THE NATURAL TRANSMISSION OF *SALMONELLA* TYPHIMURIUM IN POULTRY WITH AND WITHOUT ANTIMICROBIAL SELECTIVE PRESSURE

by

JENNIFER ELIZABETH BAUER GARLAND

(Under the Direction of Mark Harrison)

ABSTRACT

Salmonella Typhimurium is an important pathogen of humans and animals. Recently, *Salmonella* strains have arisen that are resistant to multiple antimicrobials including third generation cephalosporins. It is unclear whether these multiple resistant isolates have a selective advantage for transmission between hosts and whether antimicrobial selective pressure adds to this advantage. This study was designed to investigate the transmissibility of a resistant strain and a sensitive strain of *S.* Typhimurium with and without antimicrobial selective pressure in a poultry model. The data from these studies indicated that multiple antimicrobial resistance in *Salmonella* isolates do lead to increased transmissibility under antimicrobial selective pressure, the sensitive *Salmonella* strain survived and was transmitted efficiently between animals even with antimicrobial selective pressure at MIC levels, and the sensitive *Salmonella* strain is capable of acquiring genes for resistance in as little as 7 days with or without exposure to antimicrobials.

INDEX WORDS: *Salmonella* Typhimurium, multiple antimicrobial resistance, transmission

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CHAPTER 1

Introduction

The challenge of reducing foodborne infections has been made more difficult with the increase in multi-drug resistant bacteria. Antibiotic resistance arises rapidly and spreads quickly throughout bacterial populations. Plasmids containing genes for resistance are perhaps the most dangerous method that bacteria can acquire antimicrobial resistance. It was believed that limiting the use of antimicrobials in livestock would decrease the development of antibiotic resistant bacteria. The biological cost of antibiotic resistance genes in the absence of antimicrobials was thought to give the sensitive strain the ability to out compete the resistant isolate. Unfortunately, the antibiotic resistant strains are often able to compensate for the biological cost by mutating while retaining their original resistance. The elimination of antibiotic resistant bacteria has shown to be a complicated task due to a bacterium's ability to gain resistance easily, while losing the resistance genes is more difficult (32).

In general, more research concerning the mechanisms of antibiotic resistance in bacteria is necessary to stop the spread of resistant genes in livestock (34). Pathogen spread or transmission, such as with *Salmonella* Typhimurium, is believed to be influenced by the acquisition of virulence genes and by antimicrobial selective pressure. Since *S.* Typhimurium can reside in the intestines of food producing animals, from there it can contaminate the food chain. Infections caused by *S.* Typhimurium are usually self-limiting diarrhea but rare cases involve infections of the blood, or systemic infection. Infections caused by multiple drug resistant *S.* Typhimurium increase mortality rate as well as increase systemic infections in patients (22). This phenomenon is believed to be

due to the increase in virulence of multi-drug resistant bacteria. To further understand the relationship of resistance and virulence, scientific research is necessary to understand the effect of virulence in antibiotic resistant pathogens with and without antimicrobial selective pressure. Research should also be conducted to see if new virulence genes are also acquired with resistance genes. Since little is known about the correlation of resistance and virulence, many assume the two are mutually occurring events. In addition, it is thought that antibiotic resistant pathogens have increased throughout an environment, thus, becoming more able to spread rapidly to animals. The rapid spread of antibiotic resistant bacteria is believed to be due to the increased virulence of the pathogen. To further understand the ability of a multi-drug resistant pathogen to spread through food producing animals, the transmission rate of antibiotic resistant strains compared to antimicrobial sensitive strains should be studied in an animal model.

In this study, broiler chickens were used to measure the spread of a multiple resistant and a sensitive *S.* Typhimurium. Since, 8 billion broiler chickens are sold per year and broiler chickens can contain *S.* Typhimurium within their gastrointestinal tract, the contamination of poultry with an antimicrobial resistant pathogen is an important consumer issue (6). It is believed that an antimicrobial resistant pathogen will colonize more of a flock than an antimicrobial sensitive pathogen when treated with an antimicrobial (32).

CHAPTER 2

Literature Review

Salmonella

Foodborne pathogens, *Salmonella, Escherichia coli* O157*,* and *Shigella,* are part of the Enterobacteriaceae family (34). Bacteria that belong to the Enterobacteriaceae family can cause self-limiting diarrhea or enter the blood stream causing systemic infections (34). Seventy percent of diarrheal diseases are caused by foodborne pathogens (27). Furthermore in the United States, there are 76 million cases of foodborne diseases per year, from which 325,000 hospitalizations and 5,000 deaths occur (27). Fifteen percent of these foodborne infections in the U.S. are due to contamination in food or water with *Salmonella* (18). *Salmonella* are gram-negative, facultative anaerobic, motile rods that are widespread in animals, including poultry and swine (34). Since, *Salmonella* live within the food producing animal it can easily contaminate the food chain. For example, recalls of foods containing pathogens have increased from 150 in 1988 to 315 in 2000 with a majority of these foods making it to the grocery stores before being recalled (27).

The ability of *Salmonella* contamination in food to cause infection within a human host is dependent on the serovars or serotype involved (34). *Salmonella* are grouped into two species, *Salmonella enterica* and *Salmonella bongori,* of which each contains several serotypes (34). *Salmonella* can be serotyped based on the capsular, flagellar, or envelope antigens by agglutination with antisera (34). *Salmonella enterica*

serotype Typhimurium (*S*. Typhimurium) is a common cause of self-limiting diarrhea (34). *S.* Typhimurium has a broad-host range and can cause disease in humans, cattle, pigs, horses, sheep, poultry, and rodents (34). In addition, *S.* Typhimurium is found in water, soil, insects, food plants, animal feces, and raw foods (3). Since the natural microflora of animal's intestinal tract can contain *S.* Typhimurium, controlling *Salmonella* in food-producing animals is of great concern due to the high rate of food contamination.

Multiple antimicrobial drug resistance has developed in a large percentage of *S.* Typhimurium making it more difficult to treat foodborne infections (12). Infections from antibiotic resistant bacteria are more likely to need medical treatment than infections from antimicrobial sensitive bacteria (42). *Salmonella* isolates from 8387 patients were sent to the Center for Disease Control and Prevention (CDC) to determine if the more virulent isolates were resistant or sensitive to antimicrobial agents (42). *S.* Typhimurium was among the most common recovered serotype (26%) (42). Also, isolates that were resistant to one or more antimicrobials were more likely to be recovered from the blood and patients with antibiotic resistant bacterial infections were more likely to have septicemia, infection of the bloodstream (42). In addition, hospitalization rates were found to be increased in patients that had an antibiotic resistant bacterial infection compared to a pansensitive isolate which are sensitive to all antibiotics (42). Resistance to extended-spectrum cephalosporins and fluoroquinolones are rare in *S.* Typhimurium, therefore they are the suggested antimicrobials for *S.* Typhimurium infections (1). However, children with systemic salmonellosis are given ceftriaxone intravenously because fluoroquinolones can cause cartilage damage (1, 36). Recently, *S.* Typhimurium

have acquired a CMY-2 gene which encodes for a AmpC like beta-lactamase providing resistance to the extended-spectrum cephalosporins (19).

Salmonellosis

Annually, an estimated 1.4 million people in the U.S. are affected by salmonellosis which is usually a self-limiting diarrhea (24). Salmonellosis may also cause serious systemic infection that can infect many organs and require antibiotic treatment (3). The pathology of infections caused by *Salmonella* depends on the host age and health, but 15-20 cells ingested with food can be enough to cause infection (3). Infection dosage is higher, 10^6-10^9, if not accompanied by the ingestion of food because food protects S*almonella* from the stomach acid allowing it to reach the epithelial cells of the intestines (34). Salmonellosis can occur due to improper cooking or handling of contaminated food in foodservice industries or in the home (34). Symptoms, including nausea, vomiting, diarrhea, abdominal pain, and sometimes fever, usually start 24 to 48 hours after ingestion of contaminated food or water and typically last for 2 to 7 days (34). Severity of infection varies according to the individual's age and immune system. The young, old, and immunocompromised are susceptible to severe infection and may experience more severe complications due to dehydration (18). *Salmonella* onset is as rapid as its disappearance from the intestinal tract with the exception of about 5% of the population who become carriers (34).

Pathogenesis and Virulence

Salmonellosis usually begins after the ingestion of 10^5 cells from contaminated food (34). *S.* Typhimurium is able to adjust it's pH level to survive the acidic environment by pumping the protons out of the cell allowing it to survive at pHs as low

as 3 (34). After the *S.* Typhimurium survives the acidic stomach, it colonizes the epithelial cells of the intestines, invades the M cells, and replicates within the macrophages (34). Receptors on the host cell's ganglioside recognize the *S.* Typhimurium allowing attachment to the host cell (34). Once the host cell recognizes the *S.* Typhimurium, a ruffling effect develops on the host cell surface resulting in the engulfment of the bacteria (34). Contact between the host cell and bacteria cause the host to recruit poly-morphonuclear (PMN) lymphocytes (34). Inflammation is caused by the release of prostaglandins by the PMN (34). The release of prostaglandins increases the chloride secretion and inhibits the uptake of sodium by infected cells thus producing diarrhea (34). Some *S.* Typhimurium infections enter the blood stream causing septic shock (34). These *Salmonella* are able to invade the host macrophages and survive while protecting themselves from the bactericidal compounds in the macrophage by secreting effector proteins which prevent phogosome-lysozome fusion inside the macrophage (34).

The invasiveness of a *Salmonella* strain is determined by the virulence genes the bacteria contains (34). *S.* Typhimurium has several genes for adherence and invasion (34). The different types of adhesion help the bacteria bind to the microvilli of the absorptive cells lining the lumen of the small intestines (34). Some of the genes for adhesion are located on a virulence plasmid (pSLT) that is found in all virulent strains of *S.* Typhimurium (34). Almost 90% of *S.* Typhimurium carries the *Salmonella* pSLT plasmid (34). The plasmids are 50 to 100 kb in size and contain genes that allow the bacteria to survive in macrophages and cross the mucosa (34). The pSLT plasmid is often a self-transmissible plasmid which may explain why most strains of *S.* Typhimurium possess this plasmid (34).

Most *Salmonella* also have pathogenicity islands 1 and 2 (SPI1 and SPI2), containing 42 of the 60 known virulence genes found in *S.* Typhimurium (34). The initial colonization of the gut by *Salmonella* is accomplished by the SPI1 genes (34). The *inv* gene, located on the SPI1, is responsible for membrane ruffling effect (34). The *inv* gene along with other SPI1 genes help to a make up a type III secretion system which injects proteins into host cells inducing phagocytosis and diarrhea (34). The type III secretion system causes the rearrangement of the host cell surface engulfing the *Salmonella* bacterium into non-phagocytic cells (34). Unlike SPI1 which is needed for the survival in the host immune system, SPI2 is necessary for the growth of the bacteria within the host cell (4). SPI2's type III secretion system aids the *Salmonella* bacterium in the prevention of the phagosome lysosme fusion within the macrophage (34).

