

PERSISTENCE, RECOVERY AND CHARACTERIZATION OF ANTIMICROBIAL
RESISTANT *SALMONELLA* SEROTYPES FOLLOWING *IN VIVO* CHALLENGE IN
BROILER CHICKENS

by

DOUGLAS EARL COSBY

(Under the Direction of Mark A. Harrison)

ABSTRACT

Salmonella is a major foodborne pathogen linked to poultry and poultry products. However, limited research regarding the *in vivo* interactions of various non-host specific salmonellae in broiler chicks is available. To evaluate the *in vivo* competitive effect between serotypes, 600 day of hatch chicks were obtained from a local commercial hatchery and divided between four treatment rooms (n=150/room). Seeder chicks inoculated with 1.8×10^4 cfu of *Salmonella* Kentucky resistant to tetracycline (SK^{TetR}) and seeder chicks inoculated with 2.3×10^4 cfu of *Salmonella* Heidelberg resistant to streptomycin (SH^{StrR}) were placed into three replicate treatment rooms; one room was maintained as a negative control. Ceca were removed on days 7 and 28 (n=15) and day 42 (n=30) and cultured for the presence of *Salmonella*. All control chicks were negative for *Salmonella* while 74, 77 and 67% of the ceca sampled from the treatment rooms were positive for SK^{TetR} , SH^{StrR} , or both strains of *Salmonella*, respectively. After processing with and without chlorine, 23, 15 and 5% were recovered from the chlorinated rinsates,

and 46, 20 or 15% from the non-chlorinated rinsates of SH^{StrR} , SK^{TetR} or both strains, respectively. Twenty SH^{StrR} isolates and 21 SK^{TetR} isolates were randomly selected to ensure representatives over sampling days and groups and were characterized by various genotypic and phenotypic tests; parent and inoculum strains were also characterized. Pulsed-field gel electrophoresis (PFGE) profiles using *XbaI* revealed a minimum of 96.7% and 97.2% genetic similarity within the SH^{StrR} and SK^{TetR} isolates, respectively. Antimicrobial resistance profiles ranged from three pan-susceptible isolates to 11 multidrug resistant profiles ($R \geq$ two classes of antimicrobials). Streptomycin resistance was detected in 18 of the 20 SH^{StrR} isolates and tetracycline resistance was detected in 20 of the 21 SK^{TetR} isolates. Four resistance genes, *tetA*, *tetB*, *strA* and *aadA1*, were probed by PCR and detected in 11, 10, 15 and 19 of the SH^{StrR} strains, respectively and in 19, 21, 16 and 14 of the SK^{TetR} strains, respectively. The recovered SH^{StrR} and the SK^{TetR} isolates had a > 96.6% genetic similarity when compared to the *Salmonella* strains used to inoculate the seeder chicks.

INDEX WORDS: *Salmonella*, Pulsed-field gel electrophoresis, Antimicrobial resistance, Broilers

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DEDICATION

I would like to dedicate this dissertation to my beloved family. Achieving this accomplishment would not have been possible without the support and love of my wife, Linda Cosby, my three sons, Benjamin, Daniel and Nicholas, my parents Ed and Jackie Cosby and my in-laws, Melvin and Virginia Fleming. Your love, support and understanding are what enabled me to reach this milestone in my life. Without all of your encouragement and sacrifices, I would not have been able to complete this monumental achievement. To all of you, my beloved family, I will be forever in your debt and you have all of my love.

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Sincerest thanks,

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CHAPTER 1

INTRODUCTION

The prevalence of *Salmonella enterica* associated with poultry and poultry meat products has been well documented (2, 4, 6, 7, 8) and these prevalence have both public health and economic implications. Scharff (18) estimated that the total cost for non-typhoidal *Salmonella* to be in excess of 14 billion dollars a year in the United States. Non-typhoidal *Salmonella* spp. were confirmed by laboratory analysis in almost 41,930 cases of domestically acquired foodborne salmonellosis annually, and the estimated total number of cases of foodborne salmonellosis is projected to be in excess of one million due to either physicians under reporting or by the afflicted individuals not seeking medical care (17).

Salmonella enterica is a zoonotic pathogen which can readily pass from animal to man through the consumption of contaminated food. It can also be acquired through contact with infected animals as well as through consumption of contaminated water (3, 6, 14, and 16). Clinical manifestations of human salmonellosis from non-Typhi *Salmonella* range from self-limiting gastroenteritis to severe bacteremia, while *Salmonella* Typhi is a non-zoonotic *Salmonella* and is responsible for typhoid fever. Generally, milder cases of disease (i.e., self-limiting gastroenteritidis) are not treated with antimicrobials and resolve within 5-7 days; however, extended duration of illness, cases resulting in septicemia, or cases involving immune-compromised individuals, may warrant antimicrobial therapies (1). Infections caused by antimicrobial resistant strains

may compromise treatment outcomes thus resulting in increased morbidity and mortality (9).

Of the six *S. enterica* subspecies, the majority of human and animal infections are caused by strains in subspecies I. Despite the close genetic relationship of serovars in subspecies I, major differences in virulence, host adaptation and host specificity have been characterized. These differences are important when categorizing salmonellae into three groups: broad-host range, host-adapted and host-restricted serovars.

Virulence determinants that define host range and degree of pathogenicity in a particular animal host are not fully understood. Four factors affect the virulence and pathogenicity in animal hosts (3). First, *Salmonella* pathogenicity and/or host range is complicated by the fact that some strains within a serotype are capable of asymptomatic colonization and/or persistence in a particular animal species while causing acute disease in other animal species. Second, most infections of livestock are sub-clinical as evidenced by the differences in the recovered isolates from surveillance studies vice the recovered isolates from clinical submissions due to animal illness. Third, some of the relative differences in pathogenicity and/or host range may be attributed to differential gene regulation. Fourth, pathogenicity and shedding in livestock are often dependent on both animal management and environmental events (such as increased population densities in grow out facilities; mechanical failures; and the amount of compliance by the farm laborers with known biosafety practices and regulations) that contribute to compromised animal health and/or increased pathogen exposure (3).

Any assessment of pathogenicity and risk to human and animal health depends on parameters such as the diversity of salmonellae present on the farms, clinical and sub-

clinical infections, the potential for regulatory and virulence differences within and among serovars, and management and environmental events that increase exposure to *Salmonella* and/or compromised host immunity (2). Also, differences between human lifestyles and animal rearing may lead to the selection of serovar variants that exhibit enhanced bacterial fitness in the present host when compared to the fitness in a previous host (12). Heithoff et al., (12) demonstrated distinct differences in the level of virulence present in *Salmonella* isolates recovered from animal hosts and those recovered from human salmonellosis patients when placed into mice to determine virulence attributes. Collectively, data generated from these types of studies will enhance our ability to devise treatment and intervention strategies that are host/species specific.

Jones et al., (13) in a review of data from all cases of *Salmonella* infection in FoodNet states during the 1996-2006 time period found six hundred-eighty-seven serotypes isolated from 46,639 cases. Eighty-nine percent of the isolates were from stool specimens, five percent were isolated from blood and four percent were from urine. The top five serotypes in human illness were *S. Typhimurium*, *Enteritidis*, *Newport*, *Heidelberg* and *Javiana*. *S. Heidelberg* was fourth on the list with 2,830 identified cases, 26% requiring hospitalization, 0.4% resulting in deaths and 13.4% resulting in invasive illnesses. *S. Kentucky* was 52nd on the list of serotypes from more than 50 identified cases with only 25% of patients requiring hospitalization. No deaths or invasive illnesses were reported.

S. Enteritidis emerged as a one of the more common serotypes reported worldwide and was an important cause of human illness in the United States in the 1980's acquired through contaminated eggs, egg products and other poultry products. An

estimated one in 20,000 eggs is internally contaminated and a one in 250 broiler chicken is contaminated with *S. Enteritidis* (5).

The emergence of antimicrobial resistant *Salmonella* recovered from meat products has heightened concerns regarding antimicrobial use in food animal production. Since 1997, the National Antimicrobial Resistance Monitoring System (NARMS) has collected data on antimicrobial resistant bacteria implicated in foodborne outbreaks. Using broth microdilution techniques, NARMS provides information regarding antimicrobial susceptibility to a custom made panel of antimicrobials for *Salmonella*, *Campylobacter* spp., generic *E. coli* and enterococci (15). These analyses also include information regarding multi-drug resistance (MDR), defined as resistance to 2 or more classes of antimicrobials. MDR is of particular concern as treatment options (when indicated) may be limited. Berrang et al., (2) found common resistance to tetracycline, ampicillin, streptomycin and cephalosporin derived antibiotics in *Salmonella* isolates recovered from broilers post-slaughter. Almost 45% of isolates were resistant to one or more antimicrobials, and 36% were MDR.

Since 1997, NARMS data reported the five predominant serotypes recovered from the carcass rinses of broiler chickens obtained after processing (in decreasing order of frequency) are *Salmonella enterica* subspecies *enterica* Kentucky, Enteritidis, Heidelberg, Typhimurium var. 5-, and Typhimurium. Three trends have also been observed: 1) from 1997 through 2006, *S. Kentucky* increased in number of isolates recovered for any year prior to declining in 2008 and 2009, 2) during the same period, *S. Heidelberg* decreased in number prior to increasing in 2008, and 3) the percent of *S. Enteritidis* has not varied over the same time period. In 2005 the poultry industry, in an

effort to control the increasing prevalence of *S. Kentucky*, implemented a variety of control measures including the vaccination of broilers for *S. Kentucky* (9). Whether by coincidence or effect these control measures corresponded with a decrease of *S. Kentucky* and an increase of *S. Heidelberg* isolates. Interestingly, *S. Enteritidis* has not been affected by the implementation of these rigorous control measures for the control of *S. Kentucky*. Dorea et al., (10) found a lower prevalence of *Salmonella* in the ceca and reproductive tracts of vaccinated breeder chickens when compared with non-vaccinated chickens. Dorea et al., (10) also reported a lower prevalence of *Salmonella* in the broiler chicks from vaccinated breeders and that broiler farms populated with the chicks from vaccinated breeders had fewer *Salmonella* positive environmental samples when compared to chickens and farms from non-vaccinated breeders. Gast (11) stated that vaccination can enhance the short-term responsiveness of control programs to address problems involving specific serotypes of elevated significance.

In 2003, the United States Department of Agriculture (USDA) established USDA VetNet, a program modeled after PulseNet USA, the national molecular subtyping network for foodborne disease surveillance. The objectives of VetNet are three-fold: 1) to use PFGE to subtype zoonotic pathogens submitted to the animal arm of NARMS; 2) to compare VetNet and PulseNet PFGE patterns; and 3) to use the data for surveillance and investigation of suspected foodborne illness outbreaks. The data from the NARMS Program identified two distinct areas for investigation which serve as the goals of this research. These goals are as follows (in no particular order): 1) Is the difference in prevalence rates for two of the top serotypes in poultry and poultry products due to

bacterial competition between *S. Kentucky* and *S. Heidelberg* *in vivo*? 2) Are there changes in recovered isolates after passage through the host animal(s)?

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CHAPTER 2

LITERATURE REVIEW

***Salmonella* taxonomy and history**

In the 19th century, the causative agent for typhoid fever was identified, which eventually became known as *Salmonella* (Andrew, et al., 2001). Salmon and Smith (1885) first isolated *Bacillus cholera-suis*, now called *Salmonella Choleraesuis* from swine diagnosed with hog cholera (Le Minor, 1981). While Smith was the first to actually identify the organism, Salmon was credited with the discovery which came to bear his name.

Bacteria of the genus *Salmonella* are Gram-negative, facultatively anaerobic, non-spore forming, usually motile rods (peritrichous flagella) belonging to the Enterobacteriaceae family which are associated with the alimentary tract of animals. Salmonellae reduce nitrates to nitrites; carbon-dioxide and hydrogen gases are usually produced from D-glucose; and hydrogen sulfide is typically produced by most salmonellae. Nearly all salmonellae are aerogenic except for *S. serovar Typhi* which never produces gas. Tests for indole production (tryptophanase), oxidase and urease are negative and 16S rRNA sequence analyses indicate that *Salmonella* belong to the Gammaproteobacteria (Popoff and LeMinor, 2005). The two *Salmonella* species, *S. enterica* and *S. bongori* were further separated by 16S rDNA sequence analysis and found to be closely related to the *Escherichia coli* and *Shigella* complex by both 16S and 23S rDNA analyses (Popoff and LeMinor, 2005). *Salmonella* species have an optimal

growth temperature of 35 to 40°C with a growth range of 2 to 54°C depending on the serotype and growth matrix involved.

Most of the *Salmonella* isolates recovered from cases of human infection belong to *Salmonella enterica* subspecies *enterica*. Mazzotta (2000) determined the D- (decimal reduction time or the time required at a certain temperature to kill 90% of the organisms being studied) and z- (thermal reduction time or the temperature required for the thermal destruction curve to move one log cycle) values of the commonly isolated *Salmonella* serotypes in ground chicken breast meat using an end-point procedure in pouches with a 7D process for *Salmonella* in chicken of approximately 3 s at 71.1°C and a z-value of 5.7°C. Salmonellae do not grow well at low temperatures (Sörqvist, 2003). However, salmonellae are hardy and not always killed by freezing (Obafemi and Davies, 1986). Most salmonellae survive well in acidic foods ($\text{pH} \leq 4.6$, Food and Drug Administration (FDA), 2011) and resist dehydration. They have long been considered some of the most important causal agents of foodborne illness throughout the world. Foodborne salmonellosis still occurs in developed, developing and under-developed countries, giving testimony to the importance of this bacterial genus in terms of human morbidity and mortality contributions (Bell, 2002). Many reports of salmonellosis are recognized as being sporadic in nature and often occur as isolated cases. However, improved methods for investigating foodborne disease combined with advancements in the collection and sharing of data on foodborne illnesses has enabled the identification of the etiologic agent linking individual illnesses into larger outbreaks.

Salmonella subspecies (serotypes) are antigenically differentiated by agglutination reactions with homologous antisera, and the combination of antigens

possessed by each strain is referred to as its antigenic formula; this antigenic formula is unique for each *Salmonella* serotype. Presently, the Kauffman-White scheme is used for assigning the serotype name to the unique antigenic formula (LeMinor, 1984). The antigens present on the surface of the bacterial cell include the somatic (O) or outer membrane antigens, the flagellar (H) antigen; and the capsular (Vi) antigen (Popoff and LeMinor, 2005). More than 2,500 *Salmonella* serotypes are recognized and this number increases every year (WHO, 2007). Additional methods for further differentiating *Salmonella* strains include phage typing (Rankin and Platt, 1995), pulse-field-gel-electrophoresis (PFGE) analysis (Swaminathan et al., 2001), PCR ribotyping (de Oliveira et al., 2007), antimicrobial resistance patterns (de Oliveira et al., 2007) and multilocus sequencing of DNA (Kotetishvili et al., 2002).

The ability of *Salmonella* species to cause human infection involves attachment and colonization of intestinal columnar epithelial cells and specialized micro-fold (M) cells overlying Peyer's patches (Ponka et al., 1995). Symptoms of salmonellosis include diarrhea, abdominal pain, nausea and vomiting lasting 1 to 7 days, and the illness is generally self-limiting in healthy adults with a mortality rate of < 1% (Andrews et al., 2001). In severe cases, infection may progress to septicemia and death, unless the person is promptly treated with the appropriate antimicrobials, presently fluoroquinolones, macrolides and third-generation cephalosporins (Klotchko and Wallace, 2011). Individuals who are immune-compromised, children, infants and elderly are most likely to require antimicrobial treatment. Infections with antimicrobial resistant strains may compromise treatment outcomes thus resulting in increased morbidity and mortality (WHO, 2011b). In rare instances, some individuals can develop chronic conditions

including reactive arthritis, Reiter's syndrome and ankylosing spondylitis (National Institute of Allergy and Infectious Disease, 2011).

The infective dose for salmonellosis in adult humans is estimated to be in the range of $10^4 - 10^6$ cells or colony forming units (cfu) or higher, but can be as low as $10^1 - 10^2$ cells in highly-susceptible individuals or if contained in a food with a high fat matrix, (i.e., chocolate, cheese, salami or peanut butter) (Angulo et al., 2000; Bell, 2002). The prevalence of *Salmonella enterica* associated with raw poultry and poultry meat products have been well documented (Bell, 2002, Cox et al., 2005, Bailey et al., 2010; Cox et al., 2010; Chai and Mahon, 2011), and have both public health and economic implications.

Salmonella enterica is a zoonotic pathogen which can readily pass from animal to humans through the consumption of contaminated meat, animal products or other food products after contamination with animal fecal material. Salmonellosis can also be acquired through direct or indirect contact with colonized animals as well as through consumption of contaminated water (de Oliveira et al., 2004; Bauer-Garland et al., 2006; Lammerding, 2006; Chai and Mahon, 2011). Salmonellae can also be considered a common commensal of the gut microflora of animals, including mammals, birds, reptiles, amphibians, fish and shellfish (Heinitz et al., 2000; Bailey et al., 2010). Fecal contamination is the main source of food and water contamination playing a large role in the dissemination of salmonellae in the environment and subsequently the food supply chain. Meat animals can be infected and act as reservoirs of salmonellae.

