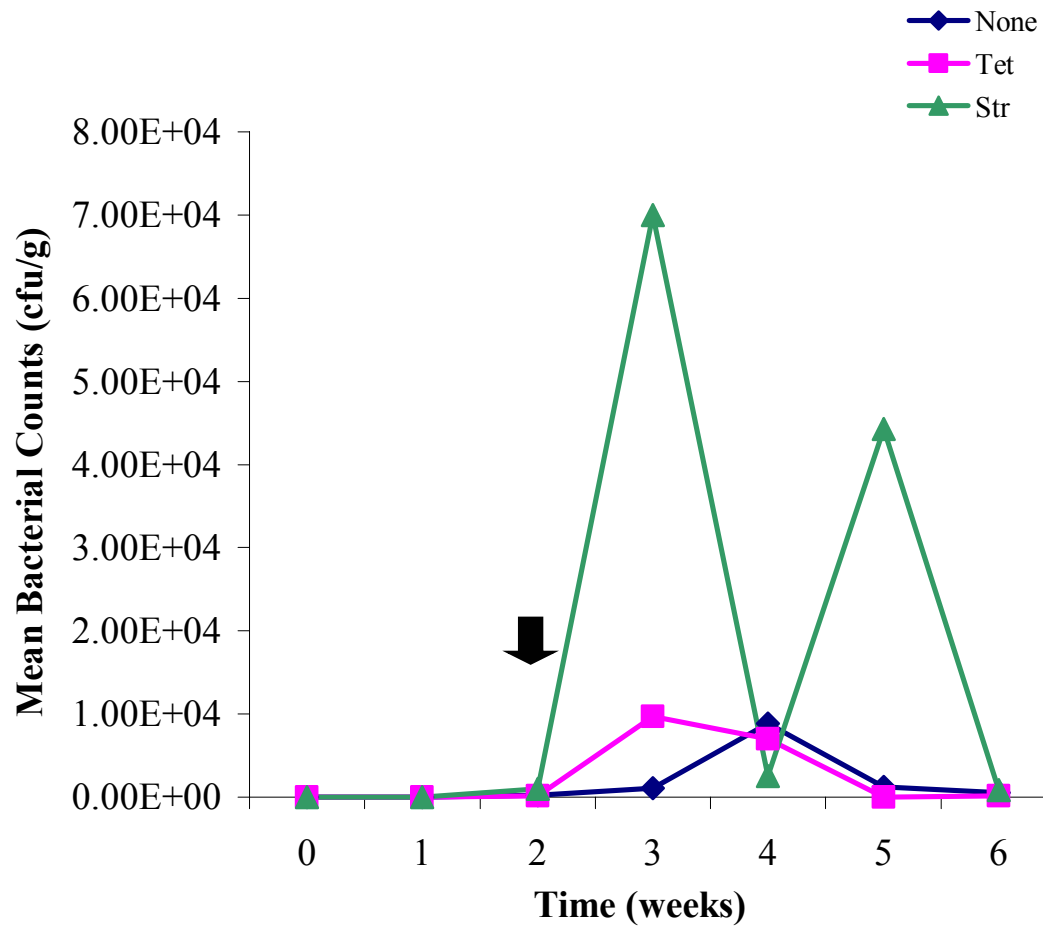


Figure 5. Level of *Salmonella* in commercial broiler chickens exposed to antibiotics chlortetracycline or streptomycin. Mean bacterial counts were obtained for salmonellae by plating samples onto MacConkey agar supplemented with naladixic acid and rifampicin. Identity of *Salmonella* was confirmed by whole cell agglutination with *Salmonella* group B-specific antisera. Birds were administered, orally, with 10^8 CFU salmonellae at day of hatch. Arrow indicates antibiotic treatment administered to broiler chickens.