S. Typhimurium can also acquire virulence genes by conjugation, transduction or transformation with genes from other bacteria. Transfer of DNA via bacteriophage transduction or transformation, the uptake of DNA from the environment, usually occurs within the same bacterial species due to the necessity of specific receptors on the recipient bacteria's surface (34). Transduction or transformation involves a narrow range of bacteria and antibiotic resistance is not spread rapidly through an environment with these methods. However, the transfer of genes via conjugation can spread genes for virulence as well as antibiotic resistance to members of different bacterial species (34). Conjugation, the transfer of DNA directly from one bacterial cell to another, can be mediated by conjugative plasmids or by conjugative transposons (34). Plasmids capable of transferring themselves contain genes necessary for conjugation and are referred to as self-transmissible plasmids (34). Plasmids such as *S.* Typhimurium virulence plasmid

contain genes that confer virulence as well as antibiotic resistance (34). Conjugative transposon, on the other hand, are located within the bacterial chromosome and can excise themselves and transfer to another bacteria (34). Antibiotic resistance via conjugative transposons most likely accounts for just as much resistance as conjugative plasmids (34). Interestingly, the conjugative plasmid of *S.* Typhimurium has also been found to contain class 1 integrons that encode multiple antimicrobial resistance (39). Integrons are similar to transposons except that they contain an integrase gene. Intregrase genes can insert DNA fragments into sites of a recipient bacteria's chromosome and can acquire sequential integrons (34). Thus, plasmids, transposons and integrons are important in the transmission of multiple antimicrobial resistances in *Salmonella.*

Epidemiology

Salmonella is one of the most reported foodborne illness in the United States (24). The reported cases could be even higher but confirmation of *Salmonella* infection is difficult because most people are unaware that they have a foodborne infection. A specimen is not typically obtained by the health care provider, or proper test are not preformed in the laboratory (29). Thus, of the 1.4 million cases of salmonellosis in 2003, only 33,589 were confirmed and reported to the CDC (29). Based on the 2003 census population in the U.S., 11.6 people per 100,000 reported a *Salmonella* infection (29). Twenty-seven percent of the 33,589 people infected with *Salmonella* were hospitalized and 34 (0.6%) people died from the infection (24).

Confirmed human *Salmonella* infections are reported to the National *Salmonella* Surveillance System which obtains the data from states and public health laboratories (29). The CDC further analyzes isolates that are abnormal for additional verification

(29). Also, animal and environmental sources of *Salmonella* are reported from the U.S. Department of Agriculture (USDA) and Food Safety and Inspection Service (FSIS) (29). According to the National *Salmonella* Surveillance System, *S.* Typhimurium is the most common serotype reported from human sources in the 2003 annual summary (29). Furthermore, chickens are the most frequent non-clinical source of *S.* Typhimurium (29).

Reports from the National Antimicrobial Resistance Monitoring System (NARMS), a collaboration of CDC, FDA, and USDA, monitor antimicrobial resistance in humans and animals to study the trend of resistance in bacteria (29). A large percentage of *S.* Typhimurium were reported to be resistant to multiple antimicrobials (29). Resistance to 1 or more antimicrobials was found in 21% of the isolates while resistance to 5 antimicrobials was found in 30% of the *S.* Typhimurium isolates (29). According to NARMS in 2002 4% of *Salmonella* isolates were resistant to ceftiofur, the extended-spectrum cephalosporin used in animals, while 4% had decreased susceptibility to ceftriaxone, the extended-spectrum cephalosporin used in humans and 0.2% were resistant to ceftriaxone (40).

Transmission

Humans and animals, including birds, are carriers of *Salmonella* and can occasionally become shedders (18). Shedders do not completely rid themselves of the bacterium but instead the bacteria can reside within the intestines where it is released through the host feces. Once the animal is slaughtered, the bacteria from the intestine can contaminate the carcass, gaining access into the food chain (37). S*almonella* is transmitted by the oral-fecal route and is can be found in food and water contaminated with animal feces (18). In a study of retail ground beef, resistant strains of *Salmonella*

were found to be a common occurrence (44). Twenty percent of the 200 ground beef samples collected had *Salmonella* contamination (44). Of the positive *Salmonella* isolates, resistance to at least 1 antibiotic was found in 84% and 53% were resistant to more than 3 antibiotics (44). Generally it is also believed that animal feed contaminated with *Salmonella* can infect animals (15). In one study, the factory that produced poultry meal was determined to be the primary source of *Salmonella* contamination (15).

Poultry houses contain up to 22,000 birds in close contact, increasing the spread of pathogens (5). Poultry that carry *Salmonella* can shed the bacteria through feces while experiencing no symptoms through out their lifetime (34, 36). In general, the chicken and the *Salmonella* bacterium have a commensal relationship since the bacterium does not harm the chicken and the chicken does not rid itself of the bacteria (36). In birds positive for *Salmonella, t*he ceca which is part of the gastrointestinal tract, usually contains the *Salmonella* bacterium (34). Infected birds shed *Salmonella* for weeks to months, thus continuously contaminating the environment (36). Once in the environment, *Salmonella* can survive there for up to 13 months unless the area is properly cleaned (36).

Use of Antibiotics

The transmission of *S.* Typhimurium is further complicated by the development of resistance to antimicrobials. It is not known if there is a link between the resistance of a bacteria and its ability to spread to other animals. Antibiotic resistant *Salmonella,* however, are believed to have a selective advantage over sensitive strains. Since the antimicrobial sensitive strain is killed or inhibited by antimicrobial use, the resistant bacteria can out compete and spread to the areas that once contained the sensitive

isolates. The increase frequency of antibiotic resistant bacteria within the animal could multiply the amount of resistant bacteria that shed into the environment, thus, possibly amplifying the spread to other animals. To further understand the complications due to antibiotic resistance, the cause of resistance needs to be investigated.

One example of the use of antibiotics in animals and the development of resistance in humans includes the use of avoparcin in Europe (33). Bacteria resistant to vancomycin are rare in animals in the U.S. where the use of avoparcin, an analog to vancomycin, is banned in animals (33). However, in Europe where avoparcin was at one time used as a growth promoter, the development of resistance to vancomycin began to appear in the bacteria found in the intestines of Europeans (33). Soon after this discovery the use of avoparcin in animals in Europe was banned (33). This pattern of development of resistance and the use of antimicrobials in livestock can be seen in *S.* Typhimurium which resides in livestock. Consequently, antimicrobial use in livestock production is believed to increase the resistance to antibiotics in *S.* Typhimurium. Since the 1950s half of the antibiotics used in agriculture are used for animal growth promotion at subtherapeutic levels (26). Antibiotics used at subtherapeutic levels are considered too low to dramatically effect the growth of the bacteria, but enough to allow the bacteria to accumulate mutations or acquire new DNA for resistances (32). Antibiotics used in livestock at levels designed to kill the bacteria, or therapeutic levels, are believed to be better for the prevention of antibiotic resistance (26). Subtherapeutic levels used in agriculture are believed to increase the resistance to antibiotics in humans making it more difficult to treat human illnesses (26).

It is still unclear what mechanisms are involved in promotion of animal growth by antimicrobial use. Some studies show that bacteria residing in the natural microflora of the animal's stomach compete with the animal for energy by metabolizing the carbohydrates in the animal's diet (17). It is also believed that the animal will have more surface area available to absorb nutrients once the bacteria residing in the intestines are reduced by antimicrobial treatment (17). Furthermore, adhesion and the production of toxin by bacteria causes the gut wall in chickens to thicken which can be prevented with some antibiotics (21). Therefore, the use of antibiotics in animal feed is believed to save energy by preventing the bacterial metabolism of carbohydrates thus improving profits and decreasing the consumer costs for food (21).

Another issue surrounding antibiotic use in animals is that the acute illness by one bird means treatment for all birds that may have come into contact with the sick bird (36). Animals with diseases like pneumonia or gastroenteritis require therapeutic treatment, either as individuals or as a whole group. Treatment is usually supplied in the animal's feed or water leading to possible over or under dosing with the antibiotic (36). Antibiotics, such as cephalosporin, used in livestock are identical to or related to drugs used in humans (38). Treatment of foodborne infections in humans can become difficult when the pathogen is resistant to the drug used to treat the patient (38). Forty percent of antibiotics used in the United States are reported to be used for veterinary purposes and three fourths of those are estimated to be used for nontherapeutic use in animal feed (36). Europe has banned the use of some antibiotic growth promoters and has proposed to ban the rest by the year 2006 (31). Although the Scientific Committee on Animal Nutrition (SCAN) in Europe took precautionary steps despite having little scientific evidence to

show the transfer of antibiotic resistant bacteria in animals to humans (20). After the elimination of some antibiotic growth promoters, there was a decrease in enterococci resistance to antimicrobials in animals and humans (20). However, there was also a decrease in animal health which caused an increase in veterinary use of therapeutic levels of antibiotics (20). As discussed above, although therapeutic levels are considered better for the prevention of antibiotic resistance than subtherapeutic levels, the administration of the antibiotic can still lead to the development of resistance in bacteria.

The increase in bacterial resistance is also due to the human use of antimicrobials. Inappropriate use of antibiotics for non-bacterial illnesses and not completing the prescribed antibiotic regimen for the duration of the infection both contribute to the increase in bacterial resistance (28). An example of abuse of antibiotics is the unnecessary use for non-bacterial infections such as virus. This is due to an empirical diagnosis and lack of proper clinical analysis of infections (28). Improper education of the public also leads to misuse and abuse of antibiotics. The general public fails to take the entire prescription which leaves some microbes alive with subtherapeutic levels of the drug in the system of the patient as the drug clears the system. Within six years, the abuse of antibiotics increased 21% within patients treated in hospitals (28). The need for guidelines to decrease the use of antibiotic abuse is expressed by many including CDC's *Get Smart: Know when Antibiotics Work* program (28).

Companion animals can also cause the development of antimicrobial resistance since they too require the use of antibiotics for infections. A study concerning cats and their risk for transmission of antimicrobial resistance found that the cats shed resistant strains of *S.* Typhimurium (41). Healthy cats are not a threat while sick cats receiving treatment

did shed resistant strains of *S.* Typhimurium that could become a potential hazard (41). Additionally, a study of the prevalence of antimicrobial resistance in pets that included mammals, reptiles, fish and birds, found resistance in all animal types (35). *Salmonella* isolation was low in these animals but resistance to streptomycin was found in 83.3% isolates from birds and 100% from the other animals, while 50% of the isolates from fish were resistant to cephalothin (35). Even though the transmission of antibiotic resistant pathogens from the pet to the owner is minimum due to the low amount of *Salmonella,* it is a reported occurrence (35). However, the transmission of antibiotic resistant pathogens from poultry products to humans is reported more often since the contamination of poultry product usually supplies higher doses that are necessary for infection (6).

Poultry

There is approximately 358 poultry hatcheries in the U.S. which produce 8 billion broiler chickens a year (6). The poultry industry places broiler chickens in separate housing facilities based on their age and species to prevent the spread of pathogens (6). Approximately, 9 million cases of foodborne illness occur each year in the U.S. of which 8% might be due to contaminated poultry products (6). Infections caused by *Salmonella* in poultry are dependent on the poultry age, the poultry stress level, serovar, and bacterial strain virulence (6). *Salmonella* has many serotypes with a few that can cause disease in poultry (6). *S. pullorum,* pullorum disease, and *S. gallinarum,* fowl typhoid, are both able to cause disease and even death in poultry (6).

Treatment for these diseases involve antibiotics given to poultry supplied in the feed, drinking water, or as an injection (6). Normally a bird that is sick will not eat, but it will continue to drink water, thus drugs placed in the water is the best method for treatment of

infection (6). Antimicrobial treatment in water can have varied dosage rates within birds depending on environmentally factors that may lead to increased or decreased drinking (6). It is also important to understand how the antimicrobials behave in the body of the chicken (6). For example, the antimicrobial tetracycline is absorbed from the intestines and then distributed throughout the body of the chicken (6). Some drugs pass through the intestines into feces while others are absorbed into the bloodstream (6). Thus the recommended dose is determined by manufacturers to ensure proper administration of the antibiotic within the chickens (6). Dosage level is also determined so that the maximum level is reached for which the antibiotic is active within the bloodstream or target organ (6). There is also a withdrawal period for antimicrobials to prevent the deposit of drugs in the muscle or eggs of the chicken which can be harmful to humans (6). Furthermore, the age of a chicken is important in the colonization of bacteria (6). Young chicks are not able to fight off invading bacteria as well as the older chickens that have well developed immune system (6). However, as the bird gets older the immune system begins to diminish (6).