Scallan et al., (2011) estimated that of the 9.4 million cases of foodborne illnesses, 5.5 million (59%) were caused by viruses, 3.6 million (39%) by bacteria and 0.2 million (2%) by parasites in the United States. Non-typhoidal *Salmonella* accounted

for approximately 1.0 million (11%) of these illnesses, resulting in approximately 42,000 laboratory confirmed illnesses, 19,000 hospitalizations, and approximately 400 deaths (Scallan et al., 2011). Scallan et al., (2011) estimated that cases of salmonellosis were reported only half of the time and under diagnosed by a factor of 29.3. Using these factors combined with the confirmed case reports gives an estimate of almost 1.3 million cases of foodborne salmonellosis in the United States each year. The annual cost associated with salmonellosis in the U.S. has been estimated to be approximately \$14.6 billion (Scharff, 2010). Scharff (2010) estimated that the health-related economic cost of each foodborne illness in the U.S. is approximately \$2,000, taking into account quality of life (pain and suffering) calculations.

***Salmonella* in poultry and poultry products**

Among *Salmonella*-contaminated poultry carcasses, total numbers of *Salmonella* are generally low (FSIS, 2011). From the 2007-2008 baseline survey for young chicken, upon enumeration of the 1500 re-hang carcass samples qualitatively confirmed as positive, 11% were below the limit of detection (LOD), 42% ranged from 0.0301 to 0.3 MPN/mL, 34% ranged from 0.301 to 3.0 MPN/mL and only 11 samples were above 30 MPN/mL. From the 170 post-chill samples (n=3275) qualitatively confirmed as positive, none exceeded 30 MPN/mL, 46% of the positives ranged from 0.0301 to 0.3 MPN/mL, 14% ranged from 0.301 to 3.0 MPN/mL and 5% were in the 3.01 to 30 MPN/mL range (FSIS, 2008). However, human salmonellosis is often attributed to small numbers of *Salmonella* replicating through temperature abuse during storage, poor handling or improper cooking techniques and temperatures which are insufficient to kill the salmonellae prior to ingestion. The *Salmonella* serotypes most often isolated from young

chicken during the 2007-2008 Nationwide Microbiological Baseline Data Collection Program: Young Chicken Survey were *S. Kentucky*, Heidelberg, Typhimurium and Typhimurium (*var 5-*) (FSIS, 2008).

Salmonella accounted for 1,335 foodborne outbreaks and 36,490 associated illnesses in outbreaks reported to Food Disease Outbreak Surveillance System (FDOSS) from 1999-2008. Poultry accounted for a higher percentage of *Salmonella* outbreaks of infection compared to other food commodities. A single food source was reported in 35% (468) of the outbreaks; 29% (137) were due to poultry with 71% (97) of those due to chicken. Most reported cases of *Salmonella* infection are sporadic and outnumbered outbreak associated cases by more than 15 to 1 (Scallan et al., 2011). *S. Enteritidis* and Typhimurium were the serotypes most commonly reported in human illness (CDC, 2010f). *S. Kentucky* is the serotype most frequently recovered from carcass surveillance programs, while *S. Enteritidis* and *S. Typhimurium* are the first and second most common serotypes recovered from human illness (Jones et al., 2008).

Exposure to poultry meat has also been linked to *Salmonella* illness. A review of the Centers for Disease Control and Prevention (CDC) outbreak data from the past six years shows that seven out of twenty-five outbreaks were related to live poultry, shell eggs or further processed poultry products (Table 2.1). All of these outbreaks occurred over multiple states and Canadian provinces infecting more than 6,000 individuals and created multiple public health scares which resulted in several recalls and corrective actions. These outbreaks represent the individuals actually linked to an outbreak of salmonellosis and did not include all of the individual cases of salmonellosis within the outbreak, and not officially linked to the outbreak. Inclusion of these cases increases the

total numbers overall. Therefore we can conclude that poultry and poultry products are an important vehicle for human *Salmonella* infections in the United States.

Factors affecting *Salmonella* colonization in chickens

Factors known to affect *Salmonella* colonization include 1) age of the chicken, 2) environmental and physiological stressors (feed and water deprivation, dramatic temperature changes, etc.), 3) survival of *Salmonella* through the gastric barrier, 4) animal health and disease status of the chicken, 5) use of antimicrobials and or coccidiostats, 6) diet, and 7) genetic background of the chicks (Tilquin et al., 2005, Beaumont et al., 2009). Bacterial colonization and invasion are influenced by parameters specific to *Salmonella* and the effects of environmental stimuli (avian gastrointestinal tract) on gene expression (Dunkley et al., 2008).

One of the most important factors is the age of the birds. Newly hatched chicks are most susceptible to *Salmonella* colonization because they lack mature gut microflora or feed in the alimentary tract (Snoeyenbos et al., 1978). While very low doses of *Salmonella*, as low as 10 cells, can readily infect day-old chicks, the susceptibility of chicks to infection with *Salmonella* tends to decrease with age (Milner and Shaffer, 1952). Cox et al., (1990) found that 38% of intracloacally inoculated day-old chicks could be colonized with as few as two cells of *Salmonella*. Similarly it was determined that through oral and intracloacal inoculation, the number of cells required for a Colonizing Dose₅₀ (CD₅₀) was 100 times fewer than that of 3 day old chicks that had been fed. Gast and Holt (1998) challenged day old chicks to evaluate the persistence of *S. Enteritidis* through maturity (24 weeks of age) and demonstrated that although *S. Enteritidis* was usually cleared from internal organs within 8 weeks post-inoculation, the

production of internally contaminated eggs by a hen that was not shedding *S. Enteritidis* in her feces suggest that very extended persistence in internal organs can occur at a low frequency. Beal et al., (2005) determined that age and genetics affect the ability of chickens to resist *Salmonella* colonization.

One approach used to help control *Salmonella* colonization in chicks, particularly those which lack mature intestinal microflora, is competitive exclusion (CE). First reported by Nurmi and Rantala (1973), CE as a treatment involves the oral administration of intestinal microflora from healthy, salmonellae-free adult chickens to newly hatched chicks. This CE intestinal microflora is used to accelerate the maturation of the chicks' gut and can be either defined (known bacterial strains) or undefined (a complex of unknown bacterial cultures from an adult chicken's intestinal tract). Both defined and undefined CE cultures increase subsequent resistance to *Salmonella* colonization. The concept behind the use of probiotics is similar to that of competitive exclusion with the distinction that probiotics are intended to enhance the functions of the existing microflora (Wagner et al., 2009 and Wagner, 2006).

A second factor that can affect colonization is the ability of *Salmonella* to survive the passage through the pH of the gastrointestinal tract. Natural infection occurs mainly through the oral route and, in poultry, *Salmonella* encounter the acidic (pH ~4.5-5) environment of the crop (Farner, 1942). *Lactobacillus* strains present in the crop assist in maintaining the low pH associated with the crop environment, but upon feed withdrawal, a decrease in the lactobacilli population causes the crop pH to increase to approximately pH 6.0-6.3 (Durant et al., 1999 and Humphrey et al., 1993), providing a more suitable environment for survival of *Salmonella*.

Salmonella must survive passing through the proventriculus and gizzard which are also acidic environments. The pH of the proventricular contents becomes acidic (pH 2.0 to 4.0) about the 20th day of incubation and is indicative of the considerable secretion of hydrochloric acid by the proventricular glands with the actual onset of secretions beginning between days 11 and 13 of incubation in response to the ingestion of albumin by the embryo (Hill, 1971). In an *in vitro* study, Cox et al., (1972) reported a decreased survival rate for *Salmonella* spp. at pH 4.4 which corresponds to the proventriculus, with limited survival at pH 2.6 which is encountered in the gizzard. Finally, the pH of the small intestine (6.2) and large intestine (6.3) are closer to neutral and therefore more suited for *Salmonella* survival and proliferation in 3-week-old chickens (Jayne-Williams and Fuller, 1971). As with lactobacilli colonization, antimicrobial or anticoccidial feed additives may also influence *Salmonella* colonization by altering or reducing normal intestinal microflora (Cox et al., 2003). Regardless of what initiates the change, alterations in the protective gut microflora can increase a chicken's susceptibility to *Salmonella* colonization.

A third factor associated with colonization includes both the dose and strain of *Salmonella* to which the chickens are exposed (Milner and Shaffer, 1952, Sadler et al., 1969), including the ability of the strain to attach, colonize, and invade the various intestinal tissues (D'Aoust et al., 1991). Higher levels (10^4 - 10^5 cfu) of *Salmonella* are more likely to colonize chickens, and some *Salmonella* serotypes can colonize the avian intestinal tract more efficiently at lower levels than others (Barrow et al., 1988). However, *Salmonella* must first attach themselves to the host epithelial cells to initiate the processes of colonization and invasion (Finlay and Flakow, 1989; Khan et al., 2003).

Attachment is mediated by cell surface proteins known as adhesins, with the *Salmonella enterica* serovars possessing several fimbrial and non-fimbrial adhesins that are capable of binding to intestinal epithelial cells (Korhonen, 2007). The *Salmonella* Pathogenicity Island (SPI) 1 (discrete genetic units) contributes to colonization of the chicken with *Salmonella*, while SPI2, in the absence of SPI1, inhibits colonization (Dieye et al., 2009). *Salmonella* invasion is mediated by genes located on SPI1 (Bohez et al., 2006). Several studies have shown that mutations in these SPI1 specific genes can affect the intestinal colonization of young chicks (Porter and Curtiss, 1997; Turner et al., 1998; Morgan et al., 2004).

Rabsch et al., (2000), Callaway et al., (2008) and Foley et al., (2011) all analyzed epidemiological data collected through surveillance studies from the last half of the 20th century in the United States and Europe to explain the reduction of host specific *Salmonella*, specifically *S. Gallinarum* and *S. Pullorum*, in poultry production. These three studies support the theory that the increase in the prevalence of *Salmonella* Enteritidis and other non-host specific *Salmonella* serotypes in poultry and poultry products might be the result of the reduction and/or elimination of the host specific *Salmonella* serovar Gallinarum which includes the two biovars, Gallinarum and Pullorum. Rabsch et al., (2000) proposed that the increase in prevalence of *S. Enteritidis* was a result of the industry's actions which resulted in the reduction in the prevalence of *S. Gallinarum* and *S. Pullorum*. Since *S. Gallinarum* has no animal reservoirs other than domestic and aquatic fowl, the eradication left a niche which was filled by non-host specific *Salmonella* serovars, Heidelberg, Typhimurium and Enteritidis in particular (Foley et al., 2011). Thomson et al., (2008) sequenced the genomes of *S. Enteritidis* PT4

isolate P125109, a host-promiscuous serovar, and *S. Gallinarum* isolate 287/91, a chicken-restricted serovar. Genomic comparisons between these two genomes indicate that *S. Gallinarum* 287/91 is highly related to and likely a direct descendent of *S. Enteritidis* which has undergone extensive degradation through deletion and pseudogene formation (Thomson et al., 2008) which might explain the increase in *S. Enteritidis* colonization of chickens following the reduction and/or elimination of *S. Gallinarum* in the poultry industry.

Other studies looking at the competition between *Salmonella* serotypes in the gut of broiler chicks are almost non-existent. Nógrády et al., (2003) examined the growth suppression of *Salmonella* Hadar, *in vitro* under strict anaerobiosis and *in vivo* in the intestine of day-old chicks. Four strains were selected for evaluation of their ability to suppress the growth of *S. Enteritidis*, Typhimurium, Virchow and Saintpaul. Nógrády et al., (2003) were able to show that pre-colonization of the chicken with *S. Hadar* prevented the super-infection with any of the four mentioned serotypes. Ngwai et al., (2006) looked at the *in vitro* growth suppression of antibiotic resistant *Salmonella* Typhimurium DT-104 by non-DT104 strains. The non-DT104 strains were able to prevent the multiplication of the antimicrobial resistant DT104 strain when the DT104 strain was added in low numbers to 24 h cultures of the non-DT104 strains. The implication is that one *Salmonella* serotype might be able to prevent the colonization of another *Salmonella* serotype.

Horizontal transmission of *Salmonella* in poultry

Horizontal transmission of salmonellae among broiler and layer chickens has been demonstrated in studies conducted worldwide (Byrd et al., 1998; Liljebjelke et al., 2005;

Thomas et al., 2009; De Vylder et al., 2011 Roll et al., 2011). Byrd et al., (1998) found that after colonizing a minimum of 5 chicks per treatment pen with as few as 10^2 cfu per chick of *S. Typhimurium*, approximately 57% of the remaining birds became colonized with \log_{10} 2.2 cfu of *S. Typhimurium* per g of cecal contents by d 17 of grow-out. This population of salmonellae in the ceca increased when the seeder chicks were orally gavaged with larger concentrations of *S. Typhimurium*. Byrd et al., (1998) also recovered *S. Typhimurium* from litter samples at d 17, which indicates the potential for horizontal transmission of salmonellae from seeder chicks to contact chicks through the litter.

Liljebjelke et al., (2005) recovered *Salmonella enterica* from two integrated poultry systems over seven consecutive flocks isolating 15 different serotypes. *S. Typhimurium* and Enteritidis isolates from poultry carcasses shared the same PFGE pattern as those isolated from the rearing environment and from rodents caught in the same house implicating horizontal transmission as one means of spread of these *Salmonella* serotypes (Liljebjelke et al., 2005). However, indistinguishable PFGE types of *S. Typhimurium*, Enteritidis and Heidelberg were isolated from carcasses, the broiler chicken environment and chick-box liners which also implicates the hatchery as a source for these persistent serotypes on this farm (Liljebjelke et al., 2005).

Detection and characterization of *Salmonella*

Pulsed-field gel electrophoresis has been used and widely accepted as the gold standard for tracking outbreaks of foodborne illness since 1995 when the Centers for Disease Control and Prevention (CDC) selected four state public health laboratories for a national molecular subtyping network for foodborne bacterial disease surveillance

(Swaminathan et al., 2001; Jackson et al., 2007; Lawson et al., 2011; Wattiau et al., 2011). This network later became known as PulseNet (Stephenson, 1997) and has expanded to include countries all over the globe, from northern Canada to islands in the Pacific (CDC, 2011g).

For over 80 years, subtyping of *Salmonella enterica* for epidemiological surveillance has been performed by serotyping (Burr et al., 1998; Wattiau et al., 2011). Serotyping is a method in which surface antigens are used to indentify *Salmonella* serotypes based on agglutination reactions with specific antibodies. This has allowed for the long-term epidemiological surveillance of *Salmonella* in the food chain and in public health investigations (Wattiau et al., 2011). However, in epidemiological investigations, identification and tracking of salmonellosis outbreaks require the use of more sensitive methods for determining the causative strains at a taxonomic level than is achieved by serotyping alone (Herikstad et al., 2002; Lawson et al., 2011; Tabe et al., 2010; Wattiau et al., 2011). Pulse-field gel electrophoresis (PFGE) profiling is a DNA fingerprinting method based on the restriction digestion of purified genomic DNA and is currently considered the gold-standard for the subtyping of foodborne pathogens, especially *Salmonella* (Gerner-Scmidt et al., 2006; Swaminathan et al., 2006; Whittam and Bergholz, 2007). PFGE is the platform used by PulseNet, a national molecular subtyping network that was established in 1996 by the Centers for Disease Control and Prevention, (CDC) (Gerner-Scmidt et al., 2006; Stephenson, 1997). PulseNet is now utilized by all state public health laboratories and food safety laboratories at the Food and Drug Administration (FDA) and the United States Department of Agriculture (USDA) (Xia et al., 2009). Currently, PFGE data are considered a reliable and sensitive way to detect

differences between closely related strains (Wattiau, et al., 2011). Isolates with indistinguishable PFGE profiles can be classified as epidemiologically linked with a high degree of confidence (Whittam and Bergholz, 2007; Xia et al., 2009).

PFGE can be used to assess relatedness within *Salmonella* serotypes and has been useful during outbreak investigations (Swaminathan et al., 2006). The ability to track *Salmonella* serotypes through an animal model gives researchers the ability to follow the adaptations of *Salmonella* strains and to answer questions regarding the complex interactions between *Salmonella* serotypes in the animal hosts and/or the environment.

Definition of antibiotics and antimicrobial resistance

Antibiotics (chemical substances produced by various microorganisms), synthetic chemicals, disinfectants or drugs, collectively referred to as antimicrobial agents, have been used since the time of antiquity to treat patients with a variety of bacterial diseases (Saylers and Whitt, 2005). Since the 1940's, antibiotics have greatly reduced morbidity and mortality from infectious diseases. During the Second World War, the use of penicillin and sulfa drugs greatly improved the survival rate of injured and ill soldiers, sailors and marines fighting in less than hospitable locations (Saylers and Whitt, 2005; Alanis, 2005). Penicillin was the first used antibiotic to be discovered by Fleming in 1928 (Fairley, 2007). Since that time, scientists have discovered and developed a number of different classes of antimicrobials exerting bactericidal or bacteriostatic effects (Croft et al., 2007).

Although heralded as “wonder drugs”, antimicrobials can lose some level of efficaciousness as resistance develops. Antimicrobial resistance is a result of microbes changing to reduce or eliminate the effect of an antimicrobial to which it had previously

been susceptible. Soon after Fleming's discovery, he cautioned everyone that resistance to penicillin might not be long in developing and within a year of penicillin widespread use, he was proven correct as a number of strains developed resistance (Alanis, 2005). The pharmaceutical industry easily kept pace with the rapidly evolving resistant microorganisms that emerged during the middle part of the 20th century by developing new forms of the existing antibiotics and/or entirely new classes of antimicrobial drugs (Alanis, 2005; Croft et al., 2007).