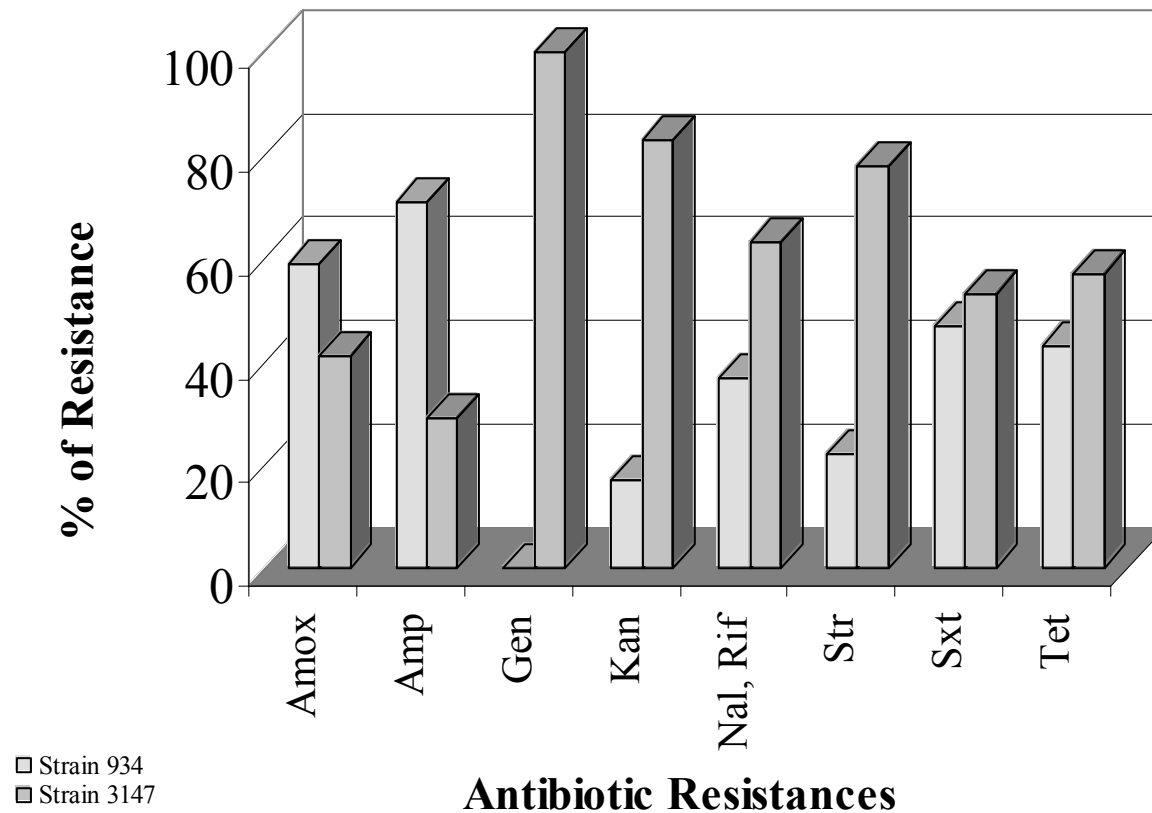


Figure 6. Antimicrobial resistances for *Salmonella* strains 934 and 3147 recovered from the poultry environment. Percentage of isolates (n= 40) bearing resistance phenotypes. Percent resistance was calculated as the number isolates with a particular resistance phenotype. *Salmonella* recipient strains inoculated into day of age broiler chicks were made resistant to nalidixic acid and rifampicin. Antibiotic abbreviations: Nal, Rif= nalidixic acid and rifampicin; Str = streptomycin; Sxt = sulfisoxazole; Amp = ampicillin; Amox = amoxicillin; Gen = gentamicin; Kan = kanamycin; and Tet = tetracycline.

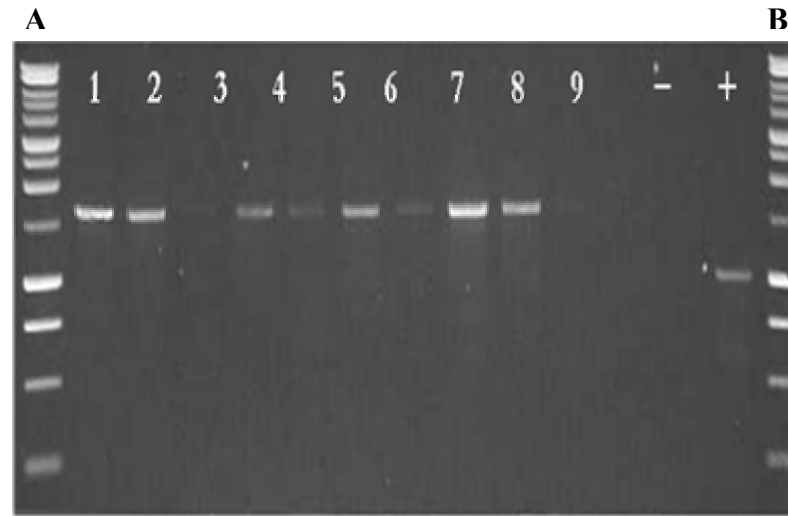


Figure 7. Class 1 integron gene cassette(s) of *S. enterica* Typhimurium strain 3147 recovered from poultry environment, as assessed by PCR. PCR using primers to sequences 5' and 3' of the *attC* integration site (59 base element) was used to amplify gene cassette(s) present with in the integron's integration site. Lanes A and B: 1kb ladder used as a molecular weight marker; lanes 1: integron-positive *Salmonella* 3147; *E. coli* K12 strain SK1592 (Tn21:: pDU202), positive control for 1.0 kb, *aadA1* gene cassette, denoted by (+); and *S. enterica* Typhimurium SR11, integron-negative PCR control, denoted by (-).