Acquisition of resistance in *Salmonella*

Discovery of antimicrobials is quickly followed by the development of antibiotic resistance in bacteria. For example, *Esherichia coli* became resistant to penicillin while the original production of the drug was still under way (33). There are many ways the pathogen *Salmonella* can develop resistance to antimicrobials. As discussed above for virulence genes, antimicrobial resistance genes can be acquired, via horizontal gene transfer (32). Horizontal gene transfer occurs by transfer of plasmids and by conjugative transposons which can transfer to the chromosome or integrate into plasmids in the

recipient bacteria (32). Plasmids and conjugative transposons can be acquired easily by other bacteria thus increasing the spread of antimicrobial resistance (32). A study of the *in vivo* acquisition of ceftriaxone resistance in *Salmonella,* found horizontal gene transfer occurs even in the presence of antimicrobials that are supposed to kill the bacteria (37). It was shown that sensitive strains could develop antimicrobial resistance within hours through the conjugation of plasmids from resistant strains (37).

Bacteria that become resistant in the antibiotic treated animals can be transfered to humans through contaminated food (21, 37). Resistance to extended-spectrum ß-lactams in humans has no known source but is believed to be acquired from contaminated food (11). In this study, patients with extended-spectrum resistant bacteria acquired the resistant bacteria while in the United States (11). Previous infections from extended-spectrum resistant bacteria were acquired outside of the U.S. (11). In one case, a child became infected with the same ceftriaxone-resistant strain that infected calves on his father's farm (13). Based on similar susceptibility test and pulsed-field gel electrophoresis (PFGE) the child became infected with the same *Salmonella* that were infecting his father's cattle. Furthermore, the isolates from the child and cattle encoded an AmpC-like ß-lactamase that conferred resistance to extended-cephalosporin, Ceftriaxone (13). Thus, the transmission of antibiotic resistant strains of *S.* Typhimurium from livestock to humans is a reported threat.

Cephalosporins resistance

ß-lactam antibiotics are used to inhibit the synthesis of bacterial cell walls causing cell death. Bacteria can protect themselves by producing ß-lactamase which hydrolyzes the ß-lactam ring causing the antibiotic to be inactivated (34). ß-lactamase produced by

the bacteria are very specific to certain ß-lactams that they are active against, thus, new ß-lactam drugs are produced that are structurally different and resistant to hydrolysis by some ß-lactamses (34). Soon after the development of new ß-lactams, new ß-lactamases are produced by the bacteria (34). For example, cephalosporins were created in 1980 to have the same actions as other ß-lactams, but they were not at that time affected by ß-lactamases (30). Bacteria soon became resistant to extended-spectrum cephalosporins by producing large amounts of their chromosomal AmpC ß-lactamase (30). *Salmonella* that did not have the chromosomal AmpC ß-lactamase, acquired resistance mediated through a plasmid from other bacterial species (45). ß-lactamase inhibitors, such as clavulanic acid, can be used with ß-lactamases to prevent the ß-lactamase from inactivating the antimicrobial (34). Unfortunately, extended-spectrum ß-lactamases are resistant to both clavulanic acid and extended-spectrum cephalosporins (1).

In the U.S., resistance to extended-spectrum cephalosporins is linked to a plasmid mediated AmpC-like ß-lactamase that is encoded by the bla_{cmy} gene, CMY-2 gene (9). CMY-2 is located on a transferable plasmid with multiple drug resistance and is the most common method that *Salmonella* acquires resistance to extended-spectrum cephalosporins (19). Gray *et. al.*, found extended spectrum cephalosporin resistance spread throughout the environment in low levels suggesting that it was an up-and-coming problem (9, 19). The CMY-2 AmpC-like ß-lactamase is similar to the CMY gene found in *Citrobacter* (45). This strong similarity further illustrates the ability of genes for resistance to transfer between bacterial species (45).

Salmonella isolates positive with a CMY gene are resistant to tetracycline (9). Tetracycline resistance in *Salmonella* isolates is common with 41.3% of the animal origin

Salmonella isolates resistant to tetracycline in 2003 (12). Bacteria resistant to tetracycline have been found frequently in the gastrointestinal tract of humans and animals (10). Genes for antibiotic resistance can be genetically linked to other genes that confer resistance to different antibiotics such as the link between tetracycline and extended spectrum cephalosporin (2). Tetracyclines, such as chlortetracycline, belong to a family of broad-spectrum antibiotics that inhibit protein synthesis in gram-negative and gram-positive bacteria (10, 34). Protein synthesis is inhibited in gram-positive and gram-negative bacteria by preventing the binding of the aminoacyl-tRNA molecules to the 30S ribosomal subunit by tetracyclines (10). Resistance to tetracyclines is found in both gram negative and gram-positive bacteria which are capable of transferring resistance to each other. Tetracyclines are used in both human and veterinary medicine, and their effectiveness has been limited due to microbial resistance. Tetracycline is also used at subtherapeutic levels in animal feed to act as a growth promoter (10). In poultry, chlortetracycline is added in various low quantities to large amounts of feed (6).

Concerns have arisen over use of antibiotics in animal feed which is shown to contribute to the increase of resistant bacteria in animals treated with the antibiotics. Emergence of resistance in naturally occurring environmental bacteria pre-and-post-tetracycline use by humans has shown that resistance is a relatively new incident (10). Protection of ribosomes by large cytoplasmic proteins and efflux pumps provide the mechanisms for bacterial resistance to tetracycline (10). Levy *et. al.* fed tetracycline supplemented feed to chickens and found that resistance developed in their intestinal microflora within one week (23). Furthermore, plasmids with multiple antibiotic resistance were identified in the resistant bacteria that were found to be resistant to

tetracycline, ampicilllin, streptomycin, and carbenicillin (23). Chickens not treated with tetracycline also excreted resistant organisms though at lower levels than the treated groups (23).

Effects of Antibiotics on Bacterial Virulence

In 1980, 13% of *S.* Typhimurium were resistant to one or more antibiotics; this percentage increased to 51% in 2001 (14, 19). *S.* Typhimurium resistant to one or more antibiotics is more likely to occur in the blood stream and the individual will need antibiotic treatment (14). Sepsis, endocarditis, meningitis, and even death are a result of infection of the blood with *S.* Typhimurium (14, 19). Increased pathogenesis of *S.* Typhimurium may be due to the acquisition of virulent genes along with the resistant genes (43). Since virulence genes and resistance genes are both found on plasmids, bacteria are able to acquire new genetic material that can increase the pathogenicity of the organisms as well as the resistance to antimicrobials.

Antibiotic resistance confers a selective advantage to resistant bacteria; however, it is not clear if resistant bacteria are more pathogenic. Previous work by Carlson *et. al.* found that multiple resistant *S*. Typhimurium phagetype DT104 did not have an increased ability to enter tissue culture cells (8). Non-resistant *S*. Typhimurium DT104 was found in some cases to be more invasive than multi-drug resistant *S*. Typhimurium DT104 (8). Further analysis of *S.* Typhimurium DT104 was performed with adherence assays which looked at the ability of the bacteria to adhere to the tissue culture cells (8). The adherence to tissue cells was not different in antibiotic resistant strains compared to sensitive strains and was determined not to be the cause of the impaired invasion of the multi-drug resistant bacteria (8). It was determined that the multi-drug resistant *S.*

Typhimurium DT104 may be less invasive due to the integron for the DT104 phenotype interrupting proteins for expression of certain invasion properties (8).

However, the strains which produced the AmpC B-lactamase formed larger cells, diplobacilli, and filaments (25). Epithelial cells from canine kidney were used to determine the invasiveness of the *S.* Typhimurium that contained the CMY gene (25). Bacteria that produced AmpC ß-lactamase had flattened and rough colonies compared to bacteria that did not have the AmpC gene (25). The *S.* Typhimurium with the AmpC gene also produced cells that were larger or diplobacili which is caused by the lack of segregation of the daughter cells (25). Therefore, it was discovered that the overproduction of the CMY-2 gene had a biological cost on the bacteria which decreased the cell growth and its ability to invade cells (25). Without antibiotic selective pressure, antibiotic resistant bacteria are considered to be less fit than the antibiotic sensitive strain as shown in the previous study (25). Thus, the removal of antibiotics in livestock production is believed to decrease the frequency of antibiotic resistant bacteria occurrences. However, Bjorkman and Anderson found that decreasing the use of antibiotics did not deter resistant bacteria because the resistant bacteria were able to evolve without the loss of their resistance (7). In most cases the mutations in the antibiotic resistant bacteria increased their fitness level compared to that of the original strain *in vitro* and in animal models (7). Furthermore, resistance is easy for a bacteria to obtain from different species and genera and is difficult for the bacteria to lose (16).

Currently, it is known that patients with infections from multi-drug resistant pathogens have increased visits to hospitals, prolonged hospital stays, and require antibiotic treatment, especially extended-spectrum cephalosporins. It is believed that the

use of antibiotics for infections from antimicrobial resistant pathogens increases the virulence of the pathogen, thus causing a more severe infection. At this time, there are no studies of the multiple drug resistant strains colonization rate in a flock with and without antimicrobial selective pressure. Due to the current rise in multi-drug resistant *S.* Typhimurium it is believed that these strains are more virulent and thus more capable of colonizing a flock. It is not known if the increase in antibiotic resistant bacteria is due to the use of antimicrobials in the livestock industry or due to other selective pressures. In the current effort of controlling the increase in antibiotic resistant pathogens, selective pressure used in agriculture is studied as a major source of multi-drug resistant pathogens.

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CHAPTER 3

NATURAL TRANSMISSION OF *SALMONELLA* TYPHIMURIUM IN POULTRY WITH AND WITHOUT ANTIMICROBIAL SELECTIVE PRESSURE: STUDY 1[1]

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Abstract: *Salmonella* Typhimurium is an economically important pathogen of humans and animals. Recently, *Salmonella* strains have arisen that are resistant to multiple antimicrobials including third generation cephalosporins. It is unclear whether these multiple resistant strains have a selective advantage for transmission between hosts under antimicrobial selective pressure. This experiment was designed to study the transmissibility of a resistant strain (8381r) and a sensitive strain (8382s) of *S.* Typhimurium with and without antimicrobial selective pressure, in a poultry model. The multiple antibiotic resistant phenotype of *S.* Typhimurium 8381r is resistant to 12 of 17 antimicrobials on the NARMS panel. Strain 8382s was pansensitive to all antimicrobials on the NARMS panel. One bird in each pen of ten was inoculated with 10^9 CFU of *S.* Typhimurium 8381r or 8382s per *os*. Treatment groups for each isolate included no antimicrobials and 120 µg/ml chlortetracycline (tet), constant dose in water for a total of 4 treatment groups. Three replicates of each treatment were run. Cloacal swab and litter samples were collected on day (D) 1, 3, 5, and 7, and birds were necropsied on day 10 post inoculation. Upon necropsy percent positive tissue samples for strain 8381r were ileum-cecum-junction (ICJ) (4%), colon (0%), cecum (15%) for tet treated and no *Salmonella* was recovered from the ICJ, colon, or cecum for non-treated birds. Strain 8382s positive tissues were ICJ (30%), colon (15%), cecum (56%) for tet treated and ICJ (48%), colon (87%), cecum (100%) for non treated birds. This data indicates that multiple antimicrobial resistance in *Salmonella* isolates does not necessarily lead to increased transmissibility regardless of antimicrobial selective pressure. Additionally, a sensitive *Salmonella* strain can survive and transmit efficiently between animals even with antimicrobial selective pressure at minimum inhibitor concentrations (MIC) levels.