Antimicrobial resistance can be intrinsic (part of the normal architecture of a bacterium) or acquired through exchange of DNA (Croft et al., 2007). Intrinsic resistance results through spontaneous mutation of genetic material which confers some new adaptation allowing the organism to resist the lethal effects of the antimicrobial agent. Spontaneous mutations can be either base-substitutions, frame shift mutations, deletions of genetic material or insertions of large DNA elements and can occur naturally at an average frequency of 1×10^6 per base pairs (Smith, 1992; Drake et al., 1998; Tenaillon et al., 2001). In acquired resistance, resistance factors in the form of plasmids, transposons or integrons move between bacteria either through conjugation, transformation or transduction (Saylers, 2002). Common drug resistant microorganisms include methicillin-resistant *Staphylococcus aureus* (MRSA) (Deresinski, 2005; Foster, 2004), multidrug resistant *Salmonella* spp. (Threlfall, 2002; Alcaine, et al., 2007), and multidrug resistant *Mycobacterium tuberculosis* (Alanis, 2005), all of which can be linked to increases in morbidity and mortality, especially in immune-compromised patients. This resistance can lead to longer, more expensive hospital stays and increased mortality from bacterial infections (World Health Organization, 2011a).

Some important factors in the development of resistance include selective pressures, proliferation of multiple resistant clones, and the inability to detect emerging phenotypes. These selective pressures can include overuse or misuse of antimicrobials in the treatment of human disease, in agriculture and in home disinfectants (Rybak, 2004).

In the past 60 or so years, physicians and pharmaceutical companies have been constantly challenged to stay one step ahead of bacteria which are adapting rapidly to antimicrobial drugs which have been developed for their control. While initially expected to virtually wipe out infectious diseases and deaths related to these pathogenic organisms by the middle part of the twentieth century (Croft et al., 2007), overuse and misuse of antimicrobials has resulted in their decreased efficacy. More and more of these pathogens have acquired or are acquiring the genetic material (either chromosomal DNA or plasmids) to effectively block the actions of these drugs and some bacteria have even become resistant to multiple drugs and classes of drugs, making them almost “pan-resistant” (Alanis, 2005). Infections resulting from resistant organisms once only found in hospitals and health care facilities are now commonly found in the community, creating a potential crisis for the future control of these pathogenic species (*i.e.*, methicillin resistant *Staphylococcus aureus*) (Pray, 2008). Additionally, the reduction of development of new antimicrobial drugs and classes of drugs by the pharmaceutical companies has virtually ceased due to 1) the increased cost associated with development, 2) the ethics and negative public opinion of animal and/or human testing and 3) an increase in government regulations required for the approval of any new antimicrobial drug or new use for an existing drug (Croft et al., 2007).

According to the CDC, over 47 million cases of domestically acquired foodborne illness occur annually in the United States, of which at least 70% of the pathogenic organisms involved are resistant to at least one antimicrobial drug. Approximately 3,000 people die in the U.S. each year from these illnesses. According to the CDC's website, drug resistant infections lead to longer hospital stays and more expensive treatments which may be less effective and even toxic to the patient (CDC, 2011f). This problem appears to be increasing rather than decreasing as more bacteria acquire multiple drug resistance (MDR).

In the mid to late 1980s, the medical community and consumers realized antimicrobial drugs might not be the "magic bullet" for control of bacterial infections and illnesses as once believed. Public and scientific interest in the administration of therapeutic and sub-therapeutic antimicrobials to animals increased due to the emergence and dissemination of MDR zoonotic bacterial pathogens (McDermott et al., 2002). The definition for MDR varies by laboratory and has been reported as resistance to 3 or more antimicrobials (CDC, 2010e). Currently, the National Antimicrobial Resistance Monitoring System (NARMS) defines MDR as resistance to 2 or more classes of antimicrobials (NARMS, 2009). Regardless, treatment of resistance to multiple classes of antimicrobials, particularly those involving the cephalosporins and fluoroquinolones (Angulo et al., 2000), has severely limited treatment options.

Mechanisms of drug resistance

The two primary routes which bacteria use for the development of antimicrobial resistance are spontaneous (natural) and acquired. Both mechanisms are forms of genetic modification of a microorganism for survival, Darwinism at work. In spontaneous

mutation, a genetic mutation naturally occurs conferring on the organism the ability to resist the lethal effects of an antimicrobial; the trigger for spontaneous mutation is unknown but exposure to the antimicrobial agent may provide selective pressure for antimicrobial resistance (Alanis, 2005).

Mechanisms of bacterial resistance vary and can be described as follows: 1) the oldest known mechanism of resistance is for the bacteria to produce specific proteins, usually enzymes, which alter the antimicrobial into a form which no longer has the intended mode of action. One example is the production of β -lactamases by *Salmonella* which inactivate the β -lactam class of antimicrobials (Foley and Lynne, 2008). 2) A second mechanism of resistance is the efflux pump which actively pumps antimicrobials out of the bacterium so that antimicrobial concentrations in the cell never reach the threshold necessary to interfere with the cell's metabolic processes (Croft et al., 2007). Tetracycline and chloramphenicol resistance in *Salmonella* isolates are examples of energy-dependent efflux pumps which remove the tetracycline and chloramphenicol from the bacterial cell before it can prevent the binding of tRNA to the A site of the 30 S ribosomal subunit, thus inhibiting protein synthesis (Mascaretti, 2003; Foley and Lynne, 2008). 3) A third mechanism of resistance is to chemically change or mutate the target which the antimicrobial works on, preventing binding of the antibiotic to the target, also known as receptor modification (Croft et al., 2007). This is observed for vancomycin-resistant enterococci which mutate the terminal peptides from D-Ala-D-Ala to D-Ala-D-Lac which has a lower affinity to vancomycin (Croft et al., 2007). One thing is certain; bacteria have demonstrated an extraordinary capability to survive.

Antimicrobial resistance mechanisms in *Salmonella* by antimicrobial class

Aminoglycosides

Aminoglycosides were first discovered in 1943 when streptomycin was isolated from *Streptomyces griseus* (Gonzales and Spencer, 1998). Other commonly known compounds in this class of drugs include gentamicin, neomycin, amikacin and kanamycin (Gonzales and Spencer, 1998). These drugs are effective for treating infections caused by Gram-negative bacilli and are usually used in combination with glycopeptides and β -lactams to ensure a broad spectrum of action (Gonzales and Spencer, 1998; Graham and Gould, 2002). Aminoglycosides bind to conserved sequences within the 16S rRNA of the 30S ribosomal subunit (Mascaretti, 2003) which leads to codon misreading and translation inhibition. Most aminoglycosides are bactericidal with the exception of spectinomycin which is bacteriostatic (Mascaretti, 2003). Primary mechanisms for non-typhoidal *Salmonella* to resist aminoglycosides are (i) decreased drug uptake, (ii) drug modification and (iii) modification of the ribosomal target of the drug (Alcaine et al., 2007).

Beta-lactams

Penicillins, cephalosporins and carbapenems are the three major groups of beta-lactams. The antimicrobial effects of these drugs are mediated by their ability to interfere with a group of proteins known as penicillin-binding proteins, which are involved in the synthesis of peptidoglycan, a component of the bacterial cell wall. Beta-lactams are generally bactericidal, but the activity varies among beta-lactams, organisms and target penicillin-binding proteins (Alcaine et al., 2007). Beta-lactams must cross the bacterial outer membrane to reach their penicillin-binding protein targets. This passage is

facilitated by two porins, OmpC and OmpF (Alcaine et al., 2007). While changes or loss of the porins are uncommon mechanisms of resistance, some cases have been documented where a decrease in either OmpF or OmpC porin concentrations resulted in observable increases in resistance to beta-lactams such as ampicillin, cefoxitin and other cephalosporins (Alcaine et al., 2007).

In *Salmonella*, an inhibition of the essential penicillin-binding proteins leads to bactericidal activity. With the widespread use of penicillins, resistance to ampicillin, methicillin and other penicillin drugs are common (Angulo et al., 2000). The most common mechanism of resistance is the secretion of beta-lactamases into the periplasmic fluid for gram-negative microorganisms and into the environment for gram-positive microorganisms. These enzymes hydrolyze the beta-lactam ring into beta-amino acids which has no antimicrobial activity. The genes encoding for beta-lactamase production are typically carried on plasmids (Mascaretti, 2003). *Staphylococcus* resistance to methicillin has become particularly worrisome as MRSA has emerged as a serious problem (Pray, 2008). In response to beta-lactam resistance, a second class of beta-lactams, the six-member ringed cephalosporins was developed. Carbapenems are the latest group of beta-lactams containing a five-member ring without sulfur bound to the four-member beta-lactam ring (Mascaretti, 2003). They have become particularly important in treatment of acute otitis media, an important health problem in early childhood and the most frequent condition for which antimicrobials are prescribed for children in the U.S. (Arrieta et al., 2003; Holten and Onusko, 2000). Beta-lactams have a broad range of activity against gram-negative and gram-positive bacteria, with the later generations having the broader spectrum of activity.

Phenicol

Chloramphenicol, once the drug of choice for the treatment of typhoid fever, and florfenicol, the newest phenicol, are included in this class of antimicrobial drugs (Mascaretti, 2003). Chloramphenicols produced by *Streptomyces venezuelae* were discovered in 1947 and work by binding to the peptidyltransferase center of the 50S ribosomal unit, preventing the formation of peptide bonds (Mascaretti, 2003).

Chloramphenicols have a broad range of activities against both Gram-positive and Gram-negative bacteria and are able to cross the blood-brain barrier making them a powerful choice in systemic infections (Alcaine et al., 2007). However, chloramphenicols are limited in use except in developing countries due to the widespread resistance and toxicity.

Resistance in *Salmonella* isolates is conferred by two mechanisms: (i) enzymatic inactivation of the antibiotic by chloramphenicol O-acetyl-transferase and (ii) removal of the antibiotic by an efflux pump. Neither chloramphenicol acetyltransferase (CAT), the enzyme responsible for most of the plasmid mediated resistance to chloramphenicol (Cannon et al., 1990), nor the known non-enzymatic chloramphenicol resistance genes (*cmlA* and *cmlB*) confer resistance to florfenicol (Dorman and Foster, 1982; Keys et al., 2000). However, both mechanisms are known to be effective in conferring chloramphenicol resistance in *Salmonella* serotypes, especially Typhimurium and Agona (Schwarz and Chaslus-Dancla, 2001). Development of florfenicol for use in animal husbandry was intended to decrease the resistance to chloramphenicol in humans. Florfenicol was approved by the Food and Drug Administration (FDA) in 1996 for the treatment of bovine respiratory pathogens and is not currently approved for use in

humans (White et al., 2000). Chloramphenicol was banned from veterinary use in Europe in 1994, while florfenicol was approved for use in 1995 in France (Arcangioli et al., 1999).

Quinolones and Fluoroquinolones

Quinolones and fluoroquinolones are synthetic bactericidal drugs and nalidixic acid was the first medically approved quinolone (Mascaretti, 2003). The early quinolones targeted DNA gyrase while the later generations of quinolones target DNA gyrase and DNA topoisomerase IV (Wolfson and Hooper, 1989). The mode of action for quinolones is complex and not fully understood (Mascaretti, 2003). High level resistance to quinolones is still rare (Casin et al., 2003; Olsen et al., 2001), but some *Salmonella* isolates with resistance to nalidixic acid and low-level resistance to other quinolones have been documented (Molbak et al., 1999; Breuil et al., 2000).

Two mechanisms of resistance occur. The first mechanism is mediated by target mutations in the quinolone resistance determining region of gyrA and gyrB in the parC subunit of topoisomerase IV (Cloeckaert and Chaslus-Dancla, 2001; Baucheron et al., 2004). The second mechanism involves alterations in the expression of the AcrAB-TolC efflux system through mutations in the genes encoding the system regulators resulting in the over expression of this efflux system and decreasing quinolone sensitivity (Baucheron et al., 2004; Oliver et al., 2005). No single mutation confers high-level resistance to the quinolones; instead, it is the result of an accumulation of various mutations (Heisig, 1993).

When fluoroquinolones were first licensed for human therapy, no immediate rise in *Salmonella* resistance was observed. After the licensing of fluoroquinolones for

animal use, the rates of fluoroquinolone-resistant *Salmonella* in animals and food and subsequently in human infections rapidly increased in several countries (WHO, 2011b). Currently, six fluoroquinolones have been approved for animal use in the U.S., enrofloxacin, danofloxacin, orbifloxacin, difloxacin, marbofloxacin, and sarafloxacin (Martinez et al., 2006). However, two of these drugs, sarafloxacin and enrofloxacin which were licensed for treatment of respiratory diseases in poultry, have been removed from the approved list due to increased antimicrobial resistance in *Campylobacter* and *Salmonella* species recovered in human illnesses (Nelson et al., 2007).

Tetracycline

Chlortetracycline was isolated from *Streptomyces aureofaciens* in the 1940s and this family of drugs became popular because of their broad spectrum of activity with minimal adverse effects (Alcaine et al., 2007). Tetracyclines act by preventing the binding of tRNA to the A site of the 30S ribosomal subunit, thus inhibiting protein synthesis (Mascaretti, 2003). Tetracycline resistance in *Salmonella* isolates is generally attributed to the production of an energy-dependent efflux pump, which removes tetracycline from the bacterial cell. Other mechanisms of tetracycline resistance have been documented in other bacterial species but not yet reported among *Salmonella* isolates (Chopra and Roberts, 2001).

There are at least 32 different genes that confer resistance to tetracycline and oxytetracycline with *tet(A)*, *tet(B)*, *tet(C)*, *tet(D)*, *tet(G)* and *tet(H)* most often found in *Salmonella* isolates (Chopra and Roberts, 2001; Mascaretti, 2003). The most commonly reported of these is *tet(A)* which is located within *Salmonella* genomic island 1 (Carattoli et al., 2002), on integrons (Briggs and Fratamico, 1999) and on transferrable plasmids

(Frech and Schrawz, 1999; Pezzella et al., 2004; Gebreyes and Thakur, 2005). The *tet(B)* gene is also relatively common and located on transferable plasmids (Guerra et al., 2002). These genes appear to be easily transferred and widespread among *Salmonella* isolates and are almost always present in isolates that display multidrug resistance (Carattoli et al., 2002; Chen et al., 2004; Pezzella et al., 2004) which might make them important markers enabling the identification of potentially serious *Salmonella* infections.

Tetracycline and 31 other antimicrobials were approved for use in broiler feeds in the U.S. without a veterinary prescription for the treatment of coccidiosis, growth promotion and other purposes in 1951 (Jones and Ricke, 2003). Beginning in the late 1950s and 1960s each European state has approved its own national regulations concerning the use of antibiotics in animal feeds (Castanon, 2007). Diarra et al., (2007) found that isolates recovered from broiler chickens over a 35 day grow-out period showed some degree of multiple antibiotic resistances. The consequences of poultry production for environmental, food safety, and animal welfare issues are now part of consumers' opinions and demands (Dibner and Richards, 2005). Decreased use of antimicrobial growth promoters is both consumer and legislative driven (Dibner and Richards, 2005; Diarra, et al., 2007; Rahmani and Speer, 2005).

Sulfonamides and Trimethoprim

These two classes of antimicrobials have been used in combination for the treatment of bacterial infections since the late 1960s. They are bacteriostatic and competitively inhibit enzymes involved in synthesizing tetrahydrofolic acid (Alcaine et al., 2007). Sulfonamides are structural analogues of *para*-amino benzoic acid (PABA) and compete with PABA in the synthesis of dihydrofolic acid effectively inhibiting

dihydrofolate synthetase (DHPS) in bacteria which synthesize folate (van Duijkeren et al., 1994). As a result, sulfonamides do not affect mammalian cells because mammals do not synthesize folate; instead folate is taken up directly from food (Bushby, 1980).

Trimethoprim inhibits dihydrofolate reductase (DHFR) (Mascaretti, 2003). Sulfonamide resistance in *Salmonella* isolates has been attributed to the presence of an extra *sul* gene, which expresses an insensitive form of DHPS (Antunes et al., 2005; Mascaretti, 2003).

Trimethoprim resistance is attributed to the expression of DHFR which does not bind trimethoprim (Mascaretti, 2003).

Combinations of trimethoprim and sulfonamides have been used in veterinary practice since 1970 because of their wide spectrum of activity, clinical efficacy and relatively low cost (Bushby, 1980). Trimethoprim/sulfonamides combinations are used in the treatment of diseases caused by gram-positive and gram-negative bacteria to include infections of the respiratory tract, urogenital tract, alimentary tract, skin, joints and wounds (van Duijkeren et al., 1994).

Transmission of antimicrobial resistance in *Salmonella*

Two mechanisms are implicated in the spread of antimicrobial resistance in *Salmonella* populations, (1) horizontal transfer of genes for antibiotic resistance and (2) clonal spread of antimicrobial drug-resistant *Salmonella* isolates (Molbak et al., 1999; Butaye et al., 2006). Resistance genes can be horizontally transferred between *Salmonella* strains or from other bacterial species to the *Salmonella* strains (Chen et al., 2004). In *Salmonella*, plasmids and class I integrons are primarily responsible for horizontal transmission (Chen et al., 2004; Dieye et al., 2009). Other species can contribute resistance genes not currently found in the *Salmonella* gene pool through this

mechanism. Resistance genes for the various antimicrobial drug classes can be found on several different plasmid types and many of these plasmids carry multiple antimicrobial resistance genes which can be transferred to other *Salmonella* and other bacterial species (Guerra et al., 2001; Doublet et al., 2004; Villa and Carattoli, 2005). Integrons are elements that contain the genetic determinants of components of a site-specific recombination system that recognizes and captures mobile gene cassettes (Fluit, 2005). Integrons contain the gene for an integrase (*int*) and an adjacent recombination site. Although gene cassettes are not necessarily part of the integron once incorporated, they become part of the integron (Villa and Carattoli, 2005). Two integron classes exist, resistance and super-integrons. Nearly all gene cassettes from resistance integrons encode resistance to antibiotics or disinfectants (Fluit, 2005). Class I and class II integrons have been found in *Salmonella*. Class I integrons are primarily in the *Salmonella* genomic islands (Fluit, 2005) while class II integrons are embedded in the TN7 family transposon but have not been fully described (Carattoli, 2003).