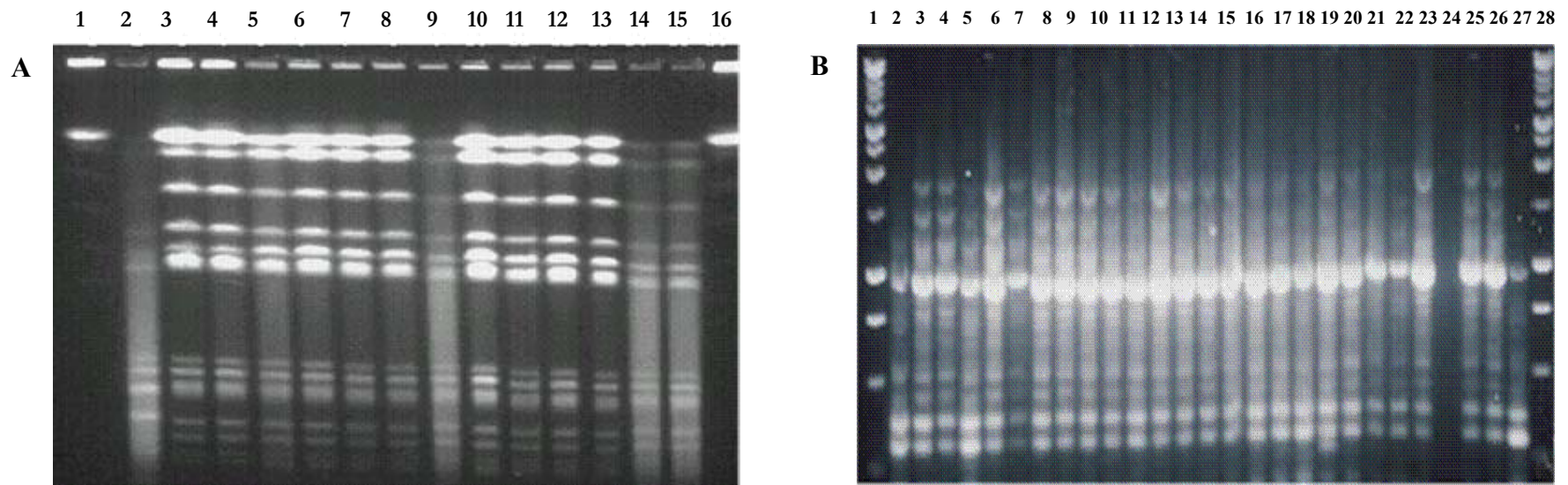


Figure 8. Identity of antibiotic resistant *S. enterica* Typhimurium recovered from the poultry environment as strains 934 and 3147 confirmed by PFGE (A) and RAPD (B). (A) Bacterial strains were typed using restriction enzyme, *Xba*I. Lanes 1 and 16: MW DNA standard, *S. cerevisiae* chromosomal DNA; lanes 3 and 4: *S. enterica* Typhimurium strains 934 and 3147, respectively; and lanes 5-15: *S. ser.* Typhimurium recovered from the poultry environment. (B) Isolates were typed by RAPD PCR. Lanes 1 and 28: MW standards, 1 kb DNA ladder; lanes 3-23: *S. ser.* Typhimurium recovered from poultry environment; lanes 25 and 26: *S. enterica* Typhimurium strains 934 and 3147, respectively; and lanes 2 and 27: *S. ser.* Typhimurium SR11, which served as quality control for assessing reproducibility of RAPD typing.