Introduction

Salmonella infections continue to be an important health concern in both developed and developing countries. Diseases caused by multiple antibiotic resistant *Salmonella* can have a poor response to treatment and are theorized to be more virulent than sensitive isolates (18). This is especially evident in *Salmonella enterica* ser. Typhimurium which is one of the most common foodborne pathogens. Although the majority of *S.* Typhimurium infections cause self-limiting diarrheal illnesses, severe and even life-threatening infections can occur. Resistance to extended-spectrum cephalosporins and flouroquinolones are rare in *S.* Typhimurium, therefore these drugs are the suggested antimicrobials for treatment of *S.* Typhimurium infections. However, children with systemic salmonellosis are given ceftriaxone intravenously because fluoroquinolones can cause cartilage damage (2, 15). Recently, a proportion of *S.* Typhimurium strains have acquired resistance to third-generation cephalosporins (17). In *Salmonella* from North America, resistance to third generation cephalosporins is linked to a plasmid encoded AmpC-like ß-lactamase that hydrolyzes cephalosporins (7). The bla_{CMY} gene which confers this resistance to extended-spectrum cephalosporins encodes a cephalomycinase, CMY-2 (7). It has been shown, that cephalosporin resistant bacteria can be found in low numbers, throughout a range of animal host species, suggesting this may be a developing problem (7). It was also shown in this study that 100% of the CMY-2 positive isolates were also resistant to tetracycline (7). Tetracycline resistance in *Salmonella* isolates is common with 41.3% of animal origin *Salmonella* strains found to be resistant to tetracycline in 2003 (6). The development of antibiotic resistance has limited the effectiveness of tetracycline in both human and veterinary medicine. The

tetracycline group includes antibiotics such as chlortetracycline, oxytetracycline, demeclocycline, and doxycycline (13). Chlortetracycline is a common antimicrobial used in poultry production, both therapeutically and for growth promotion and is believed to increase the development of antibiotic resistance (5).

Antibiotics are used in the livestock industry for treatment of animal diseases and for animal growth promotion (8). Therapeutic doses of antibiotics are necessary to kill or inhibit bacteria and are considered to be less of a threat for the development of resistance compared to subtherapeutic levels that are often used for growth promotion. In order to affect the bacteria at the site of infection, antimicrobials need to reach therapeutic levels at that site (2). Subtherapeutic levels are considered too low to kill the bacteria but may be present in sufficient levels so that the bacteria can develop resistance (12). Antimicrobials for poultry can be supplied in the drinking water, food, or given as an injection at therapeutic levels to treat infections (2). Antibiotics are supplied at subtherapeutic levels for long periods of time in poultry for growth promotion (2). Since subtherapeutic levels can contribute to bacterial resistances, antimicrobial resistance emergence has been associated with the use of antimicrobial agents in livestock, including chickens (2).

Antibiotic resistant *S.* Typhimurium is thought to have a competitive advantage when in the presence of selective agents, such as antibiotics, in comparison to sensitive bacteria. Also, the maintenance of antimicrobial resistant genes without antimicrobial selective pressure is believed to be too much of a biological cost on the *S.* Typhimurium (11). It is thought that decreasing the use of antibiotics in animals will help to eliminate the spread of antimicrobial resistant organisms. However, it has been found in previous

studies that the prevalence of antibiotic resistant bacteria was not altered by decreasing the use of antibiotics and the spread of resistant bacteria was not affected (3, 9). Thus, it is not clear if the use of antibiotics in animals increases the spread of antimicrobial resistant bacteria.

To explore the relationship between *S.* Typhimurium antimicrobial resistance and competitive advantage under common antimicrobial selective pressure, the natural transmission of *S.* Typhimurium in poultry was determined with and without antimicrobial selective pressure. Two transmission studies were conducted for *S.* Typhimurium in poultry. A pansensitive, sensitive to all antimicrobials, *S.* Typhimurium and a multidrug resistant *S.* Typhimurium were examined independently for their ability to colonize and spread in a broiler flock, with and without chlortetracycline selective pressure.

Materials and Methods

Experimental Design. For these experiments, 120 one day old chickens were obtained from a local hatchery and randomly separated into four biosecure rooms with 3 pens in each room. Pens were separated by solid panels creating a 5 x 6 area that prevented any litter or birds from crossing into other pens. Each pen had separate feed, water, and heat supplied. Birds were permanently marked with color for future record keeping and to distinguish the inoculated seeder chicks from the naïve chicks.

Bacterial Strains and Challenge Culture. Two strains of *S.* Typhimurium were obtained from the United States Agriculture Department (USDA) Bacterial epidemiology antimicrobial resistance (BEAR) research unit where they were used for antimicrobial susceptibility testing used in the national antimicrobial resistance research monitoring

(NARM) program. The resistant strain (8381r) that is resistant to 12 antimicrobials listed in Table 1.1 and a pansensitive strain, which is sensitive to all antimicrobials (8382s), also listed in Table 1.1. As depicted in Table 1.1, isolate 8381r was found to be resistant to one of the extended spectrum cephalosporins, ceftiofur, based on the commonly used breakpoint of 8 µg/ml. Nalidixic acid (Sigma, St Louis, MO) was chosen as a selective marker for recovery from the background microflora. We induced nalidixic acid resistance in challenge isolates 8381r and 8382s by passing on nalidixic acid gradient plates. Gradient plates were produced by growing isolates 8381r and 8382s on Luria Bertani (LB) agar (BD) plates that contained 16 µg/ml of nalidixic acid for 24 h. The isolates that grew on the 16 µg/ml nalidixic acid LB plates were streaked onto LB plates containing 32 µg/ml nalidixic acid and allowed to grow for 24 h. Isolates that grew on the 32 ug/ml nalidixic acid LB agar plates were struck once onto LB plates containing 64 µg/ml nalidixic acid and allowed to grow for 24 h. The isolates were then passed 3 times on 32 µg/ml nalidixic acid and lastly isolates were passed 6 times on plates that did not contain antibiotics. To verify resistance in the isolates that were grown on plates without antibiotics were passed again on plates containing 32 µg/ml nalidixic acid and then isolates were frozen an stored at -80ºC.

To produce the challenge inoculum, strains were separately grown in 2.5 ml of LB broth (BD) for approximately 17 h at 37ºC in a shaker bath at about 180 rpm. One ml of this culture was inoculated into 150 ml of fresh LB broth and incubated in a shaker bath for 5 h. The OD_{600} was checked with a spectrophotometer and the OD was adjusted to obtain culture concentrations of 10^9 CFU/ml (OD_{600}=0.8) by diluting with phosphate-buffered saline (BD). Isolates were enumerated by spiral plating onto Briliant Green

Sulfur (BGS; BD) with 32 µg/ml nalidixic acid with spiral autoplater (Spiral Biotech, Behesda, MD). Colonies were counted using a Q count (Spiral Biotech).

Determination of Antimicrobial Susceptibility. Antimicrobial susceptibility testing was done using a semi-automated broth micro-dilution system (Sensititre, TREK Diagnostics, Inc., Westlake, OH) as per manufacturer's directions. Custom 96-well plates were used from the National Antimicrobials Resistance Monitoring System (NARMS) program and included antimicrobials used in both human and veterinary medicine listed in Table 1.1. Clinical and Laboratory Standards Institute (CLSI; Wayne, PA) standards were followed throughout the testing procedure. Quality control strains *Esherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, and *Pseudomonas aeruginosa* ATCC 27853 were used. MIC values were evaluated pre-and post-exposure to nalidixic acid. Also, one isolate from each treatment groups that was recovered from the ceca, for a total of 12 isolates, were analyzed for their post-infection MIC values.

Determination of Serotype and Phage-Type. Serotyping and phage-typing were performed using standardized CLSI methods on all *Salmonella* isolates (National Veterinary Services Laboratories, NVSL, Ames, Iowa) and cephalosporin resistant isolates were phage-typed.

PCR Analysis. PCR analysis was done on *S.* Typhimurium 8381r and 8382s as previously described by Gray et al (7).

Experimental Design: Treatments. Birds were divided into 4 biosecure rooms with each room having 3 pens of 10 birds given the same treatment. Two rooms were used to study the transmission of the resistant strain by inoculating one seeder bird in each pen with 10^9 CFU/ml of 8381rs. The other two rooms were used to study the transmission of

the sensitive strain by inoculating one seeder bird in each pen with 10^9 CFU/ml of 8382s. Treatment groups for each isolate, 8381r and 8382s *S.* Typhimurium, included no antimicrobials and chlortetracycline (tet; Fort Dodge Animal Health, Fort Dodge, Iowa) at a constant dose given in water. Fresh water with or without tet was supplied daily. Hence, there were 2 sensitive *S.* Typhimurium, 8382s, treatments, one with and one without antimicrobials and 2 resistant *S.* Typhimurium, 8381r, one with and one without antimicrobials. Three replicates of each treatment were performed. One hundred and twenty µg/ml of chlortetracycline per gallon of water was added according to directions for therapeutic dose of antibiotics in chickens for a full 24 h period. One seeder bird from each pen of ten birds was inoculated with 10^9 CFU/ml of 8381r or 8382s per os. Cloacal swabs were taken from each bird and 3 fecal samples were collected from the pen floors and cultured on day 1, 3, 5, and 7 post-inoculation. Birds were sacrificed and necropsied on day 10 post-inoculation; cecum, ileum cecum junction (ICJ), and colon were qualitatively cultured from the challenge strain. The ICJ and colon were also quantitatively cultured for the challenge strain.

Qualitative and Quantitative Bacteriology. Qualitative bacteriology was achieved by individually placing fecal swabs, litter samples, colon, ICJ, and cecum into GN broth (BD) with 32 µg/ml nalidixic acid (GN/Nal). Fecal swabs and cecum were placed directly into GN broth while litter samples, colon, and ICJ were placed in bags with 50 ml of phosphate buffered saline (PBS; BD). Samples were placed in a Mini Mix (Interscience, Belgium) stomacher for 2 min and 100 µl of the homogenized samples were placed into GN/Nal. For further verification, samples from GN/Nal were spread plated onto BGS with nalidixic acid. Presumptive positive samples were inoculated into

triple sugar iron agar (TSI) and lysine iron agar (LIA) slants. Quantitative bacteriology of litter samples, colon, and ICJ was done by spiral plating (Sprial Biotech, Behesda, MD) 100 µl stomached solution of samples onto BGS with 32 µg/ml of nalidixic acid (BGS/Nal). The Q count (Spiral Biotech, Behesda, MD) auto plate imager was used for quantitative analysis to determine the colony forming units (CFU) per ml of homogenized cecum. The weights of the organs were previously determined in order to calculate the CFU/g.

Statistical Analysis. CFU/g of bacterial counts was logarithmically transformed prior to analysis. Differences in *Salmonella* transmission and colonization of the organs were determined using Analysis of Variance (SAS/English statistical package version 9.1) with significance expressed at $P<0.05$ level.

Results

Antimicrobial Susceptibility. After induced nalidixic acid resistance of the antimicrobial resistant isolate (8381r) was resistant to 13 of the antimicrobials on the NARMS panel as shown in Table 1.1. Isolate 8381r was still resistant to ceftiofur. After induced nalidixic acid resistance, 8382s was resistant to only nalidixic acid as shown in Table 1.1.