Antimicrobial resistance as a global problem

Antimicrobial resistance is widespread according to the American Academy of Pediatrics (Shea et al., 2004). It has been elevated by major world health organizations as one of the top health challenges of the 21st Century (CDC, 2010e; FDA, 2000). Antimicrobial resistance is also increasing among human pathogens. Bacteria resistant to multiple antimicrobials are of particular concern. In some cases, few or no antibiotics are available to treat resistant pathogens (Institute of Medicine (IOM), 1998; Molbak et al., 1999). The escalating resistance has raised concern that we are entering the “post-antibiotic era,” meaning we may be entering a period where there would be no effective

antimicrobials available for treating many life-threatening infections in humans (Gilchrist et al., 2007). If this is true, deaths due to infection will once again become a very real threat to substantial numbers of children, young adults, sick and elderly individuals.

Overuse and/or misuse of antimicrobials in both veterinary and human medicine is responsible for the increasing crisis of antimicrobial resistance (Gilchrist et al., 2007). In 2001, the Union of Concerned Scientists estimated that over 11.2 million kilograms (kg) of antimicrobials were used as growth promoters in animals compared to 1.4 million kg of antimicrobials for human medical use (Union of Concerned Scientists, 2001). Volumes have been written on direct and indirect evidence linking animal use of non-therapeutic antimicrobials to the antimicrobial resistance now confronting humans (Marshall and Levy, 2011).

One of the most effective ways to select for resistance genes in bacteria is to expose bacteria to low doses of broad-spectrum antimicrobials (Shea et al., 2004). Levy et al., (1976) examined the effect of low-dose tetracycline in feed on the intestinal flora of chickens. When comparing the antimicrobial resistance of bacteria isolated from chickens feed low doses of tetracycline to bacteria isolated from birds feed a diet without tetracycline, resistance increased after 36 hours on a diet with low levels of tetracycline and after two weeks approximately 90% of the chickens in the experimental group were excreting bacteria all of which were resistant to tetracycline (Levy et al., 1976). Another trend observed was that feeding tetracycline to the chickens in the experimental group resulted in the development of multidrug resistance among the microorganisms recovered. Resistance to not only tetracycline, but also to sulfonamides, streptomycin, ampicillin and carbenicillin developed through plasmid transfer (Shea et al., 2004). This

resistance extended over time to the control birds although at lower levels and subsequently to the farm workers. Six months after the removal of tetracycline from feed on the farm, no tetracycline-resistant bacteria were isolated from 8 of 10 farm workers tested (Levy et al., 1976).

When animals become colonized by resistant organisms, these organisms spread to other animals and eventually humans either through the food chain, direct contact or contamination of the environment with animal excreta (Witte, 1998). The increasing industrialization of food animal production increases the stress on the animals which causes increased bacterial shedding and the inevitable contamination of hides, carcasses and meat with fecal bacteria (Barkocy-Gallagher et al., 2001; Millemann et al., 2000). There is also an increase the amount of active antimicrobials detected near waste lagoons, surface waters and river sediments (Halling-Sorensen et al., 1998). The presence of these antimicrobials in the environment raise concerns that microbial populations might be under selective pressure stimulating horizontal gene transfer and amplifying the number and variety of organisms that are resistant to antimicrobials (Shea et al., 2004). Chee-Sanford et al., (2001) found resistance genes identical to those found in swine waste lagoons, in groundwater, and in soil microbes hundreds of meters downstream.

While it was hoped by many that the years of experience following the bans on antimicrobials as growth promotants in Europe would precede an end to the use of antimicrobials as growth promotants in the U.S., arguments continue based on the lines of cost-to-benefit ratios and perceived deficits in solid scientific evidence (Marshall and Levy, 2011). The European Common Market began by issuing a ban against the use of tetracycline in the mid-1970s and the bans continued until a total ban on the use of

antimicrobials as non-therapeutic growth promotants was enacted in 1999 by the European Union (Marshall and Levy, 2011). Industry voiced concern that the total withdrawal of antimicrobials from non-therapeutic uses would lead to an increase in the disease rate of the food animals and thus to an increase in the use of therapeutic antimicrobials (Marshall and Levy, 2011). In Denmark, a different result seems to have appeared after initial negative after-effects. Farmers have modified their animal husbandry practices accommodating for the loss of the banned antimicrobials resulting in improved immunity and reduced infection rates leading to fewer demands for therapeutic antimicrobials (Marshall and Levy, 2011).

Conclusions

Salmonella species continue to be one of the major causes of bacterial illnesses in the United States causing an estimated 1.4 million cases a year. These cases are linked to foodborne outbreaks, live animal contact, poor hygiene and environmental exposure. Much research has been conducted on virulence, pathogenicity and invasiveness of the various serotypes in humans and animals. With the emergence of antimicrobial resistance, the pathogenicity and virulence of certain *Salmonella* serotypes have increased and treatment options are decreasing and becoming more expensive.

The effectiveness of antimicrobials, long considered “wonder drugs” and “silver bullets” for the treatment and control of bacterial infections, has rapidly been decreasing due to the development of resistance mechanisms. Bacteria are able to obtain genetic material which allows for the survival and selection of antimicrobial resistant cell lines. The acquisition of resistance has been linked to the selective pressure applied when antimicrobials are either overused (too often and in the wrong concentrations) or misused

(the wrong antimicrobial selected for use) in animal production or human medicine. Politicians, farmers, scientists and consumers are becoming more concerned with the increase in antimicrobial resistance and measures are being taken to reduce the amount of antimicrobials used in animal husbandry either through regulation or education of producers, doctors and consumers.

In 2002, the Facts about Antimicrobials in Animals and Their Impact on Resistance (FAAIR) made the following recommendations: 1) Antimicrobial agents should not be used in agriculture in the absence of disease, 2) Antimicrobials should be administered to animals only when prescribed by a veterinarian, 3) Quantitative data on antimicrobial use in agriculture should be made available to inform public policy, 4) The ecology of antimicrobial resistance should be considered by regulatory agencies in assessing human health risk associated with antimicrobial use in agriculture, 5) Surveillance programs for antimicrobial resistance should be improved and expanded, and 6) The ecology of antimicrobial resistance in agriculture should be a research priority (FAAIR Scientific Advisory Panel, 2002). Implementation of these six recommendations along with further research into the mechanisms and the ecology of antimicrobial resistant bacteria, especially *Salmonella* species, may provide a return to the effectiveness of antimicrobials in treating infections caused by pathogenic bacteria.

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Table 2.1. Reported salmonellosis outbreaks in the U.S. and Canada 2006-2011

Source	Year	Location	No. of cases	Serotype	Reference
Ground Turkey	2011	Multistate (26) ^a	78	<i>S. Heidelberg</i>	CDC, 2011b
Cantaloupe	2011	Multistate (9)	20	<i>S. Panama</i>	CDC, 2011c
Chicks and Ducklings	2011	Multistate (16)	49	<i>S. Altona</i>	CDC, 2011e
Chicks and Ducklings	2011	Multistate (12)	22	<i>S. Johannesburg</i>	CDC, 2011e
Turkey Burgers	2011	Multistate (10)	12	<i>S. Hadar</i>	CDC, 2011d
Alfalfa Sprouts	2010-2011	Multistate (27)	140	<i>S. I 4,[5],12:i:-</i>	CDC, 2011a
Alfalfa Sprouts	2010	Multistate (11)	44	<i>S. Newport</i>	CDC, 2010a
Alfalfa Sprouts	2009	Multistate (14)	234	<i>S. Saintpaul</i>	CDC, 2009c
Shell Eggs	2010	Multistate (11)	≥ 1,939	<i>S. Enteritidis</i>	CDC, 2010b
Frozen Entrée	2010	Multistate (18)	44	<i>S. Chester</i>	CDC, 2010c
Red and Black Pepper / Italian Style Meats	2009-2010	Multistate (44)	272	<i>S. Montevideo</i>	CDC, 2010d
Peanut Butter and Peanut Butter Products	2008-2009	Multistate (46) and Canada	714 (U.S.) 1 (Can)	<i>S. Typhimurium</i>	CDC, 2009b
Raw Produce (Jalapeno Peppers)	2008	Multistate (43), DC and Canada	1,442 (U.S.) 5 (Can)	<i>S. Saintpaul</i>	CDC, 2008c
Malt-O-Meal Rice/Wheat Cereal	2008	Multistate (15)	28	<i>S. Agona</i>	CDC, 2008a
Cantaloupes	2008	Multistate (16)	51	<i>S. Litchfield</i>	CDC, 2008b
Banquet™ Pot Pies	2007	Multistate (35)	> 272	<i>S. I 4,[5], 12:i:-</i>	CDC, 2007a
Veggie Booty	2007	Multistate (20)	65	<i>S. Wandsworth</i>	CDC, 2007d
Peanut Butter	2007	Multistate (44)	425	<i>S. Tennessee</i>	CDC, 2007b
Live Poultry (chicks)	2007	Multistate (2)	65	<i>S. Montevideo</i>	CDC, 2009a
Live Poultry (chicks)	2007	Multi-state (23)	64	<i>S. Montevideo</i>	CDC, 2009a
Live Poultry (chicks)	2006	Michigan	21	<i>S. I 4, 5, 12, i:-</i>	CDC, 2006b
Live Poultry (chicks)	2006	Multistate (21)	56	<i>S. Montevideo</i>	CDC, 2006b
Live Poultry (chicks)	2006	Oregon	4	<i>S. Ohio</i>	CDC, 2006b
Tomatoes	2006	Multistate (21)	183	<i>S. Typhimurium</i>	CDC, 2006a
Poultry Vaccine Production	2006	Maine	21	<i>S. Enteritidis</i>	CDC, 2007c

^a Number in parenthesis indicates the number of states involved in the outbreak.

CHAPTER 3

COLONIZATION OF DAY OF HATCH BROILER CHICKS WITH
ANTIMICROBIAL RESISTANT STRAINS OF *SALMONELLA* HEIDELBERG AND
KENTUCKY¹

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ABSTRACT

Salmonella is a major foodborne pathogen linked to raw poultry and poultry products. However, limited research regarding the *in vivo* interactions of non-host adapted *Salmonella* serotypes in broiler chicks is available. In order to evaluate the effect of two serotypes on co-colonization, 600 day of hatch chicks were obtained from a local commercial hatchery and divided between four treatment rooms (n=150/room). Forty-five seeder chicks were orally inoculated with 1.8×10^4 cfu of *Salmonella* Kentucky resistant to tetracycline (Tet, SK^{TetR}) while an additional 45 seeder broilers were inoculated with 2.2×10^4 cfu of *Salmonella* Heidelberg resistant to streptomycin (Str, SH^{StrR}). Fifteen wing-banded seeder broilers of each serotype were placed into three replicate treatment rooms; one additional room was maintained as a negative control with the same bird density and 30 of these chicks gavaged with sterile saline. On days 7 and 28 post commingling, the ceca of 15 non-seeder broilers per room were aseptically removed and cultured, and on day 42, the ceca of 30 non-seeder broilers were removed and cultured for the presence of *Salmonella*. Ceca from all control room chicks sampled were negative for *Salmonella* while on day 42, the ceca from the treatment rooms containing seeder chicks were *Salmonella*-positive, 83% SK^{TetR}, 96% SH^{StrR}, and 82% both serotypes of *Salmonella*. After processing on day 43, a statistically significant difference in the recovery of SK^{TetR} and SH^{StrR} was detected from post chill carcass rinsates ($P < 0.05$). After processing and immersion chilling with or without chlorine, carcass *Salmonella* recovery was 23, 15 and 5% for chlorine chilled carcass rinsates (n=60) and 46, 20 and 15% from non-chlorine chilled rinsates (n=59) for SH^{StrR}, SK^{TetR} or both serotypes. Since there were no overall differences detected from the cecal

samples during grow-out and the fact that all ceca were positive for both serotypes on day 28, no exclusion between these serotypes was observed in this study during grow-out. Additionally, *Salmonella* serotype prevalence levels in ceca samples (90%) collected prior to the day of processing (pre-feed withdrawal) may not directly be predictive of the levels of recovered *Salmonella* serotypes from post-chill carcasses (19% chlorine chilled and 33% non-chlorine chilled).

Key words: Antimicrobial Resistance, Colonization, *Salmonella*, Broilers

INTRODUCTION

Salmonella continues to be a leading cause of foodborne enteric disease in many countries and is responsible for significant human suffering, loss of productivity and even mortality (Scallan et al., 2011). There is a plethora of information regarding the prevalence of *Salmonella*-contaminated raw poultry, with contamination of poultry and poultry products being commonly reported as being between 20 and 50% (Cox et al., 2005; Foley et al., 2008).

The increased isolation of non-host-specific *Salmonella* Enteritidis from both poultry products and human salmonellosis is of concern (Altekruse et al., 2006; Jones et al., 2008; Frederick and Huda, 2011). These concerns extend globally and efforts to control *Salmonella* in the poultry industry have become more intensive (Mead et al. 2010). However, control of *Salmonella* in the poultry industry is a difficult prospect. There are multiple constraints (presence of *Salmonella* in chicks obtained from local hatcheries, presence of rodents, insects and other birds on the farm, environmental stressors during grow-out, cross contamination in the transport coops, etc.) in the production of live poultry and poultry products that limit the ability to either reduce or eliminate the microbial hazards associated with raw poultry and poultry products (Gustafon and Kobland, 1984; Goren et al., 1988; Curtiss et al., 1993; Byrd et al., 2001; Humphries et al., 2001; Line, 2002; Van Immerseel, 2005; Burkholder et al., 2008; and Dorea et al., 2010).

Literature can provide any number of examples regarding *Salmonella* persistence and transmission in poultry production (Byrd et al., 1998; Bailey et al., 2001; Liljebjelke et al., 2005; Altekruse et al., 2006). However, despite global efforts to control

Salmonella, one of the biggest hurdles involves our limited knowledge regarding the interactions of multiple serotypes in the chickens and/or the environment. The purpose of this study was to utilize two *Salmonella* serotypes with different antibiotic resistance profiles to study the interactions between these *Salmonella* serotypes in the lifespan of a broiler.

MATERIAL AND METHODS

Bacterial Strains, Growth and Maintenance.

Strain selection. Two *Salmonella* serotypes (*Salmonella* Kentucky and *Salmonella* Heidelberg) were selected for use from the FY2004 NARMS collection of cultures based on a single resistance to separate classes of antimicrobial compounds (aminoglycosides and tetracycline). The strains were re-examined by the broth microdilution methods of NARMS to ensure no change in resistance pattern had occurred during storage.

Maximum level of resistance to the appropriate antimicrobials, Str for *S.* Heidelberg and Tet for *S.* Kentucky was determined by plating the isolates on BGS containing the appropriate antimicrobials in 25 ppm increments from 50 to 400 ppm (150 ppm Str for SH^{StrR} and 275 ppm Tet for SK^{TetR}). Each strain was plated on BGS plates with both antimicrobials to ensure no cross resistance was observed (data not shown).

S. Kentucky was selected because it was consistently in the top five serovars recovered from raw poultry and meat products and has a high prevalence rate in chicken carcass rinse samples (FSIS, 2010). Whereas, *S.* Kentucky is frequently isolated from broiler chickens it is infrequently recovered from human clinical isolates associated with foodborne illness (Jones et al., 2008). *S.* Heidelberg was selected because it is also among the top five serovars listed for raw poultry and meat products as well as from

human clinical isolates submitted to Centers for Disease Control and Prevention (CDC) (CDC, 2009). The use of these two *Salmonella* serovars provided the ability to differentiate between the serovars based on both serogrouping methodology (B for SH^{StrR} and C3 for SK^{TetR}) and through the use of antimicrobials in the media based on the resistance pattern of the serovar.

Maintenance. *Salmonella* serovar Kentucky^{TetR} and *Salmonella* serovar Heidelberg^{StrR} strains, obtained from the National Antimicrobial Resistance Monitoring Service (NARMS, U.S. Department of Agriculture), were grown on either blood agar (BA, Remel Products, Lexena, KA) or brilliant green sulfa agar (BGS, Becton-Dickinson (B-D), Franklin Lakes, NJ) with 125 ppm streptomycin (Str, Sigma Chemical Co. (Sigma), St. Louis, MO) or 200 ppm tetracycline (Tet, Sigma) or on BGS plates without Str or Tet at 37°C. BA plates and BGS plates were incubated for 24 h before observation and re-incubated an additional 24 h. Isolates were selected and maintained on trypticase soy agar (TSA, B-D) slants for working cultures and frozen at -80°C in nutrient broth (NB, B-D) with 15% glycerol (Sigma) for long term storage.

Inoculum Preparation. Inocula were prepared from BGS agar plates containing either 125 ppm Str or 200 ppm Tet grown for 48 h at 37°C. One isolated colony was suspended in 3.0 mL of sterile 0.85% saline (Sigma) and diluted as necessary with 0.85% saline to an optical density reading of 0.20 at 540 nm with a Milton-Roy Spect-20 (Milton-Roy Spectrophotometer 20, Thermo Spectronics, Madison, WI) which correlates to approximately 2.0×10^8 cfu/mL of *Salmonella* from a laboratory standard curve. Serial dilutions were prepared to the desired inoculum level of approximately 10^5 cfu/mL and 0.1 mL of this inoculum was administered to each seeder chick by oral gavage. Both

inocula were enumerated on BGS agar plates with either 125 ppm Str or 200 ppm Tet at 37°C for 48 h.