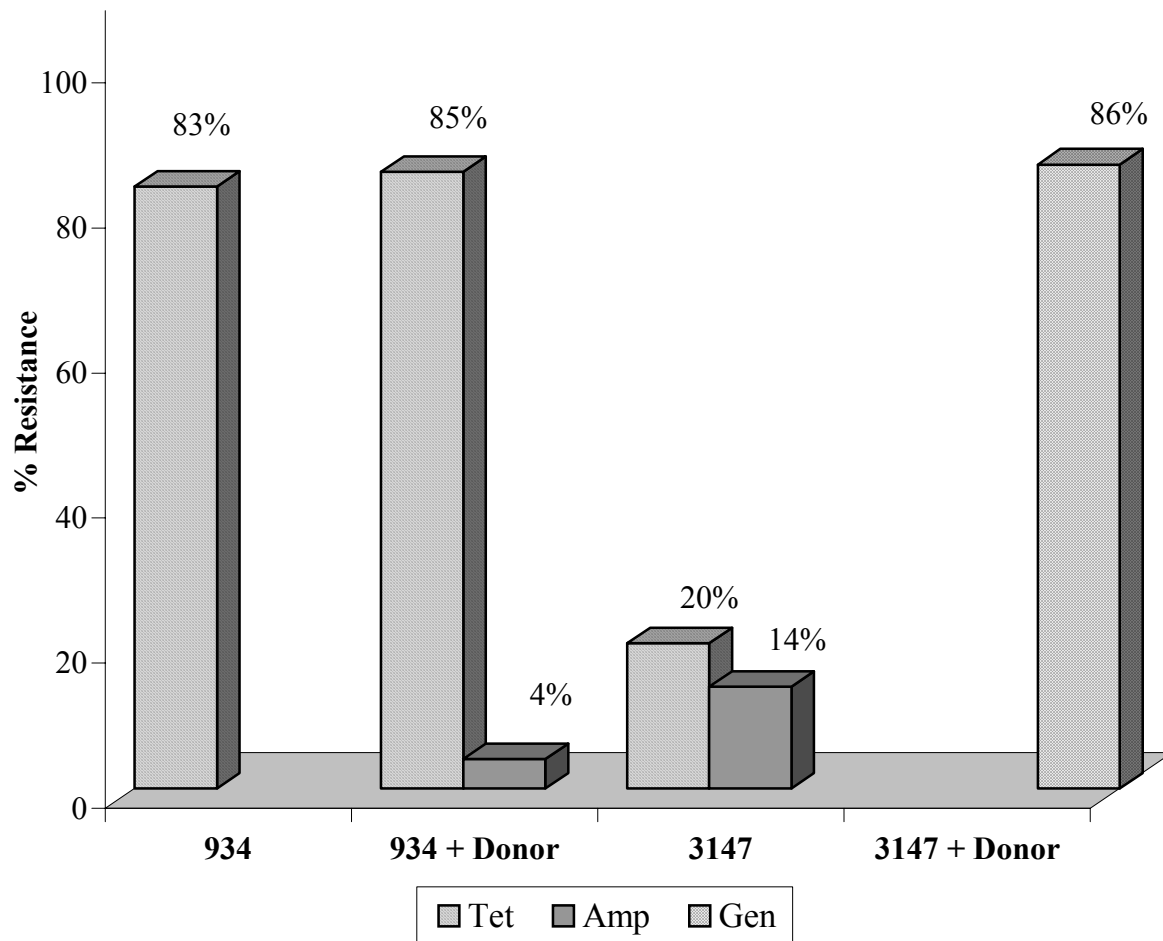


Figure 9. Percentage of antibiotic resistance of *Salmonella* recovered from broiler chickens as calculated from Most Probable Number (MPN).

Chapter 4

CONCLUSIONS

The emergence of antibiotic resistance among food- related pathogens is undoubtedly a food safety concern. The use of low- level antimicrobials in animal husbandry and agriculture are often blamed for the emergence of resistance among non-pathogenic and pathogenic organisms. One rationale for such an assumption is the rise in the incidence of organisms resistant to analogous veterinary drugs and the development of resistance to drugs most commonly used to treat human diseases.

Although antibiotics are used as both agricultural and veterinary therapeutics for the preservation of fruits, treatment and/or prevention of diseases, and for improved growth performance, there is mounting evidence that selective pressure in the form of antibiotic usage is not necessarily required for the emergence of antibiotic resistant strains of bacteria. Unfortunately, as we have evolved so have bacteria. Microorganisms have evolved complex mechanisms for avoiding just about every antibacterial compound that has been on the market. Some of these mechanisms may be inherent to a certain bacterial species, and others may have been acquired by point mutations or by horizontal gene transfer. The majority of antibiotic resistance genes are linked in multiple, tandem repeats with mobile genetic elements such as conjugative plasmids and transposons and integrons. These genetic elements have not only allowed bacteria to communicate (by conjugation, transformation, and transduction) within a bacterial genus but between distantly related genera.

Genetic elements like integrons represent important agents for evolution, not to mention the acquisition and dissemination of resistance genes. In order for resistance to be transferred from one organism to another, both bacterial cells- one being the donor and the other a recipient-

must be have physical cell-to-cell contact. There must also be a significant reservoir of antibiotic resistance genes among the commensal (donor) community, as demonstrated within the *Enterobacteriaceae* family. The evolution of antibiotic resistance is directed by several other key factors such as the level of antibiotic pressure, selection within the resident commensal microflora, persistence of both sensitive and resistant bacterial populations, horizontal transfer of resistance genes and lastly, the biological characteristics of bacteria, for instance biological fitness and mutational rate.