Analysis of Post Challenge Isolates. MIC values were determined post infections to examine any changes in the antimicrobial susceptibility patterns in *S.* Typhimurium 8381r and 8382s. Two out of the 3 randomly selected isolates recovered from the birds with the sensitive strain were further analyzed from the pens treated with tetracycline and were found to be resistant to tetracycline, based on the commonly used breakpoint of 32 µg/ml (data not shown).

Effect of chlortetracycline on the recovery of *S.* Typhimurium 8381r in litter and from cloacal swabs. To determine if the chicks had been exposed to any *Salmonella* resistant to the selective marker, nalidixic acid (nal), the transport boxes were swabbed for bacteria and plated on growth media containing nal. No *Salmonella* with nalidixic acid resistance were isolated pre-infection from the chickens, indicating that there was no detected exposure to nalidixic acid resistant *Salmonella* from the hatchery prior to challenge with *S.* Typhimurium 8381r and 8382s. To determine the spread of *S.* Typhimurium 8381r in feces, cloacal swabs were collected throughout the study. The total number of birds with positive cloacal swabs for 8381r on day 7 is listed in Table 1.2. All seeder birds had positive cloacal swabs but were not included in the determination of percent transmission. There was no 8381r recovered from cloacal swabs from birds not treated with chlortetracycline and only 1 sample from the birds treated with chlortetracycline was positive by day 7 (Table 1.2). To examine the presence of *S.* Typhimurium in the environment of the pen, litter samples were collected throughout the study. From the litter samples, 8381r was not recovered in the 3 pens where the birds were not treated with antibiotics and 8381r was recovered from 1 of the 3 pens from bird pens treated with antibiotics by day 7 (Table 1.2).

Effect of chlortetracycline on the recovery of *S.* Typhimurium 8382s in litter and from cloacal swabs. To determine the spread of *S.* Typhimurium 8382s in feces, samples were taken and analyzed as described above for strain 8381r. All seeder birds yielded positive cloacal swabs but were not included to determine the percentage of transmission. The total number of birds with positive cloacal swabs for 8382s is listed in Table 1.2. Strain 8382s was recovered from cloacal swabs of 2 birds treated with

chlortetracycline, while 7 of the birds not treated with chlortetracycline were positive by day 7 (Table 1.2). To examine the presence of *S.* Typhimurium in the environment of the pen, litter samples were collected throughout the study. From the litter samples, 8382s was recovered from 2 of the pens from the birds treated with chlortetracycline while all the pens without treatment were positive by day 7 (Table 1.2).

Effect of chlortetracycline on the recovery of *S.* Typhimurium 8381r in organs. The colon, cecum, and ICJ were cultured to determine the rate of transmission of *S.* Typhimurium 8381r in the birds. All seeder birds had at least one organ positive results but were not included in the determination of percent transmission. No *S.* Typhimurium 8381r was recovered from the colon of the birds that did not receive chlortetracycline, while 8381r was recovered from the ICJ and cecum from 3.7% (1/30) and 7% (2/30) of the birds that were not treated, respectively (Table 1.3). No *S.* Typhimurium 8381r was recovered from the colon, ICJ, or cecum of birds treated with chlortetracycline (Table 1.3). The percentage of birds positive for *S.* Typhimurium 8381r recovered from the organs is not shown and statistical analysis was not done due to the lack of recovery.

Effect of chlortetracycline on the recovery of *S.* Typhimuiurm 8382s in organs. The colon, cecum, and ICJ were cultured to determine the rate of transmission of *S.* Typhimurium 8382s within the birds. All seeder birds were positive for *S.* Typhimurium 8382s but were not included to determine the percentage of transmission. The total amount of birds with positive organs for 8382s is listed in Table 1.3. *S.* Typhimurium 8382s was recovered from the colon, ICJ, and cecum from 87% (26/30), 48% (15/30), 100% of the birds that did not receive chlortetracycline, respectively (Table 1.3 and Figure 1.1). *S.* Typhimurium 8382s was recovered from the colon, ICJ, and cecum from

15% (5/30), 30% (9/30), and 56% (17/30) of the birds that did receive chlortetracycline, respectively (Table 1.3 and Figure 1.1). The cecum was excluded from quantitative analysis because the growth of the bacterium was too numerous to count. The percent positive of *S.* Typhimurium 8382s within the organs of the birds receiving chlortetracycline was lower than the percent positive of 8382s not receiving antibiotic treatment. The treatment groups were found to be significantly different with SAS ANOVA (P=0.002). The average growth of the 8382s bacterium within the organs was found to be higher in birds not treated with antimicrobials, 3.83 CFU/g and was found to be significantly different from birds treated with chlortetracycline, 2.27 CFU/g, with SAS ANOVA (P=0.001). There was no difference in the average growth of *S.* Typhimurium 8382s in the colon or ICJ within the individual treatment groups (P=0.86).

The effect of chlortetracycline on the transmission of *S.* Typhimurium 8381r and 8382s. The multidrug resistant *S.* Typhimurium 8381r, based on the largest percentage of positive 8381r recovered from organs, colonized only 7% (2/30) of the birds that did not receive chlortetracycline and did not colonize any birds that received treatment. The sensitive *S.* Typhimurium 8382s, based on the largest percentage of positive 8382s recovered from organs, colonized 100% of the birds not treated with antibiotics and colonized 56% (17/30) the birds that were treated with antimicrobials.

Discussion

S. Typhimurium is a widespread pathogen of humans and is often found in animals, especially in poultry and swine (14). The development of antibiotic resistance has increased concerns of foodborne infections from *Salmonella* that are believed to be more virulent due to the severity of the infections (1). Multidrug-resistant *Salmonella* are

believed to be more virulent under antimicrobial selective pressure allowing them to spread more rapidly from animal to animal. In the present experiment, the results indicate little transmission of the multiple drug resistant isolate in the organs of birds treated with antibiotics. The lack of colonization made it difficult to conclude if there was a significant difference in birds treated with the therapeutic dose of chlortetracycline versus those not treated.

Transmission of the sensitive strain of *S.* Typhimurium was slightly lower under antibiotic selective pressure from chlortetracycline, while the colonization level within the organs of the birds with and without therapeutic levels of chlortetracycline was found to be significantly different. The birds treated with chlortetracycline had significantly less bacteria residing within their organs; thus indicating the overall *Salmonella* load on the flock was lower perhaps leading to lower transmission rates. Even under antimicrobial selective pressure the sensitive isolate colonized over half the flock. Given that antibiotic treatment is usually supplied in the animal's water, possible over-or-under dosing of the antibiotic can occur (15). It is also possible given that the sensitive strain of *S.* Typhimurium only decreased slightly, that *in vivo* the drug does not affect this pathogen directly or it may have inhibited or killed other bacteria in the gastrointestinal tract allowing an opportunity for the sensitive strain to grow.

Another explanation for the lack of inhibition of the sensitive strain of *S.* Typhimurium treated with antimicrobials could be that the antibiotic was unable to reach the area where the *S.* Typhimurium resided. For example, the bacteria residing in one organ, such as the colon, may not be exposed to the same antimicrobial tissue concentration as those in a neighboring organ, such as the ileum (19). The lack of

information concerning the concentration of antibiotics in a specific organ can make it difficult to determine the affect the antibiotics have on the bacteria that reside within the gastrointestinal tract (19). The bioavailability of a drug is the percentage of drug administered that reaches the systemic circulation of the animals (19). The bioavailability of oxytetracycline, an antibiotic agent similar to chlortetracycline and used frequently in a similar manner as chlortetracycline in the livestock industry, was found to be poor in animals, with only a 23% bioavailability in pigs (19). The transmission of *S.* Typhimurium 8382s in this experiment was over 60% in the birds treated with chlortetracycline which could be the result of the lack of bioavailability of chlortetracycline in chickens.

A few of the sensitive isolates became resistant to tetracycline, as shown by the evaluation of MIC values of recovered *S.* Typhimurium isolates. Previous studies have shown that resistance can develop in as little as a week under antimicrobial selective pressure (10). In that study, strains acquired a plasmid conferring multiple antibiotic resistance from neighboring bacteria within a week (10). *S.* Typhimurium 8382s in this experiment became resistant to tetracycline based on an increase in the MIC values above the breakpoint of 32 µg/ml. The *S.* Typhimurium 8382s that became resistant to tetracycline did so under therapeutic levels of antibiotics that should have inhibited the growth of the bacterium. In a study of the *in vivo* acquisition of ceftriaxone resistance, resistance gene transmission occurred even in the presence of antimicrobials that should normally kill the bacteria (16). Bacteria that are resistant to tetracycline reside in large numbers in the gastrointestinal tract of poultry and are frequently isolated from the environment (4, 5). Thus, some of the sensitive strains of *S.* Typhimurium could have

developed antibiotic resistance by acquiring plasmids from tetracycline resistant bacteria residing in the normal microflora of the chicken.

The resistant and sensitive strains of *S.* Typhimurium had very different transmission rates within the flocks. Clearly, the spread of *S.* Typhimurium in this experiment had little to do with its antimicrobial resistance genes. The sensitive isolate did not contain the resistant genes that are speculated to increase transmission, yet it spread to over half the flock with and without therapeutic doses of chlortetracycline. A resistant strain of *S.* Typhimurium in a previous study had reduced ability to be internalized by cells *in vitro* due to the overproduction of chromosomal ß-lactamases of class C, *amp*C, which was found to produce larger cells, diplobacili, and filaments (11). The expression of *amp*C is regulated by an ampR gene, thus *Salmonella* that lack *amp*R overproduce the *amp*C gene. It has been suggested that the cost of maintenance and expression of the *amp*C gene may be too much for the *Salmonella* when the gene is overproduced consequently, reducing the reproduction of the bacterium. The reduction in growth would therefore affect the spread of *S.* Typhimurium causing a reduction in transmission between birds (11). However, the isolate used in our experiment was recovered from a farm and surveillance data shows bla_{CMY} to be increasing in animal prevalence. In this experiment the *S.* Typhimurium isolate contained resistance to cephalosporins linked to a plasmid encoding AmpC-like ß-lactamase. The antibiotic resistant *S.* Typhimurium had a significantly lower colonization rate compared to the sensitive strain. These results indicate that this multiple antimicrobial resistant *Salmonella* was less fit for broiler transmission even when the gene was located on a plasmid rather than in the chromosome. Based on previous studies of chromosomal ß-

lactamases of class C, the reduced invasion rate may be due to the reduction of intracellular replication within cells due to the over expression of *ampC* (11). This may be one explanation for the lower transmission observed in this experiment.

However, the overproduction of *ampC* was found to be a problem only in *S.* Typhimurium and not in other bacteria, such as *Escherichia coli*. It is suggested the reason *E. coli* remains virulent even when expressing *amp*C is due to the deletion of genes that normally inhibit virulence within the bacteria (11). Other organisms that express *amp*C have the necessary regulatory *amp*R gene, thus they are able to regulate the amount of *amp*C produced (11). The acquisition of *amp*C and *amp*R genes together is not as common as the acquisition of only the chromosomal ß-lactamases of class C in *Salmonella* (11). *S.* Typhimurium as well as the multitude of other *amp*C-like producing CMY-2 positive *Salmonella* isolated from humans and animals remain virulent and are increasing in overall prevalence.