Animals and Animal Husbandry

Husbandry. All chicks (Cobb) were obtained from a local broiler/breeder hatchery on day of hatch as breeder off-sex, male. These chicks were selected for use in this study because these hatcheries employ additional measures to ensure the reduction or elimination of *Salmonella* in the hatched chicks (personal communication, Dr. J.L. Wilson). The parent hens were vaccinated against *Salmonella* Senftenberg, Heidelberg and Kentucky. Future work should be undertaken using commercial broiler lines of chicks from a commercial hatchery or hatched on a research farm in order to provide a comparison and to ensure no genotypic bias was introduced from the use of these broiler-breeder chickens. Chicks were transported on clean cardboard bedding pads in reusable, sanitized plastic chick transport trays. Chicks were housed in separate treatment rooms at the Bacterial Epidemiology and Antimicrobial Resistance Research Unit animal facility (Russell Research Center (RRC), USDA, Athens, GA) on fresh pine shaving litter (n=150 chicks/room) with *ad libitum* access to feed (non-medicated starter/grower crumbles and pellets, University of Georgia Feed Mill, Athens, GA) and water (nipple drinker line) on a 24 h light/0 h dark regimen in order to remove confounding factors associated with a 20 h light/4 h dark regimen. Standard husbandry practices for growth were followed with birds displaying poor growth performance from metabolic disease and/or physical abnormalities being culled upon detection.

Inoculation. Twenty percent of the chicks (15 birds for each strain, SK^{TetR} and SH^{StrR}, a total of 30 chicks per room) in the three treatment rooms were inoculated by oral gavage

at day of hatch, immediately prior to placement with 0.1 mL of a suspension of either SK^{TetR} or SH^{StrR}, containing 1.8×10^5 and 2.3×10^5 cfu, respectively.

Animal Sampling. All chicks were euthanized by cervical dislocation (for both culling and microbial sampling) or electrical stunning followed by exsanguination (in the processing plant). The seeder chicks were excluded from cecal sampling by means of double wing bands ensuring that these chicks were not selected during the catch phase of sampling.

Sampling Plan. Transport material was sampled at time of chick placement for the presence of naturally occurring *Salmonella* spp., by the addition of 500 mL of buffered peptone water (BPW, B-D) followed by overnight pre-enrichment at 37°C. The standard method for isolation of *Salmonella* spp., from meat and poultry outlined in the USDA, Food Safety Inspection Service (FSIS) Microbiological Laboratory Guide (MLG) (FSIS, 2011) and outlined below was followed. Ceca were sampled on d 7, 28 and 42. At d 43 of grow-out, all remaining birds were processed in the pilot processing plant located at RRC and approximately 30 mL of carcass rinsate per carcass was collected for *Salmonella* isolation procedures as described in the FSIS MLG.

Isolation Methods

Transport Material. Transport pads were cultured immediately following placement of the chicks. Each cardboard pad was placed into a 1-gallon Zip-Lock bag, 500 mL of sterile BPW (B-D) was added and the pads were manually massaged for approximately 60 s to ensure complete coverage and saturation of the pad. The standard USDA, FSIS isolation procedure was used for culture of *Salmonella* spp. After 16-18 h of pre-enrichment, 0.1 mL and 0.5 mL portions of pre-enriched broth were transferred to 10.0

ml of Rappaport-Vassialdis Broth (RV, B-D) and 10.0 mL of TT Broth, Hajna (TT, B-D), respectively. RV and TT tubes were incubated at 42°C for 18-24 h. All tubes were streaked for isolation onto both BGS and double modified lysine iron agar (dMLIA, Oxoid Limited, Hampshire, UK) plates. All plates were incubated at 37°C for 48 h. Isolated colonies with typical growth patterns were picked using disposable inoculations loops/needles and inoculated onto triple sugar iron (TSI, B-D) and lysine iron agar (LIA, B-D) slants for screening. All TSI and LIA slants were incubated at 37°C for 18-24 h.

Ceca. Samples were cultured as described by Bailey et al., (2001) with minor modifications as follows. After being removed aseptically from the chicks, each pair of ceca was placed into stomacher bags (Seward Laboratory Systems, Inc., Bohemia, NY). The ceca were then gently macerated by striking the outside of the stomacher bag gently with a rubber mallet. Thirty mL of BPW was aseptically added and the ceca were stomached for 60 s using a Stomacher 80 Laboratory Blender (Seward). The homogenate was evenly divided among three sterile tubes, one containing no antimicrobial, one with 125 ppm Str and one with 200 ppm Tet added. All samples were pre-enriched overnight at 37°C and isolation continued according to the U.S. Food and Drug Administration Bacteriological Analytical Manual Online (2011). After pre-enrichment, 0.1 mL of broth was transferred to 10.0 mL of RV Broth and 0.5 mL of broth was transferred into 10.0 mL of TT and all tubes were incubated 18-24 h at 42°C. After selective enrichment tubes from pre-enrichments without either Tet or Str were streaked onto BGS agar, BGS agar with 125 ppm Str, BGS agar with 200 ppm Tet plates and dMLIA plates; tubes with Str in the pre-enrichment broth were streaked onto BGS agar, BGS agar with 125 ppm Str and dMLIA plates; and tubes with Tet in the pre-enrichment broth were streaked onto

BGS agar, BGS with 200 ppm Tet and dMLIA agar plates. All plates were incubated at 37°C for 24 h, observed and re-incubated for 24 h. Isolated colonies with typical growth patterns were picked using disposable inoculation loops/needles and inoculated onto TSI and LIA slants for screening. Tubes with typical reactions were further analyzed. One colony per plate for a maximum of 20 colonies per ceca sampled were further screened for serogrouping and serotyping.

Processing and Carcass Rinses

After 12 h feed withdrawal on litter with access to water, 40 chickens for each treatment room were processed (in order by room number) in two batches of 20 chickens per batch in the Russell Research Center Pilot Processing Plant (Athens, GA). The chickens were shackled (on 6 inch/15.2 cm spacing) and electrically stunned (Simmons Engineering, SF-7000 Pre-Stunner, Dallas, GA) at 12 V DC 400 Hz for 8 s, then both carotid arteries and one jugular vein were auto-cut (Simmons Engineering, SK-5 automatic knife) followed by bleeding for approximately 2 min. Carcasses were triple tank (740 L) scalded (Stork-Gamco, SGS-3CA, Gainesville, GA) with the first tank set at 118° F (47° C), the second tank at 128° F (53° C), and the third tank at 134° F (56° C). Total immersion scald time was 2 min, resulting in a semi-hard scald. Carcasses were then de-feathered (Stork-Gamco, D-8 picker) and the feet and heads removed. Carcasses were transferred to the evisceration line and mechanically opened (Stork-Gamco, V/O-164) and eviscerated (Stork-Gamco, PNT-24) on 12-inch/30.5-cm shackle line spacing and the line was stopped before the mechanical carcass washer. The necks of each carcass were broken with a hand held neck breaker (Jarvis, Model DNB-1, Middletown, CT) and removed to open the thoracic inlet to facilitate thoracic-abdominal cavity

drainage during carcass washing. Carcasses were washed with tap water in an inside-outside carcass washer (Stork-Gamco, MBW-16) at 689 kPa (100 psi) on 6-inch/15.2-cm shackle line spacing. Following exit of the carcass washer, the first batch of 20 carcasses were placed in the first auger chiller containing 20 ppm free chlorine sodium hypochlorite and iced tap water. Following exit of the carcass washer, the second batch of 20 carcasses were placed in the second auger chiller containing only iced tap water with no added chlorine. Chlorine concentration in the first chiller was measured at 10 min increments using the DPD Chemistry method (N, N-diethyl-p-phenylenediamine) and the sodium hypochlorite was added as needed to maintain a level of 20 ppm free chlorine. All carcasses were chilled for 40 min with air agitation. No processing equipment sanitization was performed during the processing of all four rooms. Following chilling, each carcass was removed using a new set of gloves per carcass and hung by one wing on a sanitized A-frame shackle rack for 5 min to drip chiller water. Each carcass was placed in a labeled 35.5 by 90.8 cm plastic (L340, Cryovac, Duncan, SC) bag.

Four hundred mL of BPW were added to each bag and the carcasses were mechanically shaken (Simmons Engineering, MCS, Dallas, GA) for 1 min and the rinsate collected (Dickens et al., 1985). Thirty to 50 mL of rinsate was collected into 100 mL sterile, specimen cups (Fisher Scientific, Pittsburg, PA) and incubated 16 -18 h at 37°C. No antimicrobials were added to the carcass rinsates during pre-enrichment. After pre-enrichment, 0.5 mL and 0.1 mL of rinsate was transferred to TT and RV broth, respectively, followed by enrichment at 42°C for 18-24 h. After overnight enrichment, each sample was streaked for isolation onto BGS, BGS with 125 ppm Str, BGS with 200

ppm Tet and dMLIA agar plates. All plates were incubated for 24 h at 37°C, observed and re-incubated for 24 h. Multiple colonies exhibiting typical *Salmonella* growth patterns were selected and transferred to TSI and LIA slants for further screening. Tubes with typical reactions were further analyzed. A maximum of 5 colonies per plate were screened (for a total of 40 colonies per carcass) for serogrouping and serotyping.

Serogrouping and Serotyping

Isolates exhibiting typical biochemical reactions on TSI and LIA slants were screened for somatic antigens using O-antiserum (B-D) and flagellar antigens using Microgen latex agglutination (Microbiology International, Frederick, Maryland). Serotyping was conducted using the SMART Serotyping system as described by Leader et al., (2009) using an ABI31360xl Genetic Analyzer (Applied Biosystems, Foster City, California) for a representative number of isolates.

Statistical Analysis

All results were recorded and analyzed by chi-square with SAS Statistical Software (version 8.02, SAS Institute, Cary, NC) or by mathematical calculations ($P < 0.05$).

RESULTS and DISCUSSION

All control samples were negative for the presence of inoculated and/or environmental *Salmonella* spp., including the transport pads from all chicks sampled at day of hatch. On d 7, the sampled birds were positive for 31% SK^{TetR} , 16% SH^{StrR} , or 2% both in the *Salmonella* inoculated treatment rooms combined (Table 3.1), which increased to 100% positive for both serotypes of *Salmonella* in the inoculated treatment rooms combined on d 28. On d 42, the sampled chickens were positive for 83% SK^{TetR} ,

96% SH^{StrR} or 82% both serotypes in the *Salmonella* inoculated treatment rooms combined, respectively. The statistical differences for the recovered serotypes within sampling weeks are shown in Table 3.1. Recovery varied among the treatment rooms. At d 7 in treatment room 2, 73% of the chicks were positive for SK^{TetR} , 13% were positive for SH^{StrR} and both serotypes were recovered from 7% of the chicks. These values increased to 100% positive for all broilers sampled on d 28 in all rooms. At d 42, SK^{TetR} was recovered from 80% of the broilers, SH^{StrR} was recovered from 97% and both serotypes were recovered from 80% of the broiler samples from Room 2. In rooms 3 and 4, SK^{TetR} was recovered at 7 and 13% and SH^{StrR} was recovered at 20 and 13%, respectively and none of the chicks had both serotypes recovered. At d 42 SK^{TetR} was recovered from 93 and 77%, SH^{StrR} was recovered from 100 and 90%, and both serotypes were recovered from 93 and 73% of the broilers, respectively for treatment rooms 3 and 4. Overall the recovery rates from all cecal samples were 77% for SH^{StrR} , 74% for SK^{TetR} and 67% for both serotypes of *Salmonella*. Statistical differences from all cecal samples were observed between SH^{StrR} and both serotypes and between SK^{TetR} and both serotypes but no difference was observed between SH^{StrR} and SK^{TetR} .

These two serovars were selected for use to prevent the potential for cultural bias which Singer et al., (2009) observed *in vitro* when comparing four *Salmonella* strains in broth and bovine feces. By using different antimicrobials (SK^{TetR} and SH^{StrR}) in the pre-enrichment broths, it was hypothesized that the resistant *Salmonella* strain would be able to outgrow the other *Salmonella* strain and be recovered more readily from the enrichment broth and subsequent plating media. This protocol prevented the skewing of the recovery distribution of the *Salmonella* strains used in this study, which could have

occurred if both *Salmonella* strains selected had been resistant to the same antimicrobial. However, since the recovery of both *Salmonella* strains occurred from all birds sampled on d 28, this indicated that no skewing or competitive exclusion was detected.

Horizontal transmission of salmonellae among broiler and layer chickens has been demonstrated in studies conducted worldwide (Byrd et al., 1998; Bailey et al., 2001; Liljebjelke et al., 2005; Thomas et al., 2009; De Vylder et al., 2011; and Roll et al., 2011). Byrd et al., (1998) found that after challenging a minimum of 5 chicks per treatment pen of 100 chicks with as few as 10^2 cfu per chick of *S. Typhimurium*, approximately 57% of the unchallenged contact chicks were colonized with \log_{10} 2.2 cfu of *Salmonella* per g of cecal contents on d 17 of grow-out. The level of *Salmonella* in the ceca increased (\log_{10} 5.8 cfu/g) in pen mates when the seeder chicks were orally gavaged with 10^6 cfu per chick.

Liljebjelke et al., (2005) recovered *Salmonella enterica* from two integrated poultry systems over seven consecutive flocks isolating 15 different serotypes. *S. Typhimurium* and *Enteritidis* isolates from poultry carcasses shared the same PFGE pattern as those isolated from the rearing environment and from rodents caught in the same house. However, indistinguishable PFGE types of *S. Typhimurium*, *Enteritidis*, and *Heidelberg* were isolated from carcasses, the broiler chicken environment and chick-box liners which implicated the hatchery as the initial source for these persistent serotypes on this farm (Liljebjelke et al., 2005).

Rabsch et al., (2000), Callaway et al., (2008) and Foley et al., (2008) all analyzed epidemiological data collected through surveillance studies from the last half of the 20th century in the United States and Europe to explain the reduction of host specific

Salmonella, specifically *S. Gallinarum* and *S. Pullorum*, in poultry production. These three studies support the theory that the increase in the prevalence of *Salmonella* Enteritidis and other non-host specific *Salmonella* serotypes in poultry and poultry products was the result of the reduction and/or elimination of the host specific *Salmonella* serovar Gallinarum which includes the two biovars, Gallinarum and Pullorum. Rabsch et al., (2000) proposed that the increase in prevalence of *S. Enteritidis* was a result of the industry's actions which resulted in the reduction in the prevalence of *S. Gallinarum* and *S. Pullorum*. Since *S. Gallinarum* has no animal reservoirs other than domestic and aquatic fowl, eradication left a niche which was filled by non-host specific *Salmonella* serovars, Heidelberg, Typhimurium and Enteritidis in particular (Foley, et al., 2008).

Thomson et al., (2008) sequenced the genomes of *S. Enteritidis* PT4 isolate P125109, a host-promiscuous serovar, and *S. Gallinarum* isolate 287/91, a chicken-restricted serovar. Genomic comparisons between these two serotypes indicated that *S. Gallinarum* 287/91 may be a direct descendent of *S. Enteritidis* which had undergone extensive degradation through deletion and pseudogene formation (Thomson et al., 2008) which might explain the increase in *S. Enteritidis* colonization of chickens following the reduction and/or elimination of *S. Gallinarum* in the poultry industry.

Other studies looking at the competition between *Salmonella* serotypes in the gut of broiler chicks are almost non-existent. Nógrády et al., (2003) examined the growth suppression of *Salmonella* Hadar, *in vitro* under strict anaerobiosis and *in vivo* in the intestine of day-old chicks. Four strains were selected for evaluation of their ability to suppress the growth of *S. Enteritidis*, Typhimurium, Virchow and Saintpaul. Nógrády et al., (2003) were able to show that pre-colonization of the chicken with *S. Hadar*

prevented the super-infection with any of the four mentioned serotypes. Ngwai et al., (2006) looked at the *in vitro* growth suppression of antibiotic resistant *Salmonella* Typhimurium DT-104 by non-DT104 strains. The non-DT104 strains were able to prevent the multiplication of the antimicrobial resistant DT104 strain when the DT104 strain was added in low numbers to 24 h cultures of the non-DT104 strains. The implication is that an established *Salmonella* serotype might be able to prevent the colonization of another *Salmonella* serotype. On d 7, 31% SK^{TetR} , 16% SH^{StrR} and only 2% had both SK^{TetR} and SH^{StrR} , but by d 28, all samples were positive for both SK^{TetR} and SH^{StrR} . Therefore, no competition between the two serotypes was observed in our study.

Both SK^{TetR} and SH^{StrR} were horizontally transmitted from the seeder chicks to the non-seeder chicks in the treatment rooms similar to the results of Byrd et al., (1998) and both serovars were frequently recovered from the same chick. The ability of both serovars to colonize the same chick leads to the conclusion that these non-host specific *Salmonella* serovars were able to colonize the same host, and presents some questions regarding whether or not there is sufficient competition *in vivo* between other *Salmonella* serovars to eliminate one from the intestinal tract of chickens during rearing.

After processing and immersion chilling with and without chlorine in the chillers, the *Salmonella* recovery rates were 23, 15, and 5% for SH^{StrR} , SK^{TetR} , or both serotypes, respectively, in the rinsates from the chlorinated chiller and 46, 20, and 15% for SH^{StrR} , SK^{TetR} , or both serotypes, respectively, in the rinsates from the non-chlorinated chiller. Statistical differences were observed between the recovery of SH^{StrR} and SK^{TetR} and between SH^{StrR} and both serotypes in the non-chlorinated chilled carcass rinsates (Tables 3.2 and 3.3). The differences in the recovery rates of SH^{StrR} and SK^{TetR} could be the

result of SH^{StrR} to more effectively colonize the broilers or more effectively survive in the processing plant environment. Additionally, a statistical difference was observed between the total recovery rates of the non-chlorinated and chlorinated chilled carcass rinsates for SH^{StrR} with more SH^{StrR} being recovered in the non-chlorinated chilled carcass rinsates. The addition of chlorine has been demonstrated to reduce *Salmonella* recovery from carcasses after chilling in the processing plant (Lillard, 1980; Thiessen et al., 1984; Tamblyn et al, 1997). Increased recovery rates were observed among the Rooms in both the chlorinated and non-chlorinated chilled carcass rinsates (Table 3.3) with the recovery of *Salmonella* increasing in the non-chlorinated chilled carcass rinsates over the recovery from the chlorinated chilled carcass rinsates. The increase in recovery rates from the treatment rooms processed later in the day could be indicative of the increase in bacteria present in the chill water during the consecutive processing periods.

Thomson et al., (1979) found a reduction of marker *Salmonella* in a simulated commercial chilling process when 50 ppm was added to the fresh input water, but not a total elimination of cross-contamination and the chlorine levels in the chill water rapidly decreased due to the organic matter in the water, which is why free chlorine is monitored and adjusted as necessary. Yang et al., (2001) also demonstrated that through a combination of increasing the scald water and the chill water chlorine concentration cross-contamination was effectively blocked, but this treatment had little effect on *Salmonella* cells attached to the skin. However, others have shown a reduction to the point of non-detection for carcasses and chill water with the addition of chlorine and the maintenance of appropriate levels during the chilling process (Lillard, 1980; Thiessen et al., 1984; Tamblyn et al, 1997). Our results tend to follow the trend of reduced

Salmonella recovery when chlorine was added to the chill water which was experienced by Lillard (1980), Thiessen et al., (1984) and Tamblyn et al., (1997).

The pattern of recovery in our study is in agreement with the study of Lahellec and Colin (1985) where it was observed that *Salmonella* serotypes originating in the hatchery were less important in the final product than those present or introduced into the rearing facility during grow out. Blankenship et al., (1993) found in houses not treated with competitive exclusion microflora that the environment was the primary source of contaminating *Salmonella* as opposed to the hatchery. Bailey et al., (2001) in a multistate epidemiological study found that the serotypes recovered from carcasses after processing were the serotypes most frequently recovered from on-farm samples. However, *Salmonella* serotype prevalence in ceca samples collected the day prior to processing (pre-feed withdrawal) may not be predictive of the recovered *Salmonella* serotypes from post-chill carcasses or may be at lower levels of *Salmonella* than recovered in the on-farm samples.

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Table 3.1: Number and percentage of birds positive for *Salmonella* Kentucky^{TetR}, *Salmonella* Heidelberg^{StrR} or both *Salmonella* serotypes isolated by sample day and overall per treatment room

Day	Serotype	Treatment Room				Total by Serotype
		1	2	3	4	
7 n=15	SK ^{TetR}	0	11	1	2	14
	No + (%)	(0)	(73)	(6.7)	(13)	(31) ^A
	SH ^{StrR}	0	2	3	2	7
28 n=15	No + (%)	(0)	(13)	(20)	(13)	(16) ^A
	Both	0	1	0	0	1
	No + (%)	(0)	(7)	(0)	(0)	(2) ^B
42 n=30	SK ^{TetR}	0	15	15	15	45
	No + (%)	(0)	(100)	(100)	(100)	(100) ^A
	SH ^{StrR}	0	15	15	15	45
Total by Room n=60	No + (%)	(0)	(100)	(100)	(100)	(100) ^A
	Both	0	15	15	15	45
	No + (%)	(0)	(100)	(100)	(100)	(100) ^A
42 n=30	SK ^{TetR}	0	24	28	23	75
	No + (%)	(0)	(80)	(93)	(77)	(83) ^B
	SH ^{StrR}	0	29	30	27	86
Total by Room n=60	No + (%)	(0)	(97)	(100)	(90)	(96) ^A
	Both	0	24	28	22	74
	No + (%)	(0)	(80)	(93)	(73)	(82) ^B
Total by Room n=60	SK ^{TetR}	0	50	44	40	134
	No + (%)	(0)	(83)	(73)	(67)	(74) ^A
	SH ^{StrR}	0	46	48	44	138
Total by Room n=60	No + (%)	(0)	(77)	(80)	(73)	(77) ^A
	Both	0	40	43	37	120
	No + (%)	(0)	(67)	(72)	(62)	(67) ^B

^{A, B} Values within sampling periods or totals with different superscripts are statistically different (P<0.05). Values from room 1 were not included in the chi-square tests.

Table 3.2. Number and percentage of carcass rinsates positive for *Salmonella* Kentucky^{TetR}, *Salmonella* Heidelberg^{StrR} or both *Salmonella* serotypes

Room	Chlorinated Chill Water			Non-Chlorinated Chill Water		
	SK ^{TetR} No. + (%)	SH ^{StrR} No. + (%)	Both No. + (%)	SK ^{TetR} No. + (%)	SH ^{StrR} No. + (%)	Both No. + (%)
1¹	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
2	7 (35) ^{AXa}	2 (10) ^{AXb}	2 (10) ^{AXa}	2 (10) ^{AXb}	2 (10) ^{AXc}	1 (5) ^{AXb}
3	0 (0) ^{BXb}	8 (20) ^{AXa}	0 (0) ^{BXa}	3 (15) ^{BXab}	8 (40) ^{AXb}	2 (10) ^{BXb}
4	2 (10) ^{AYab}	4 (20) ^{AYab}	1 (5) ^{AYa}	7 (35) ^{BXa}	17 (85) ^{AXa}	6 (30) ^{BXa}
Total²	9 (15) ^{ABX}	14 (23) ^{AY}	3 (5) ^{BX}	12 (20) ^{BX}	27 (46) ^{AX}	9 (15) ^{BX}

¹ Total n=40 per treatment room; n=20 for chlorinated chill water per room and n=20 for non-chlorinated chill water per room except for non-chlorinated chill water in Room 4 where n=19.

² n=60 per column for the chlorinated rinsates and n=59 for the non-chlorinated rinsates, the negative control room is not included in the calculation for neither percentage nor the chi-square analyses.

^{A, B} Values with different superscripts are statistically different (P<0.05) within chlorinated or non-chlorinated treatments between SK^{TetR}, SH^{StrR}, and both serotypes.

^{X, Y} Values with different superscripts are statistically different (P<0.05) within SK^{TetR}, SH^{StrR}, or both serotypes between chlorinated and non-chlorinated treatments.

^{a, b} Values with different superscripts within columns are statistically different (P<0.05) among Rooms 2, 3, and 4.

Table 3.3. Chi-squared values for carcass rinsates

Chi-squared	P-value	
	Chlorinated	Non-Chlorinated
Recovered <i>Salmonella</i>		
Total SK ^{TetR} vs. total SH ^{StrR}	0.246	0.003*
Total SK ^{TetR} vs. total both	0.068	0.47
Total SH ^{StrR} vs. total both	0.004*	0.0003*
Room 2 SK ^{TetR} vs. SH ^{StrR}	0.058	1.0
Room 2 SK ^{TetR} vs. both	0.058	0.548
Room 2 SH ^{StrR} vs. both	1.0	0.548
Room 3 SK ^{TetR} vs. SH ^{StrR}	0.0016*	0.077
Room 3 SK ^{TetR} vs. both	1.00	0.633
Room 3 SH ^{StrR} vs. both	0.0016*	0.028*
Room 4 SK ^{TetR} vs. SH ^{StrR}	0.376	0.0007*
Room 4 SK ^{TetR} vs. both	0.548	0.732
Room 4 SH ^{StrR} vs. both	0.151	0.0003*
SK ^{TetR} Room 2 vs. Room 3	0.0036*	0.633
SK ^{TetR} Room 2 vs. Room 4	0.058	0.047*
SK ^{TetR} Room 3 vs. Room 4	0.147	0.118
SH ^{StrR} Room 2 vs. Room 3	0.028*	0.028*
SH ^{StrR} Room 2 vs. Room 4	0.376	0.0000*
SH ^{StrR} Room 3 vs. Room 4	0.168	0.0013*
Both Room 2 vs. Room 3	0.147	0.548
Both Room 2 vs. Room 3	0.548	0.031*
Both Room 2 vs. Room 3	0.311	0.095
Chlorinated vs. Non-Chlorinated	P-Value	
Room 2 SK ^{TetR}	0.058	
Room 2 SH ^{StrR}	1.0	
Room 2 Both Serotypes	0.548	
Room 3 SK ^{TetR}	0.072	
Room 3 SH ^{StrR}	1.0	
Room 3 Both Serotypes	0.147	
Room 4 SK ^{TetR}	0.047*	
Room 4 SH ^{StrR}	0.0000*	
Room 4 Both Serotypes	0.031*	

* Indicates statistical difference (P<0.05).

CHAPTER 4

GENOTYPIC AND PHENOTYPIC CHARACTERIZATION OF *SALMONELLA* HEIDELBERG AND KENTUCKY ISOLATES RECOVERED FROM POULTRY²

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ABSTRACT

Twenty *Salmonella* Heidelberg (SH) isolates and 21 *S. Kentucky* (SK) isolates recovered from broiler chickens during a 6 week grow out period after oral inoculation of seeder chicks on the day of placement were characterized by various genotypic and phenotypic tests. Two strains of SH and SK, parent and inoculum were included as reference strains. Pulsed-field gel electrophoresis (PFGE) profiles using *XbaI* revealed a 100% genetic similarity between the parent and inoculum SH strains and 19 SH isolates with one isolate having only 96.6% similarity. Genetic similarity between the parent and inoculum SK strains and 17 SK isolates was 100% with 4 other isolates exhibiting a minimum of 97.2% similarity. Antimicrobial resistance profiles ranged from three pan-susceptible isolates (two SH and one SK) to 11 multidrug resistant profiles with amoxicillin/clavulanic acid (Aug), ampicillin (Amp), cefoxitin (Fox), ceftiofur (Tio), streptomycin (Str) and tetracycline (Tet) being the most prevalent multidrug resistance profile detected. Eighteen of the 20 isolates of SH were resistant only to streptomycin while 20 of the 21 isolates of SK were resistant to tetracycline and additional resistance patterns emerged. PCR was used to probe for four resistance genes, two tetracycline (*tetA* and *tetB*) and two aminoglycoside genes (*strA* and *aadA1*). The resistance genes *tetA*, *tetB*, *strA*, and *aadA1* were detected in 11, 10, 15 and 19 of the SH strains, respectively and in 19, 21, 15 and 13 of the SK strains, respectively. The recovered SH and the SK isolates had a > 96.6% and 97.2% genetic similarity when compared to the parent and inoculum SH and SK strains, respectively. This high degree of similarity would indicate that the isolates were progeny of the inoculum strains. The variability in the isolation of resistance genes coupled with the presence of multidrug resistant *Salmonella* strains indicates that further research is necessary to fully elucidate the effect

the environment and passage through the host exerts on phenotypic and genotypic characteristics of the isolates.

Key Words: *Salmonella*, Pulsed-field gel electrophoresis, Antimicrobial resistance, Broilers

The largely unknown burden of disease caused by foodborne pathogens and the safety of the food supply is a dynamic situation heavily influenced by multiple factors in the food chain from the farm to the fork (20). Foods remain an excellent vehicle for transmission of pathogens from one host to another host which can include humans. Even with changes in food production and improvements in product formulation and processing, common foodborne pathogens such as *Salmonella*, *Campylobacter*, enteropathogenic *E. coli* and *Listeria monocytogenes* are able to survive changing conditions by adaptation (20).

Salmonella, a leading cause of foodborne enteric disease in many countries, is responsible for significant loss of productivity, human suffering and even mortality (25). Ingestion of contaminated water or food is the usual route for the acquisition of non-typhoidal salmonellosis. Raw poultry and poultry products continue to be an important vehicle for foodborne pathogens (1, 9). The prevalence of *Salmonella*-contaminated poultry has been reported as being between 20 and 50% (2, 6, and 7).

Subtyping of *Salmonella enterica* for epidemiological surveillance has been performed by serotyping for the past 80 years or more (3, 31). Serotyping is a method by which surface antigens react with specific antibodies in agglutination reactions in order to identify the *Salmonella* serotypes. This has enabled the long-term epidemiological surveillance of *Salmonella* in the food chains and in public health investigations (31). However, the identification and tracking of salmonellosis outbreaks in epidemiological investigations requires the use of more sensitive methods to determine the causative strains at a taxonomic level which cannot be achieved by serotyping alone (10, 15, 29, 31). Pulse-field gel electrophoresis (PFGE) profiling, a DNA fingerprinting method

based on the restriction digestion of purified genomic DNA, is currently considered the gold-standard when subtyping foodborne pathogens, especially *Salmonella* (8, 27, 32). PulseNet, a national molecular subtyping network established in 1996 by the Centers for Disease Control and Prevention, uses PFGE as its main platform for epidemiological tracking of foodborne pathogens (CDC) (8). PulseNet is utilized by all of the state public health laboratories and the food safety laboratories at the Food and Drug Administration (FDA) and the United States Department of Agriculture (USDA) (33). PFGE data are considered a sensitive and reliable method to detect differences between closely related strains (31). It can be said that isolates with indistinguishable PFGE profiles may be classified as epidemiologically linked with a high degree of confidence (32, 33).

PFGE has been useful during outbreak investigations to assess relatedness within *Salmonella* serotypes (28). The ability to track *Salmonella* serotypes through an animal model gives researchers the tools necessary to follow the adaptations of *Salmonella* strains. It also allows researchers to answer questions regarding the complex interactions between *Salmonella* serotypes in an animal hosts and/or the environment.

Antimicrobial use in animals selects for resistance in both commensal bacteria and zoonotic pathogens (19). The finding of resistance genes in microorganisms within antibiotic-free environments suggests that these traits occur naturally and pre-date the industrial scale production and distribution of antimicrobial drugs (17). The development of multidrug resistance is more likely to be from horizontal gene transfers vice random mutations in the bacterial genomes. A vast number of commensal and environmental bacteria continuously and promiscuously exchange genes (17). A large capacity for carrying and mobilizing resistance genes is maintained in this large and diverse group of

species (18). Conjugal mating has been identified as the most common means of genetic exchange in horizontal gene transfer studies, with few barriers to prevent this gene sharing across the multitude of dissimilar genera (13).

The purpose of this study was to characterize (phenotypically and genotypically) two *Salmonella* serotypes with two distinct antimicrobial resistance profiles (*S.* Heidelberg, resistant to streptomycin (Str) and *S.* Kentucky resistant to tetracycline (Tet)) following co-exposure in broiler chickens.

MATERIALS AND METHODS

Bacterial strains and maintenance. Twenty-one isolates of *Salmonella* Kentucky and 20 *S.* Heidelberg were recovered from the ceca or carcass of experimentally exposed broiler chickens (5). Table 4.1 shows the number of isolates collected by Cosby et al., (5) during the previous 43 day study. Cecal strains were selected randomly from pens and birds with an attempt to select equal numbers of strains from media containing the appropriate antimicrobial and from media containing the antimicrobial for the other serotypes resistance (*i.e.*, an equal number of SH^{StrR} isolates from media supplemented with Str and an equal number of SH^{StrR} isolates from media supplemented with Tet). Carcass rinsate isolates were randomly selected to have an equal number of isolates from the chlorinated and non-chlorinated chilled rinsates. A parent and an inoculum strain of SK and SH, the parent strains provided by Dr. Paula Fedorka-Cray (National Antimicrobial Resistance Monitoring System (NARMS), Agriculture Research Service, USDA, Athens, GA) and the inoculum strains (grown on the respective antimicrobial supplemented media prior to oral gavage) were used to compare against the experimental isolates. Strains and isolates were maintained on trypticase soy agar slants (TSA, Becton-Dickinson (B-D), Franklin

Lakes, NJ) or at -80°C in nutrient broth (NB, B-D) with 15% glycerol (Sigma Chemical Co., St. Louis, MO). All cultures were grown on 5% blood agar plates (BA, Remel Products, Lexana, KS) for all analyses.

Antimicrobial susceptibility testing. The antimicrobial susceptibility testing of the 41 isolates, two parent strains and two inoculum strains was conducted using broth microdilution and antimicrobials as described by the USDA, NARMS. The antimicrobial agent breakpoints are listed in Table 4.2. These breakpoints are based on those specified by the Clinical and Laboratory Standards Institute (4); where breakpoints are unavailable NARMS breakpoints are used.

Pulsed field gel electrophoresis (PFGE) and pattern analysis. PFGE was performed on each *Salmonella* isolate to assess their genotypic relatedness when compared to the parent and inoculum strains. PFGE was performed as previously described for the USDA VetNet program (11) which is a modification of the 23-24 h PFGE procedure described and used by PulseNet (22, 23). Restriction endonuclease *XbaI* (Roche Diagnostics Corporation, Indianapolis, IN) was used for restriction digestion of the cDNA. The PFGE tagged image file format (TIFF) images were analyzed using BioNumerics version 5.10 (Applied Maths Scientific NV, Saint-Martens-Latem, Belgium). Strain relatedness was determined using the different bands algorithm for clustering and the un-weighted pair grouping for arithmetic means (UPGMA) tree building approach with a position tolerance of 1.7%. Patterns were assigned by placing isolates in a comparison and arranging them by decreasing similarity. PFGE profiles were compared against the both VetNet profiles and the CDC PulseNet profiles and assigned profile names according to the PulseNet database.