In conclusion, we have shown that some strains of *Salmonella* are much more efficient at colonizing a flock than others regardless if they are sensitive or resistant to antimicrobials. In this experiment, the rapidly spreading 8382s was capable of acquiring new antibiotic resistance. The sensitive isolates of *S.* Typhimurium obtained resistance to chlortetracycline which resulted in an antimicrobial resistant 8382s that was more efficient than the resistant strain in its transmission to other birds. Concerns about the acquisition of genes from nonpathogenic bacteria that reside normally in the microflora of the animal to pathogenic microbes containing virulence genes may be warranted based on these experiments. Diseases caused by resistant *S.* Typhimurium have been documented as severe and are often believed to be related to the organism's antimicrobial

resistances. Replication and survival within a host is necessary for *S.* Typhimurium virulence; however, the antibiotic resistant microbes in this project failed to replicate or survive to high levels based on the lack of recovered *Salmonella* from the birds. The lack of recovery of the resistant strain of *S.* Typhimurium with and without antimicrobial selective pressure provides evidence that antibiotic resistance is not a necessarily component of *Salmonella* virulence as measured by transmission.

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Table 1.1. Evaluation of antibiotic resistance of *S.* Typhimurium 8381r and 8382s to the antimicrobials used in the NARMS programs.

Test agent	8381r	8382s
Amikacin	S	S
Amoxicillin/Clavulanic Acid	R	S
Ampicillin	R	S
Apramycin	S	S
Cefoxitin	R	S
Cetfiofur	R	S
Ceftriaxone	S	S
Cephalothin	R	S
Chloramphenicol	R	S
Ciprofloxacin	S	S
Gentamicin	R	S
Imipenem	S	S
Kanamycin	R	S
Nalidixic Acid	R[a]	R[a]
Streptomycin	R	S
Sulphamethoxazole	R	S
Tetracycline	R	S
Trimethoprim/ Sulphamethoxazole	R	S

[a] Resistance to nalidixic acid was induced in *S.* Typhimurium 8381r and 8382s.

Table 1.2. Positive recovery of *S.* Typhimurium from feces of broiler chicks on day 7.

Isolate	Sample	Positives/Totals
8381r-no tet	Litter	0/3
	Cloacal swab	0/30
8381r-tet	Litter	1/3
	Cloacal swab	1/30
8382s-no tet	Litter	3/3
	Cloacal swab	7/30
8382s-tet	Litter	2/3
	Cloacal swab	2/30

Table 1.3. Positive recovery of *S.* Typhimurium from organs of broiler chicks inoculated with 8381r or 8382s.

Isolate	Organ	Positives/Totals	Mean CFU/g log 10
8381r-no tet	Colon	0/30	0
	Cecum*	2/30	
	ICJ	1/30	3.2
8381r-tet	Colon	0/30	0
	Cecum*	0/30	
	ICJ	0/30	0
8382s-no tet	Colon	26/30	4.28
	Cecum*	30/30	
	ICJ	15/30	4.54
8382s-tet	Colon	5/30	3.40
	Cecum*	17/30	
	ICJ	9/30	4.04

* Due to overgrowth of bacteria in the cecum, the CFU/g was not quantified.

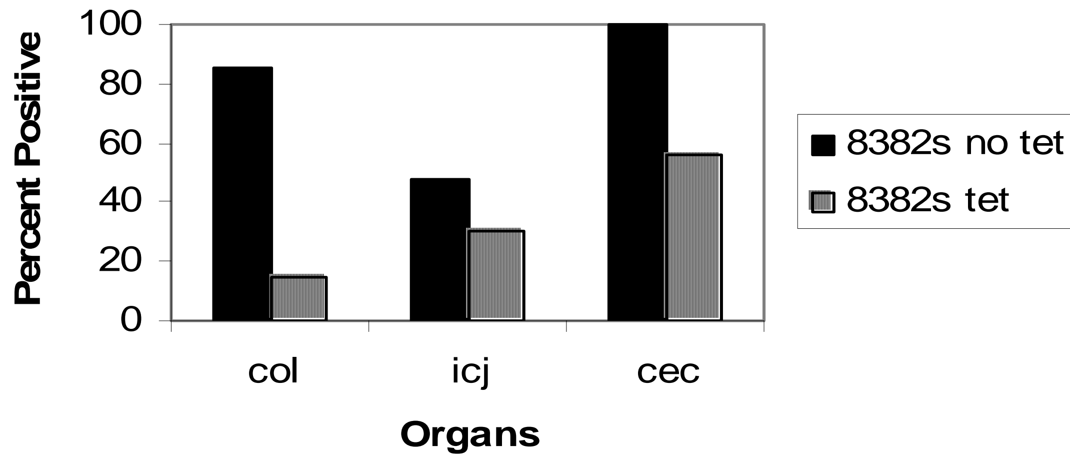

Figure 1.1. The transmission of *S.* Typhimurium 8382s in the organs of broiler chicks with and with out antimicrobial treatment.

CHAPTER 4

NATURAL TRANSMISSION OF *SALMONELLA* TYPHIMURIUM IN POULTRY WITH AND WITHOUT ANTIMICROBIAL SELECTIVE PRESSURE: STUDY 2 [1]

[1] Jennifer Garland, Paula Cray, Jeff Gray, Jonathan Frye, Mark Berrang, and Mark Harrison. To be submitted to peer review.

Abstract: Foodborne infections from *Salmonella* are a major public health problem. Further complicating these infections, some *S.* Typhimurium strains have acquired multiple drug resistance, including resistance to extended-spectrum cephalosporins. The rapid spread of multi-drug resistant *Salmonella* is believed to be due to the increased virulence of the pathogen. However, there are no studies in an animal model that show the transmission of an antimicrobial resistant *S.* Typhimurium. This experiment was designed to study the transmissibility of a resistant strain (8381r) and a sensitive strain (8382s) of *S. typhimurium* with and without antimicrobial selective pressure, in a poultry model. The multiple antibiotic resistant phenotype of *S. typhimurium* 8381r is resistant to 12 of 17 antimicrobials on the NARMS panel. Strain 8382s was sensitive to all antimicrobials. One day old broiler chicks were separated into groups of 10 birds into 8 total pens. One bird in each pen of ten was inoculated with 10^9 CFU/ml of *S.* Typhimurium 8381r or 8382s per *os*. Treatment groups for each isolate included no antimicrobials and 120 ug/ml chlortetracycline (tet), constant dose in water for a total of 4 treatment groups. Two replicates of each treatment were run. Birds were necropsied on day 6 post inoculation. Upon necropsy, percent positive ceca samples for strain 8381r was 90% for tet treated and 60% for non-treated birds. Strain 8382s was detected in 95% of the ceca from tet treated birds and 90% of the cecas were positive for strain 8382s in non-treated birds. This data indicates that multiple antimicrobial resistance in *Salmonella* does lead to increased transmissibility with antimicrobial selective pressure. Additionally, a sensitive *Salmone*lla strain can survive and transmit efficiently between animals even with antimicrobial selective pressure at MIC levels.

Introduction

Foodborne diseases caused by *Salmonella* remain a major health concern in the United States as well as other countries. The Center for Disease Control and Prevention (CDC) estimated that infections from *Salmonella* affected 1.4 million people in the U.S. in 1999 of which 22% of the people were hospitalized and 0.8% died (10). Although the majority of *Salmonella* infections are self-limiting diarrheal illnesses, severe and even life-threatening infections that require antibiotic treatment do occur. Further complicating foodborne infections, *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) are more capable of developing resistance to antibiotics and many *Salmonella* are resistant to multiple antibiotics. Multiple antibiotic resistant *Salmonella* have a poor response to treatment and are believed to be more virulent than sensitive isolates (6).

S. Typhimurium is a gram-negative, facultative, motile rod and is one of the most common causes of bacterial diarrhea in humans (19). The natural microflora of animal's intestinal tract can contain *S.* Typhimurium thus controlling *Salmonella* in food-producing animals is of great concern due to the high rate of food contamination (17). Virulent strains of *S.* Typhimurium can cause deadly infections by causing septic shock when the bacterium invades the bloodstream (19). *S.* Typhimurium with resistance to one or more antibiotics are believed to be more virulent and more likely to cause septic shock and in severe cases the individual may need antibiotic treatment (22). In 1980, 13% of *S.* Typhimurium bacteria were resistant to one or more antibiotics and this percentage increased to 51% in 2001 (22). In 2002, *S.* Typhimurium was the most common *Salmonella* serotype found in animals as reported by the National Antimicrobial Resistance Monitoring System (NARMS) (8). Of the 393 *S.* Typhimurium isolated, 27%

were found to be resistant to 5 or more antimicrobials while 40% were resistant to at least one antimicrobial (NARMS) (8). In addition, 11.6 people per 100,000 reported a *Salmonella* infection according to the 2003 census population in the U.S. (16). Twenty-seven percent of the people infected with *Salmonella* were hospitalized and 34 (0.6%) people died from the infection (14).

Salmonella with resistance to fluoroquinolones and extended spectrum cephalosporins have been uncommon in the United States, therefore they have become the suggested antimicrobial agents for treatment of severe *Salmonella* infections (21). However, children with systemic salmonellosis are treated intravenously with ceftriaxone because fluoroquinolones can cause cartilage damage (1). Extended spectrum cephalosporins act like other beta-lactam antibiotics by inhibiting synthesis of the bacterial cell wall, leading to cell death (18). Although resistance to extended spectrum cephalosporin is rare, 18.8% of the *Salmonella* animal isolates in 2003 from North America have exhibited resistance to extended spectrum cephalosporins, an occurrence linked to a plasmid mediated CMY-2 AmpC-like beta-lactamase that hydrolyzes cephalosporins (9). The *cmy*-2 gene is located on a transferable plasmid with multiple drug resistance and has been found in 21 different serotypes of *Salmonella* (9). Tetracycline resistance was also found in 100% of the *cmy* gene positive *Salmonella* (9). Bacteria resistant to tetracycline have been found frequently in the gastrointestinal tract of humans and animals (7). For example, *Salmonella* isolates with tetracycline resistance have been found in 41.3% of *Salmonella* of animal origin (8). Many genes for antibiotic resistance can be genetically linked to other genes that confer resistance to different

antibiotics, similar to this association between tetracycline and extended-spectrum cephalosporin (2).

The emergence of resistance in *Salmonella* has been associated with the use of antimicrobial agents in livestock, including chickens (3). It is not known if the spread of multiple drug resistant *Salmonella* is caused by an increase in virulence due to the selective pressure from antimicrobial treatment. Furthermore, there are few experiments that clearly study virulence traits, such as transmission, of a resistant strain of *S.* Typhimurium in an animal model. To explore the relationship between *S.* Typhimurium resistance and a competitive advantage for colonization under antimicrobial selective pressure, we examined the natural transmission of multi-drug resistant and sensitive *S.* Typhimurium in poultry with and without antimicrobial selective pressure.

Materials and Methods

Bacterial Strains. Two strains of *Salmonella* Typhimurium were used in this study. The resistant strain (8381r) was resistant to the antimicrobials listed in Table 1. As shown in Table 2.1, isolate 8381r was resistant to one of the extended spectrum cephalosporin, ceftiofur, based on the commonly used breakpoint of 8 µg/ml. The sensitive strain (8382s) was sensitive to all antibiotics (pansensitive) listed in Table 2.1. Both strains were obtained from farms in the U.S. by the United States Department of Agriculture's Bacterial Epidemiology and Antimicrobial Resistance (BEAR) research unit in Athens, GA for antimicrobial susceptibility testing included in the NARMS program.