Detection of resistance genes by PCR. Polymerase chain reaction (PCR) was used to probe for the presence or absence of the following resistance genes: *tetA*, *tetB*, *strA* and *aadA1*. All primers were obtained from Integrated DNA Technologies (www.idtdan.com, IDT, Coralville, IA) as lyophilized, desalted oligonucleotides and rehydrated in 10 mM Tris-HCl (Sigma) for a 0.1 nMol stock solution of oligonucleotides. The stock solution of oligonucleotides was diluted 1:10 in molecular grade water for a final concentration of 1mM Tris-HCl and 0.01 nMol of oligonucleotides. Primers were designed by IDT using Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>), which generates primers using Primer3 (24) and performs a BLAST (Basic Local Alignment Search Tools, <http://www.ncbi.nlm.nih.gov/pubmed/2231712>) of the generated primers against a specified database to avoid non-target amplification using the accession numbers listed in Table 4.3.

PCR reactions were carried out on whole cell templates generated by selecting an isolated colony from BA and suspending it into 0.1 ml of molecular grade water. Reactions were carried out using a GeneAmp[®] PCR System 9700 (P-E Applied Biosystems, Carlsbad, CA) with the following parameters 95°C^{10:00} [94°C^{0:30}; 65°C^{0:30}; 72°C^{0:30}]₃₅ 72°C^{7:00} 4°C[∞] using EmeraldAmp[®] Max HS PCR Master Mix (Takara Bio Inc., Mountain View, CA). Final volume of the reaction was 50.0 µl per well containing 1.0 µl of the template, 25.0 µl of the master mix, 14.0 µl of molecular grade water, and 5.0 µl each of the forward and reverse primer working solutions. Strains of *Escherichia coli* containing *tetA* and *tetB* genes, obtained from Dr. Paula Fedorka-Cray (Bacterial Epidemiology and Antimicrobial Resistance Research Unit, Agriculture Research Service, USDA, Athens, GA) and *Salmonella enterica* isolates, ARS-3085 and ARS-

C056363, positive for *strA* and *aadA1* genes obtained from Dr. Rebecca Lindsey (16) were included as positive controls. Whole cell templates of the positive control strains, a negative control (molecular grade water) and the whole cell templates of the *Salmonella* parent, inoculum and recovered strains were included in each PCR reaction. Five μl of each reaction product and 5.0 μl of a molecular ruler (Bio-Rad, EZ load 100 bp PCR molecular ruler, cat # 170-8353, Bio-Rad, Hercules, CA) was loaded into individual wells of a 1.25% Seakem[®] Gold agarose gel on a Bio-Rad Sub-Cell GT horizontal electrophoresis cell (Bio-Rad) utilizing the 25 x 15 cm tray and using four 20 well combs. Separation was carried out at 140 V for 65 min at room temperature using an E-C 250-90 (E-C Apparatus Corp., Waltham, MA) power supply.

After electrophoresis, all gels were stained in 1 L of de-ionized, distilled water with 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide (50 μl of a 10 mg/ml stock solution (Bio-Rad)) for 60 min and de-stained in de-ionized, distilled water for 60 min at room temperature. The agarose gels were documented on a Gel-Doc 1000 (Bio-Rad) and presence or absence of the appropriate sized amplicons were determined by using a standard curve and compared to the appropriate positive control strain.

RESULTS

Antimicrobial resistance profiles for SH are shown in Table 4.4a. When compared to the parent (70309P) and inoculum (70309D0) strains, which were resistant to Str, 18 of 20 were resistant to str while 2 isolates, 6-2-14-BS and CR2-39-BP were sensitive to Str. Additionally, one isolate, 1-4-14-BS, exhibited an intermediate resistance to Tet when compared to the control strains, 70309P and 70309D0. The minimum inhibitory concentrations (MIC) for the two sensitive strains were $\leq 32 \mu\text{g}$

which is well below the break point of 64 µg/ml set by NARMS as CLSI does not have a break point for Str.

The antimicrobial resistance profiles for SK are shown in Table 4.4b. When compared to the parent (71929P) and inoculum (71929D0) strains, all recovered isolates were resistant to Tet with the exception of CR-2-19-BP and CR-3-35BP which were sensitive to Tet. Unlike the parent and inoculum strains, strains passaged through the host exhibited additional resistance to amoxicillin/clavulanic acid (Aug), ampicillin (Amp), cefoxitin (Fox), ceftiofur (Tio), streptomycin (Str) and tetracycline (Tet). Five isolates exhibited the same antimicrobial resistance profile as 71929P and four isolates exhibited the same antimicrobial resistance profile as 71929D0.

Nineteen of the recovered SH isolates were clustered together with the same CDC PulseNet profile, JF6X01.0047, as the parent (70309P) and inoculum strain ((70309D0) and one isolate had a previously undetected PFGE profile which did not match any of the existing VetNet or PulseNet profiles (Figure 4.1). Nineteen of the recovered SK isolates were clustered together with the same PulseNet profile as the parent (71929P) and inoculum (71929D0) strains, JGPX01.0025 (Figure 4.2). Two isolates, CR-2-19-BP-A and CR-3-35-BP-A clustered together with the PulseNet profile (JGPX01.0002) which differed from parent and inoculum strains. Each serovar had greater than 96.6% or more genetic similarity when comparing the same serovars as a group (Figures 4.1 and 4.2).

The four resistance genes assayed for in this study were *tetA*, *tetB*, *strA* and *aadA1* (see Table 4.3 for primer sequence). SH strains 70309P and 70309D0 had the *strA* gene and 70309D0 had the *aadA1* gene present (Table 4.4a). Twenty of the recovered SH isolates had the *aadA1* gene detected and 15 of these isolates had the *strA* gene present.

Eleven of the *SH* isolates had the *tetA* gene present and 10 isolates had the *tetB* gene present with four having both *tetA* and *tetB* genes present.

SK strains 71929P and 71929D0 had both the *tetA* and the *strA* genes detect and 71929D0, also, had the *aadA1* gene present (Table 4.4b). *tetA* genes were detected in 19 of the recovered *SK* isolates and *tetB* genes were detected in all 21 of the recovered isolates. *strA* genes were present in 15 of the recovered *SK* isolates and *aadA1* genes were present in 13 isolates with 10 isolates having both *strA* and *aadA1* genes present.

DISCUSSION

In this study, 90% of the *SH* isolates recovered from the chickens (5) maintained the same antimicrobial resistance pattern as the parent and inoculation strains, while 71% of the *SK* isolates recovered from the chickens acquired additional antimicrobial resistance, 24% maintained the same resistance to Tet and 5% (one isolate) became pan-susceptible to all 15 antimicrobials tested. These two *Salmonella* strains were selected for use in this animal study because each had a single resistance to separate classes of antimicrobial compounds, aminoglycosides (Str) for the *SH* and tetracycline (Tet) for the *SK*. The theory behind using these two antimicrobial resistant strains in the host animal was to allow for the recovery and ease of differentiation of both serotypes from the chicken ceca and carcass rinsate by using antimicrobial supplemented media (pre-enrichment broth and/or selective plating media). Additionally, by choosing two serovars with different antimicrobial resistance patterns and assaying for the antimicrobial resistance of recovered isolates, any interaction between the two serovars in the avian gut might be determined by comparing the antimicrobial resistance patterns. The acquisition of Str resistance by 11 of the recovered *SK* isolates would indicate that either some

transfer of antimicrobial resistance occurred or that the avian gut and/or the isolation media provided the necessary stimulation for the SK isolates to up-regulate a gene already present on either the chromosome or a plasmid.

Two of the SH^{StrR} and one of the SK^{TetR} isolates were recovered from carcass rinsates with 20 ppm chlorine added to the chill water. In a study by Potenski et al., (21), exposure to chlorine increased the resistance of *Salmonella* Enteritidis strains to tetracycline and chloramphenicol. Potenski et al., (21) state that the exposure of chlorine in a food-processing plant may result in cross-resistance to antimicrobials used in agriculture or human therapy. Wang et al., (30) found that *S. Enteritidis* and Typhimurium responded to chlorine-based oxidative stress by the coordinated regulation of a variety of genes associated with stress response, gene regulation, metabolism and virulence. The genes involved in the cysteine biosynthesis and Fe-S cluster formation in *S. enterica* under chlorine stress were the most significantly upregulated, while the genes involved in LPS biosynthesis were downregulated (30). Of the three isolates from the carcass rinsates, the two SH^{StrR} isolates maintained the resistance profile of the inoculum strain while the SK^{TetR} isolate exhibited a resistance profile which varied from the inoculum strain by resistance to four antimicrobials and susceptibility to Tet. The number of isolates (six) compared here, three *Salmonella* isolates exposed to chlorine (20 ppm) and three *Salmonella* isolates not exposed to chlorine, is too small to make any conclusions in regards to the results of Potenski et al., (21). For the SH^{StrR} isolates, no difference was observed in the resistance profile for the two isolates recovered from the rinsates of the carcasses exposed to chlorine (CR-3-11-BP and CR-4-9-BP) when compared to the two isolates recovered from the rinsates of the carcasses not exposed to

chlorine (CR-3-24-BP and CR-4-30-BP) or strain 70309P. It is interesting to note that the antimicrobial pattern observed for the SK^{TetR} isolate (CR2-19-BP-A) which was exposed to chlorine in the chill tank exhibited a change in the resistance profile observed when compared to both strain 71929P and the SK^{TetR} isolate (CR-2-28-BP-A) which was not exposed to chlorine in the chill tank. These results indicate that more research is necessary to understand the interactions that all treatments and processes have on the antimicrobial resistance of foodborne pathogens.

Ten of the recovered SK strains were positive for the presence of *strA* and *aadA1*. SK strains, 71929P and 71929D0 displayed the *strA* gene while 71929D0, also, displayed the *aadA1* gene. Bauer-Garland et al., (2) demonstrated that strains of *S. Typhimurium* susceptible to chlortetracycline could change antimicrobial resistance patterns in the gut when therapeutic dosages of chlortetracycline were fed to the chicks as well as when no antimicrobials were fed to the chicks. Bauer-Garland et al., (2), also, demonstrated that even with the use of antimicrobials at therapeutic levels, there was a 95% colonization of chicks with the susceptible *S. Typhimurium*, with approximately the same mean numbers regardless of treatment. While this study did not include the use of antimicrobial compounds in the feed, either at therapeutic or sub-therapeutic levels, antimicrobial compounds were used in the pre-enrichment to assist in the recovery and identification of the isolates.

In our study, the Tet resistance genes *tetA* were detected in 19 out of the 21 recovered SK isolates and *tetB* genes were detected in 21 out of 21 recovered SK isolates but only *tetA* was recovered in SK strains 71929P (parent) and 71929D0 (inoculum). Lapierre et al., (14) found that resistance to Tet was more closely linked to the *tetB* gene

among 33 Tet resistant *Salmonella* strains isolated from pigs while neither *tetA* or *tetB* genes were isolated from 47 Tet resistant poultry strains. Our detection of both *tetA* and *tetB* genes from the recovered isolates as well as from the parent and inoculum strains runs counter to the findings of Lapierre et al., (14). Eleven of the recovered SK isolates were resistant for Str. Ten of the recovered SK isolates displayed both the *strA* and *aadA1* genes, five were positive for only *strA*, and two were positive only for *aadA1*. Three isolates, 1-3-13-BT, 6-2-16-BT and 6-3-16-BT, did not display either Str resistance gene assayed. These three isolates were among the 10 SK isolates susceptible to Str. Of the Str resistant isolates, five had both *strA* and *aadA1*, three had the *strA* gene and three other isolates had the *aadA1* gene detected. Of the recovered SK isolates exhibiting multidrug resistance, six were resistant to Str of which the *strA* gene was detected in four, the *aadA1* gene was detected in three while both genes were detected in only one isolate. Of the 21 recovered SK isolates only one was susceptible to Tet, CR3-35-BP (a pan susceptible isolate from a carcass rinsate). Interestingly enough, this isolate while pan susceptible displayed all four resistance gene assayed for in this study. Further study on the up regulation of these genes might provide some insight as to the reason for no resistance being detected in this isolate.

For the SH isolates, 20 of 20 were positive for *aadA1* and 15 out of 20 were positive for *strA*. Only two of the SH isolates displayed susceptibility to Str and one of these isolates displayed the *aadA1* gene, isolate 6-2-14-BS, and the other displayed both the *strA* and *aadA1* genes, isolate CR-2-39-BP. This would indicate that while the presence of the gene is an indicator of resistance, presence does not necessarily lead to the expression of the phenotypic resistance matching the genotype observed. Eleven of

the SH isolates displayed the Tet resistance genes *tetA* and 10 isolates displayed the *tetB* genes while only four isolates displayed both genes. However, the only SH isolate with an intermediate resistance to Tet did not display either of the two resistance genes. More evidence of the extremely complicated nature of comparing genotypic and phenotypic characteristics among *Salmonella* serotypes.

PFGE using a single restriction enzyme (*XbaI*) was useful to assess the similarity of isolates recovered through the grow-out cycle and processing of the chickens co-exposed to SH^{StrR} and SK^{TetR}. Our results indicate that the SH^{StrR} strain (70309D0) used to inoculate the seeder birds was in the same cluster (with a 100% similarity) as 20 of the SH^{StrR} isolates from throughout the production cycle and the SK^{TetR} strain (71929D0) used to inoculate the seeder birds was in the same cluster (with a 98.7% similarity) as 21 of the isolates recovered throughout the production cycle. The high level of genetic similarity observed in the PFGE profiles obtained by using one restriction enzyme can and should be further resolved through the use of a second restriction enzyme (either *blnI* or *SpeI*).

Zheng et al., (34) were able to discriminate 74 *Salmonella* Typhimurium strains into a nearly 1:1 ratio of nodes to strains when using two restriction enzymes (*XbaI* and *BlnI*) which indicated that almost every strain demonstrated a unique *XbaI/BlnI* pattern combination. However, in the same study (34), the discrimination of *Salmonella* Enteritidis using the same two restriction enzymes (*XbaI* and *BlnI*) resolved 76 strains into 6 unresolved strain clusters including one cluster comprised of 24 strains. Kottwitz, et al., (12) used a combination of phenotypic and genotypic traits to characterize 41 *Salmonella* Enteritidis strains from human and poultry sources. Using PFGE with two

restriction enzymes (*XbaI* and *SpeI*), Kottwitz et al., (12) were able to identify 16 distinct patterns for each enzyme and when combining the patterns from the two enzymes, 49% of the strains exhibited the same pattern. These analyses allowed the authors to conclude that there was a high similarity of PFGE patterns among the strains of poultry origin and strains isolated from patients or foods associated with salmonellosis outbreaks during the study period (12). Resolving the SH^{StrR} isolates and the SK^{TetR} isolates into more discriminatory patterns might provide more insight into the acquisition of the additional resistance acquired by the SK^{TetR} isolates.

Many factors are involved in the expression of antimicrobial resistance patterns in non-host specific *Salmonella* serovars and in the transmission of *Salmonella* from one host animal to another. The lack of correlation of gene presence to antimicrobial resistance profiles indicates that other factors may be contributing to the antimicrobial resistance profile of *Salmonella* strains and isolates. Further genetic analysis is necessary and may reveal additional fitness, resistance and/or virulence factors in *Salmonella*.

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Table 4.1. Time and number of isolates recovered in the commingling experiment

	Days Post Exposure				
	0	7	28	42	43
SH^{StrR}	2 ^a	79 (7) ^b	311 (45)	754 (86)	142 (42)
SK^{TetR}	2 ^a	167 (14)	525 (45)	620 (75)	47 (21)

^a Parent and inoculum strains of *Salmonella*.

^b Numbers in parenthesis represents total number of chicks positive with the each *Salmonella* strain.

Table 4.2. Breakpoints for antimicrobial compounds used for susceptibility testing of *Salmonella*¹

Antimicrobial Agent	Abbreviation	Susceptible Range	Indeterminate Range	Resistant Range
Amikacin	Ami	$0.5 \leq 16$	32	≥ 64
Amoxicillin / Clavulanic Acid	Aug	$1/0.5 \leq 8/4$	16/8	$\geq 32/16$
Ampicillin	Amp	$1 \leq 8$	16	≥ 32
Cefoxitin	Fox	$0.5 \leq 8$	16	≥ 32
Ceftiofur	Tio	$0.12 \leq 2$	4	≥ 8
Ceftriaxone	Axp	$0.25 \leq 1$	2	≥ 4
Chloramphenicol	Chl	$2 \leq 8$	16	≥ 32
Ciprofloxacin	Cip	$0.015 \leq 1$	2	≥ 4
Gentamicin	Gen	$0.25 \leq 4$	8	≥ 16
Kanamycin	Kan	$8 \leq 16$	32	≥ 64
Nalidixic Acid	Nal	$0.5 \leq 16$	N/A ²	≥ 32
Streptomycin ³	Str	≤ 32	N/A	≥ 64
Sufisoxazole	Fis	$16 \leq 256$	N/A	≥ 512
Tetracycline	Tet	≤ 4	8	≥ 16
Trimethoprim/ Sulfphamethoxzole	Cot	$0.12/2.4 \leq 2/38$	N/A	$\geq 4/76$

¹ Breakpoints established by CLSI (Clinical and Laboratory Standards Institute) were used when available.