Nalidixic acid (Sigma, St Louis, MO) was chosen as a selective marker for recovery from the background microflora. We induced nalidixic acid resistance in the challenge isolates 8381r and 8382s by passing the isolates on nalidixic acid gradient

plates. Gradient plates were created by growing isolates 8381r and 8382s in Luria Bertani (LB) agar (BD) plates that contained 16 µg/ml of nalidixic acid for 24 h. The isolates that grew on the 16 µg/ml nalidixic acid LB plates were streaked onto LB plates containing 32 µg/ml and allowed to grow for 24 h. Isolates that grew on the 32 µg/ml nalidixic acid were struck once on 64 µg/ml nalidixic acid. The isolates were then passed 3 times on 32 µg/ml nalidixic acid, and finally the isolates were passed 6 times on plates that did not contain antibiotics. To verify resistance, the isolates grown on plates without antibiotics were passed again on plates containing 32 µg/ml nalidixic acid and then those isolates were frozen and stored at -80ºC.

For inoculation of birds, both strains were grown overnight in 2.5 ml of LB broth (BD) for approximately 17 h at 37ºC in a shaker bath at 180 rpm to stationary phase. One ml of this culture was inoculated into 150 ml fresh LB broth and placed back into shaker bath for 5 h. The OD_{600} was checked with a spectrophotometer and the OD was adjusted to obtain culture concentration of 10^9 CFU/ml (OD_{600} = 0.8) by diluting with phosphate-buffered saline (BD). Isolates were enumerated by spiral plating onto Briliant Green Sulfar (BGS; BD), with 32 µg/ml nalidixic acid with spiral autoplater (Spiral Biotech, Behesda, MD). Colonies were counted using a Q count (Spiral Biotech, Bethesda, MD).

Determination of Antimicrobial Susceptibility. Antimicrobial susceptibility testing was performed using a semi-automated broth micro-dilution system (Sensititre, TREK Diagnostics, Inc., Westlake, Ohio) as per manufacturer's directions. Custom 96-well plates were used from the NARMS program and included antimicrobials used in both human and veterinary medicine. Clinical and Laboratory Standards Institute (CLSI;

Wayne, PA) standards were followed throughout the testing procedure. Isolates were examined for susceptibility to the antimicrobials listed in Table 2.1. Quality control strains *Esherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, and *Pseudomonas aeruginosa* ATCC 27853 were used. MIC values of the inoculated *S.* Typhimurium 8381r and 8381s were evaluated pre- and post-exposure to nalidixic acid and are listed in Table 2.1.

Antimicrobial Susceptibility Testing of Post-inoculum Isolates. Ten samples from chicks were selected from each of the 4 treatments. Thus, a total of forty random samples of *S.* Typhimurium were selected for further analysis of their post-MIC values to determine if there were any changes in the sensitivity or resistance to any antimicrobials.

PCR and PFGE Analysis. PCR analysis was performed on the *S.* Typhimurium inoculums 8381r and 8382s as described by Gray et al (9). PFGE analysis was performed on 9 of the randomly selected post-inoculum isolates as described in reference (20).

Animals. A total of 192 one-day-old broiler chickens from a local hatchery were randomly separated into two rooms with 4 pens in each room (groups of 12 birds each). Pens were separated by panels that prevented any litter or birds from crossing into other pens. Each pen had separate feed, water, and heat supplied. Seeder bird's feathers were individually colored to distinguish them from the other birds for future record keeping.

Experimental design: Birds were divided into 2 biosecure rooms with each room receiving either 8381r or 8382s. The rooms were further divided into 4 pens containing 12 birds each. Two pens from each room received a constant dose of chlortetracycline (tet) (Fort Dodge Animal Health, Fort Dodge, Iowa) in water while the other 2 pens in each room received no antibiotics, for a total of 4 treatments. The procedure was

repeated twice, thus 4 replicates of each treatment were performed. Fresh water with or without tet was supplied daily. For birds treated with tet, 120 µg of chlortetracycline per ml of water was added according to manufacturer's directions for therapeutic dose of antibiotics in chickens for a full 24 h period. Two seeder birds from each pen of twelve were inoculated with 10^9 CFU/ml of 8381r or 8382s per os. Birds were sacrificed and necropsied on day 7 post-inoculation for examination of the cecum.

Bacterial culture. The ceca were placed in bags with 50 ml of phosphate buffered saline (PBS; BD). Qualitative bacteriology was performed by stomaching with a Mini Mix (Interscience, Belgium) for 2 min and placing 100 µl into GN broth (BD) containing 32 µg/ml nalidixic acid. Samples were incubated at 37ºC for 18 h. Presumptive positive isolates were plated onto BGS plates. For further identification, isolates were inoculated onto triple sugar iron agar (TSI; BD) slants and lysine iron agar (LIA; BD) slants and xylose lysine desoxycholate (XLT-4), agar (Hardy Diagnositcs, Santa Maria, CA)'

Quantitative bacteriology of ceca was done by spiral autoplating (Spiral Biotech) the homogenized cecum and PBS onto BGS with 32 µg/ml nalidixic acid and incubated at 37ºC for 18 h. The Q Count imaging system was used for quantitative analysis. Further analysis of presumptive positives was performed the same as the qualitative analysis. The weights of the organs were previously determined in order to calculate the CFU/g.

Data Analysis. CFU/g of bacterial counts was logarithmically transformed prior to analysis. Differences in *Salmonella* transmission and colonization of the organs were determined using the using Analysis of Variance (SAS/English statistical package version 9.1) with significance expressed at $p < 0.05$ level.

Results

Antimicrobial Susceptibility of challenge stains. After induction of nalidixic acid resistance, 8381r was resistant to 13 of the antimicrobials on the NARMS panel as shown in Table 2.1. The sensitive isolate 8382s was resistant to nalidixic acid after induction as shown in Table 2.1.

Effect of chlortetracycline on the recovery of *S.* Typhimurium 8381r from the ceca. No *Salmonella* with nalidixic acid resistance was recovered from the chickens pre-inoculation, indicating that there was no detected exposure to nalidixic acid resistant *Salmonella* from the hatchery prior to challenge with *S.* Typhimurium 8381r and 8382s. Once the study was completed, ceca were examined to determine the transmission of *S.* Typhimurium 8381r in the birds. The number of birds positive for 8381r or 8382s is listed in Table 2.2. After commingling with the 8381r infected seeder bird, 90% (36/40) of naïve pen mates treated with chlortetracycline became colonized with 8381r. After commingling with the 8381r infected seeder bird, 60% (24/40) of naïve pen mates not treated with chlortetracycline became colonized with 8381r over time as shown in Table 2.2. The spread of *S.* Typhimurium 8381r was less in untreated birds compared to chickens that received a therapeutic dose of chlortetracycline (120 µg/ml) and they were found to be significantly different ($p = 0.03$). To determine the average amount of *S.* Typhimurium 8381r that resided in the ceca of the birds, quantitative data from the BGS/nal plates were analyzed. The mean growth of *S.* Typhimurium 8381r was greater in birds treated with chlortetracycline than those not treated, 4.7 and 2.8, respectively**.** Statistical analysis with ANOVA revealed that the treatments were significantly different ($p = 0.0001$).

Effect of chlortetracycline on the recovery of *S.* Typhimurium 8382s in the ceca. Ceca were examined to determine the transmission of *S.* Typhimurium 8382s in the birds. Isolate 8382s was recovered from 95% (38/40) of chickens treated with tet and from 90% (36/40) of the chickens not treated with tet (Table 2.2). The spread of *S.* Typhimurium 8382s was not significantly different in chickens that received a therapeutic dose of chlortetracycline compared to untreated chickens ($p = 0.31$). To determine the average amount of *S.* Typhimurium 8382s that resided in the ceca of the birds, quantitative data from the BGS/nal plates were analyzed. The mean growth of *S.* Typhimurium 8382s was the same in birds treated with chlortetracycline and those not treated, 6.11 and 5.7, respectively. Statistical analysis with ANOVA reveled that the treatments were not significantly different ($p = 0.30$).

Antimicrobial susceptibility testing, seorotyping, and PFGE of post-inoculum isolates. Three out of 20 *S.* Typhimurium 8382s recovered from the ceca of the birds treated with tet became resistant to antibiotics, shown in Table 3.3. All 3 isolates became resistant to tet based on the commonly used breakpoint of 32 μg/ml. Also, 2 out of 20 *S.* Typhimurium 8382s recovered from ceca of birds not treated with tet became resistant to antibiotics, shown in Table 2.3. Only 1 of the 2 isolates became resistant to tet based on the commonly used breakpoint of 32 μg/ml. *S.* Typhimurium 8382s isolates that became resistant to anything other than the nalidixic acid were serogrouped and serotyped. Both isolates 8381r and 8382s were *Salmonella* group B and serotype Typhimurium. PFGE profiles of *S.* Typhimurium 8382s recovered from the chicks generated identical profiles with 14 matching fragments to the inoculated 8382s strain, shown in Fig. 2.1. PFGE

profiles also generated identical profiles for the 8381r strain which presented 16 matching fragments as compared to the inoculated 8381r strain depicted in Fig. 2.1.

Discussion

The use of antibiotics in livestock production has been coupled with the development of antimicrobial resistance in bacteria. In addition, antimicrobial resistance in *S.* Typhimurium can create therapeutic challenges for humans and animals infected by these bacteria. Multi-drug resistant *Salmonella* are believed to be more virulent under antimicrobial selective pressure. This study was designed to determine if multi-drug resistance *Salmonella* could spread more rapidly from animal to animal under antibiotic selective pressure.

The experiments in this study found that in broiler chicks, multi-drug resistance of *S.* Typhimurium did have significantly increased transmissibility under antimicrobial selective pressure. Within 7 days, over 90% (36/40) of the birds treated with a therapeutic dose of chlortetracycline became colonized with the multi-drug resistant strain of *S.* Typhimurium versus only 60% (24/40) of the control birds. Colonization levels of birds with the resistant isolate given chlortetracycline increased 30% compared to birds not treated with antimicrobials. Furthermore, the number of the resistant *S.* Typhimurium found in the cecum was higher in the birds treated with antibiotics compared to those not treated with antimicrobials, 4.7 and 2.8, respectively. Birds not treated with chlortetracycline had significantly less bacteria residing within their organs; thus indicating the overall *Salmonella* load on the flock was lower perhaps leading to lower transmission rates. It is believed that antibiotics disrupt the microflora of animals intestines by reducing sensitive strains of bacteria and allowing the resistant strain to

overpopulate that region (12). In this experiment, higher amounts of bacteria within the ceca under antimicrobial selective pressure may result in shedding higher levels of *S.* Typhimurium thus increasing the transmission between the birds.

Interestingly, in broiler chicks challenged with a sensitive *S.* Typhimurium, therapeutic levels of chlortetracycline did not deter the spread of the isolate. The sensitive isolate spread rapidly through the chickens with almost a 100% recovery from the ceca of birds treated with chlortetracycline. The mean numbers of the sensitive *S.* Typhimurium within the ceca was approximately the same with and without tet and was determined not to be significantly different regardless of the presence of the antimicrobials. This is indicative of the fact that virulence and colonization are not usually associated with antimicrobial resistance. Further genetic analysis of these strains of *S.* Typhimurium may show additional virulence factors that were not found in the resistant strain.

Antibiotics that are used at subtherapeutic levels in the diet of livestock are considered too low to effect the growth of the bacteria but enough to allow the bacteria to accumulate mutations or acquire new DNA for resistances under the selective pressure (4). Antibiotics used in livestock at levels designed to kill the bacteria, or therapeutic levels, are believed to be better for the prevention of antibiotic resistance (11). However, the therapeutic levels of chlortetracycline used in this experiment did not deter the sensitive *S.* Typhimurium. Additionally, a low percentage of isolates became resistant to tetracycline as well as other antimicrobials, thus altering a sensitive isolate to multi-drug resistance in as few as seven days. Given that treatment is usually supplied in the animal's water, possible over-or-under-dosing of the antibiotic could have occurred (21).

Similarly, Levy *et. al.* found that sensitive bacteria treated with therapeutic levels of tetracycline acquired a plasmid that conferred resistance to multiple antibiotics within a week (13). Since the discovery of tetracycline in the 1940s, acquisition of tetracycline resistance genes has increased in pathogenic bacteria (7). Tetracycline resistance in pathogenic bacteria that reside in poultry is a common occurrence and can be transmitted easily on a plasmid containing other resistant genes (5). Obtaining chicks that are bacteria free or containing bacteria sensitive to all antibiotics is almost impossible, thus the development of resistance in the sensitive strain of *S.* Typhimurium 8382s is likely to occur naturally in environmental bacteria. Interestingly, development of resistance was found to occur even in bacteria that were not treated with antimicrobials. Previous studies have shown that development of resistance in bacteria not treated with antimicrobials is also a common incident that occurs at a much lower rate as compared to those under selective pressure (13).

Although the sensitive isolate was not found to be significantly different in transmission rates with or without antimicrobial selective pressure, its colonization levels in both treatments surpassed that of the resistant isolate. The transmission of the resistant strain of *S.* Typhimurium did increase under antimicrobial selective pressure, but it did not appear to be any more efficient at colonization than the sensitive strains of *S.* Typhimurium. Several studies have shown that resistance genes impair the growth and invasion properties of the resistant bacteria (15). It was further demonstrated that AmpC β-lactamase production impaired the intracellular replication and invasion rates in extended spectrum cephalosporin resistant *S.* Typhimurium (15). The resistant strain of *S.* Typhimurium in this experiment that was not treated with antimicrobials colonized

over half the flock, thus the biological cost of maintaining the resistant genes must have been ameliorated. Bacteria can mutate to compensate for the biological cost of maintaining the resistance genes allowing the pathogen to retain its resistance and increase its fitness (5).

Studies concerning the competition rate between sensitive and resistant *S.* Typhimurium in an animal model showed that the sensitive isolates were more virulent (4). In the present experiment, the sensitive isolate colonized nearly 100% of the flock regardless of antimicrobial pressure. The sensitive isolate spread through the flock more efficiently than the resistant isolate when treated with antibiotics that normally should have inhibited the growth of the sensitive isolate. One explanation for this phenomenon might be that *in vivo* antibiotics did not target the sensitive strain of *S.* Typhimurium but killed or inhibited the growth of other more sensitive bacteria, most likely giving more room for this pathogen to grow. Also, the sensitive strain had a larger CFU/g of bacteria growing within the ceca of the birds treated with and without tet as those compared to the resistant strain with and without tet. The higher growth of bacteria within the ceca may have increased the amount of sensitive *S.* Typhimurium in the environment of the broiler chicks. This can explain the higher colonization rate of the sensitive strain in the broiler chicks as compared to the resistant isolate.

Based on the analysis of the data in this study, concerns regarding the use of antibiotics in livestock should not focus solely on the detrimental effects of resistant strains of bacteria. In this study, the use of therapeutic levels of antimicrobials increased the transmission rate of resistant bacteria, had no effect on the transmission of the sensitive bacteria, and induced resistance in the sensitive strains of *S.* Typhimurium.

Studying the transmission of resistance under antimicrobial selective pressure has provided evidence that there is a risk associated with the use of antibiotics in poultry even at the supposed safe therapeutic levels. However, the increased transmission in the resistant isolate under antimicrobial selective pressure did not colonize the broiler chickens as well as the antibiotic sensitive pathogen. Additionally, the sensitive *Salmonella* was able to acquire genes for resistance in as little as 7 days. In conclusion, this experiment showed that other factors are involved with the ability of a pathogen to spread through a flock, which should be researched in future studies.

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Table 2.1. Evaluation of antibiotic resistance of *S.* Typhimurium 8381r and 8382s to the antimicrobials used in the NARMS programs.

Test agent	8381r	8382s
Amikacin	S	S
Amoxicillin/Clavulanic Acid	R	S
Ampicillin	R	S
Apramycin	S	S
Cefoxitin	R	S
Cetfiofur	R	S
Ceftriaxone	S	S
Cephalothin	R	S
Chloramphenicol	R	S
Ciprofloxacin	S	S
Gentamicin	R	S
Imipenem	S	S
Kanamycin	R	S
Nalidixic Acid	R[a]	R[a]
Streptomycin	R	S
Sulphamethoxazole	R	S
Tetracycline	R	S
Trimethoprim/ Sulphamethoxazole	R	S

[a] Resistance to nalidixic acid was induced in *S.* Typhimurium 8381r and 8382s.

Table 2.2. Recovery of *Salmonella* Typhimurium 8381r or 8382s from the ceca of broiler chicks with and without chlortetracycline treatment.

Isolate	Tet (positive birds/ total birds)	No tet (positive birds/ total birds)	ChangeResistance
8381r	36/40	24/40	0/20
8382s	38/40	36/40	5[a]/20

[a] Only 20 randomly picked sensitive isolates were checked for post-MIC changes.

Table 2.3. Altered MIC values of post-infection *Salmonella* Typhimurium 8382s from birds with and without chlortetracycline treatment.

Isolate/treatment	Test Agent	Altered MIC value
8382s-a/no antibiotics*	Sulphamethoxazole	512
	Trimethoprim/sulphamethoxazole	4
8382s-b/no antibiotics	Gentamicin	16
	Sulphamethoxazole	512
	Tetracycline	32
8382s-c/tet	Gentamicin	16
	Streptomycin	64
	Sulphamethoxazole	512
	Tetracycline	32
8382s-d/tet	Gentamicin	16
	Streptomycin	64
	Sulphamethoxazole	512
	Tetracycline	32
8382s-e/tet	Gentamicin	16
	Streptomycin	64
	Sulphamethoxazole	512
	Tetracycline	32

* only 40 out of 80 birds were selected for post-MIC analysis.

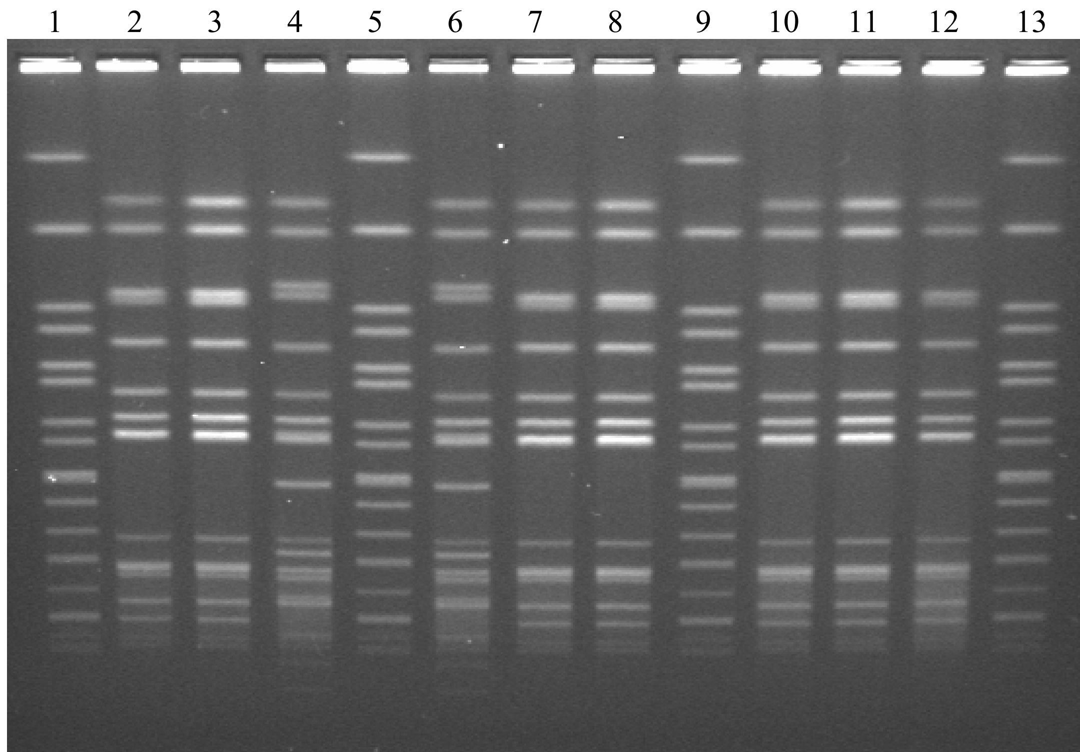

Figure 2.1. PFGE patterns of strains 8381r and 8382s pre-and-post infection. Lane 1: control-H9812, Lane2: 8382s-tet, Lane 3: 8382s-tet, Lane 4: 8381r-no tet, Lane 5: H9812, Lane 6: 8381r inoculum, Lane 7: 8382s-tet, Lane 8: 8382s inoculum, Lane 9: H9812, Lane 10: 8382s-tet, Lane 11: 8382s-no tet, Lane 12: 8382s-no tet, Lane 13: H 9812.

CHAPTER 5

Conclusion

The birds in study 1 and study 2 were challenged with the same 10^9 CFU/ml of *S.* Typhimurium, but the number of seeder birds in the second trial was increased from 1 to 2 birds. Increasing the amount of seeder birds with *S.* Typhimurium increased the amount of bacteria that were in the environment, thus improving the colonization between the birds. Multi-drug resistant *S.* Typhimurium results from the first experiment were inconclusive due to the lack of isolates recovered from the birds. In most cases only one other bird became a carrier for the resistant isolate of *S.* Typhimurium regardless of antimicrobial selective pressure. Given that the results of the first experiment contained higher percentages of *S.* Typhimurium in the ceca, the ceca was the only organ examined in the second experiment. In the second experiment both treatments had an increase in colonization compared to the first experiment. The birds were sacrificed on day 7 in both experiments so it is not possible to determine if the dose over time had an affect on the flock.

In the second experiment, the colonization of the resistant isolate within the birds treated with chlortetracycline was found to be significantly different then the colonization of birds not treated with antimicrobials. Antimicrobial selective pressure increased the amount of birds colonized with the resistant strain of *S.* Typhimurium which could persist in the environment and pass to future flocks. Even without the use of chlortetracycline the resistant strain colonized over half the flock.

Interestingly, the sensitive isolate colonization level was high in experiment 1 as well as experiment 2. Dose level had little effect on the colonization level between the flocks in either experiment. One explanation could be that the sensitive strain of *S.* Typhimurium had virulence factors that enabled it to spread through out the flock. The sensitive isolate was also able to gain resistance genes to multiple antibiotics in both experiments. The use of antibiotics in the flock created a multiple drug resistant pathogen with the ability to spread rapidly to a new host.

Based on the increasing amount of resistant bacteria, it is believed that antibiotic resistance in bacteria allows it to spread rapidly throughout an environment. However, according to the results from the sensitive isolate which spread rapidly through the flocks there are other factors involved in the transmission of a bacteria. Current studies are focused on limiting the use of antimicrobials in animals to slow the spread of resistant bacteria. However, future studies should focus on virulence genes as well as antibiotic resistance in pathogens. Also, using another antibiotic, such as extended spectrum cephalosporin, in the study of transmission may have a different effect on the spread of the antimicrobial resistant bacteria in an animal model.

In this study the sensitive isolate of *S.* Typhimurium colonized more birds than the antibiotic resistant isolate at both dosage levels. Also, the antibiotic sensitive isolate acquired genes for resistance in as little as a week. Therefore, the increase in resistant bacteria in the environment may not be due to the improved transmission of the antibiotic resistant pathogen but rather due to the ease at which sensitive bacteria can acquire resistance.