² N/A = Not applicable.

³ There is no CLSI breakpoint established for streptomycin.

Table 4.3. PCR primer sequences used for PRC determination of presence or absence of resistance genes

Gene Name	Antimicrobial Resistance	Primer Name	Sequence (5' → 3')	Amplicon size (bp)	T _m (°C)	Accession Number
<i>tetA</i>	Tetracycline	tetA(F)	GCG TTT CTG GCG CGT TTG CA	936	62.7	X00006.1
		tetA(R)	CTG CTC GCC TAC CGC GAT GG			
<i>tetB</i>	Tetracycline	tetB(F)	GCG TCG AGC AAA GCC CGC TTA	458	63.2	J01830.1
		tetB(R)	TGG GCG CCG ACC AAA TCG G			
<i>strA</i>	Streptomycin	strA(F)	GTG CTC GGC GTG GCA AGA CT	852	63.0	M28829.1
		strA(R)	ACC GCG CCT TGT TCG GTC TG			
<i>aadA1</i>	Streptomycin	aadA1(F)	CAA GGT TGC CGG GTG ACG CA	405	63.0	X02340.1
		aadA1(R)	ACG TCG GTT CGA GAT GGC GC			

Table 4.4a. Antimicrobial resistance patterns and presence of targeted resistance genes for the *Salmonella* Heidelberg^{StrR} isolates

Isolate	Antimicrobial Resistance Profile ^c														Gene Presence				
	Ami	Aug	Amp	Fox	Tio	Axo	Chl	Cip	Gen	Kan	Nal	Str	Fis	Tet	Cot	<i>tetA</i>	<i>tetB</i>	<i>strA</i>	<i>aadA1</i>
70309P ^a	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	-	-	+	-
70309D0	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	-	-	+	+
1-2-8-BS ^{b, d}	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	+	+	+	+
1-3-6-BS	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	+	-	+	+
1-3-6-BT ^e	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	-	+	+	+
1-4-14-BS	S	S	S	S	S	S	S	S	S	S	S	R	S	I	S	-	-	-	+
1-4-14-BT	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	+	+	+	+
4-2-6-BS	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	-	+	+	+
4-2-10-BS	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	+	+	+	+
4-2-13-BT	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	-	+	+	+
4-3-14-BS	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	+	-	+	+
6-2-14-BS	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	+	-	-	+
6-2-22-BT	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	-	+	+	+
6-3-5-BP	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	-	+	-	+
6-3-7-BS	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	-	+	-	+
6-4-3-BS	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	+	+	+	+
6-4-19-BT	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	-	-	-	+
CR-2-39-BP	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	+	-	+	+
CR-3-11-BP ^f	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	+	-	+	+
CR-3-24-BP	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	-	-	+	+
CR-4-9-BP ^f	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	+	-	+	+
CR-4-30-BP	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	+	-	+	+

^a Strains 70309P and 70309D0 were the parent and inoculum strains used in the commingling experiment (Cosby et al., 2012).

^b Isolates are grouped according to day of recovery (1 is equivalent to d 7, 4 is equivalent to d 28, 6 is equivalent to d 42 and CR is equivalent to carcass rinse).

^c Results in a box differed either from the parent and/or inoculum strains.

^d BS indicates that isolates were recovered from media supplemented with Str.

^e BT indicates that isolates were recovered from media supplemented with Tet.

^f Isolates recovered from chlorinated chilled carcass rinsate.

Table 4.4b. Antimicrobial resistance patterns and presence of targeted resistance genes for the *Salmonella* Kentucky^{TetR} isolates

Isolate	Antimicrobial Resistance Profile ^c														Gene Presence				
	Ami	Aug	Amp	Fox	Tio	Axo	Chl	Cip	Gen	Kan	Nal	Str	Fis	Tet	Cot	tetA	tetB	strA	aadAI
71929P ^a	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	+	-	+	-
71929D0 ^a	S	S	S	S	S	S	S	S	S	S	S	R	S	R	S	+	-	+	+
1-2-2-BS ^{b, d}	S	R	R	R	R	I	S	S	R	S	S	R	R	R	S	-	+	+	-
1-2-3-BT ^e	S	S	S	S	S	S	S	S	S	S	S	R	S	R	S	+	+	+	+
1-3-13-BS	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	+	+	-	-
1-3-13-BT	S	S	S	S	S	S	S	S	S	S	R	S	R	S	+	+	+	+	
1-4-11-BT	S	R	R	R	R	I	S	S	S	S	R	S	R	S	-	+	+	-	
1-4-11-BS	S	R	R	R	R	I	S	S	S	S	R	S	R	S	+	+	-	+	
4-2-6-BS	S	S	S	S	S	S	S	S	S	S	R	S	R	S	+	+	+	-	
4-2-7-BT	S	R	R	R	R	I	S	S	S	S	R	S	R	S	+	+	+	-	
4-3-15-BS	S	S	S	S	S	S	S	S	S	S	S	S	R	S	+	+	+	+	
4-4-2-BT	S	R	R	R	R	I	S	S	S	S	S	S	R	S	+	+	+	-	
4-4-6-BS	S	R	R	R	R	I	S	S	S	S	R	S	R	S	+	+	+	+	
6-2-16-BT	S	S	S	S	S	S	S	S	S	S	S	S	R	S	+	+	-	-	
6-3-3-BS	S	S	S	S	S	S	S	S	S	S	S	S	R	S	+	+	-	+	
6-3-4-BS	S	S	S	S	S	S	S	S	S	S	R	S	R	S	+	+	+	+	
6-3-16-BT	S	S	S	S	S	S	S	S	S	S	S	S	R	S	+	+	-	-	
6-4-21-BT	S	R	R	R	R	I	S	S	S	S	S	S	R	S	+	+	+	+	
6-4-29-BS	S	R	R	I	R	S	S	S	S	S	R	S	R	S	+	+	-	+	
CR-2-19-BP ^f	S	R	R	R	R	I	S	S	S	S	S	S	S	S	+	+	+	+	
CR-2-28-BP	S	S	S	S	S	S	S	S	S	S	R	S	R	S	+	+	+	+	
CR-3-35-BP	S	S	S	S	S	S	S	S	S	S	S	S	S	S	+	+	+	+	
CR-4-23-BP	S	R	R	R	R	I	S	S	S	S	S	S	R	S	+	+	+	+	

^a Strains 71929P and 71929D0 were the parent and inoculum strains used in the commingling experiment (Cosby et al., 2012).

^b Isolates are grouped according to day of recovery (1 is equivalent to d 7, 4 is equivalent to d 28, 6 is equivalent to d 42 and CR is equivalent to d 43, carcass rinse).

^c Results in a box differed either from the parent and/or the inoculum strains.

^d BS indicates that isolates were recovered from media supplemented with Str.

^e BT indicates that isolates were recovered from media supplemented with Tet.

^f Isolate recovered from chlorinated chilled carcass rinsate.

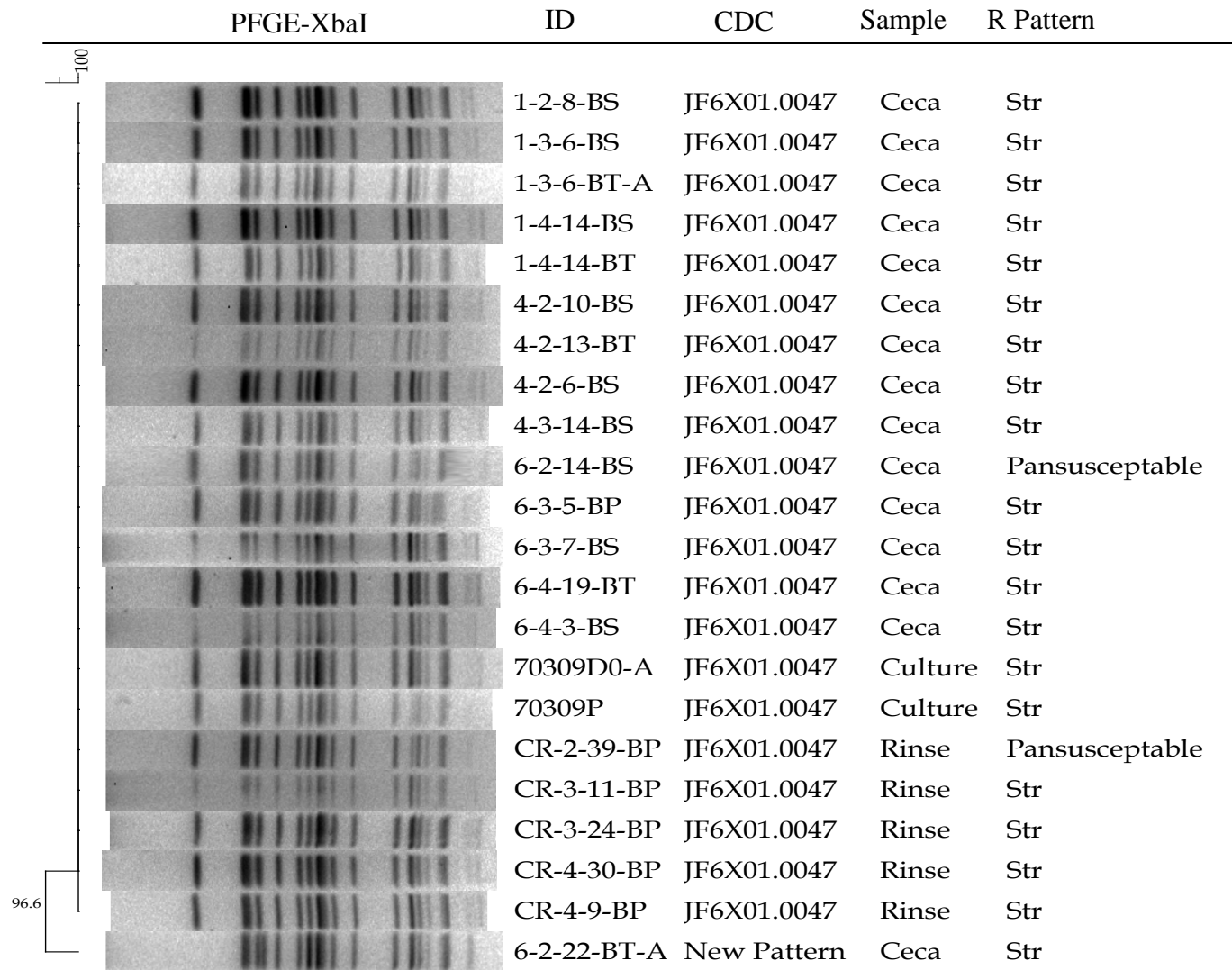


Figure 4.1. PFGE profile and dendrogram for the *Salmonella* Heidelberg^{StrR} strains 70309P, 70309D0 and the recovered *Salmonella* Heidelberg isolates

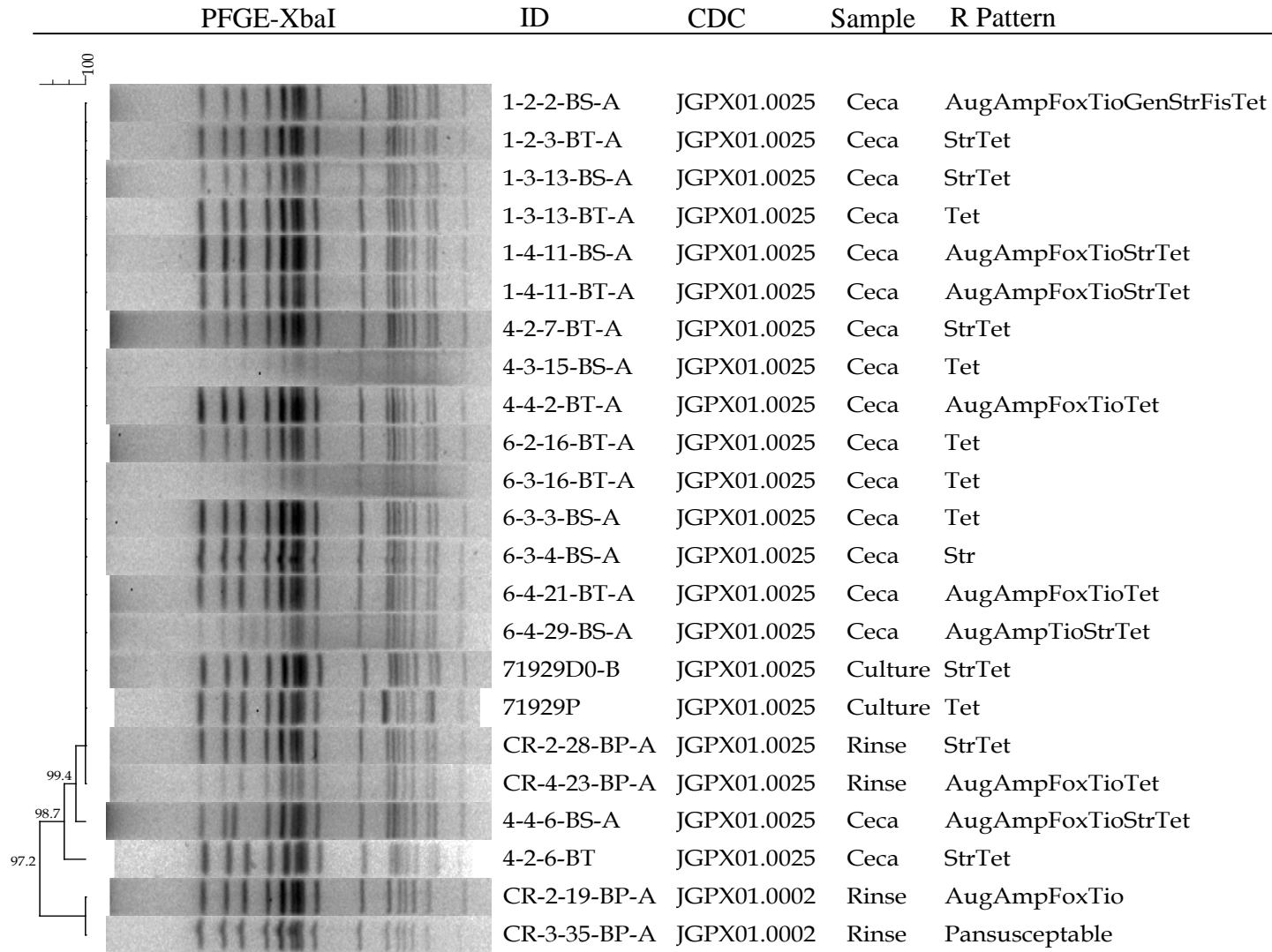


Figure 4.2. PFGE profile and dendrogram for the *Salmonella* Kentucky^{TetR} strains 71929P, 71929D0 and the recovered *Salmonella* Kentucky^{TetR} isolates

CHAPTER 5

SUMMARY AND CONCLUSIONS

The objectives of these studies were 1) evaluate the difference in the prevalence rates for two of the top serotypes isolated from poultry and poultry products to determine if the increased recovery rates for *Salmonella* Kentucky versus *Salmonella* Heidelberg is due to bacterial competition between *S. Heidelberg* and *S. Kentucky* and 2) to characterize both phenotypically and genotypically the isolates to determine if there is a genetic or phenotypic reason for the differences in the prevalence of recovery of the two *Salmonella* serotypes.

For the first objective, three replicate treatment rooms were populated with day-of-hatch chicks at the same density as found in a commercial poultry operation of which 10% were orally gavaged with SH and 10% with SK (for a total of 20% of the chickens in a treatment room) which had been selected from the National Antimicrobial Resistance Monitoring System for resistance to streptomycin and tetracycline, respectively. On days 7, 28 and 42 cecal samples were collected and cultured for the presence the two antimicrobial resistant *Salmonella*. On day 43, 40 carcasses were processed in the Richard B. Russell Research Center pilot processing plant, whole carcass rinses performed, rinsates collected and cultured for the presence of the antimicrobial resistant *Salmonella*. Among the treatment rooms, 76.7, 74.4 or 66.7% of all chicks sampled by cecal sampling were positive for SH, SK or both serotypes, respectively. After processing, 35.0, 18.0 or 4.2% of the carcass rinsates were positive for SH, SK or both serotypes, respectively. The recovery trend from this study does not follow the recovery

trend currently observed in the poultry industry where SK is the most commonly isolated serotype from carcass rinsates. However, the recovery rates of 77 and 74% for the two serotypes would imply that the bacterial competition theorized between *Salmonella* Heidelberg and Kentucky was not evident in this study.

For the second objective, antimicrobial resistance profiles, PCR probes for four resistance genes, *tetA*, *tetB*, *strA* and *aadA1* and pulsed-field gel electrophoresis for 20 SH and 21 SK isolates recovered during the first objective. Antimicrobial resistance profiles ranged from pan-susceptible isolates to 11 multidrug resistant profiles with *aug*, *amp*, *fox*, *tio*, *str* and *tet* being the most prevalent profile detected. The antimicrobial resistance profiles of the recovered SH isolates were more conserved than the resistance profiles in the recovered SK isolates, with 90% of the SH isolates exhibiting the same profile as the inoculum strain while only 24% of the SK isolates exhibited the same profile as the inoculum strain. PCR probes for resistance genes detected resistance genes *tetA*, *tetB*, *strA* and *aadA1* in 11, 10, 15 and 20 of the SH strains, respectively and in 19, 21, 16 and 14 of the SK strains, respectively. Pulsed-field gel electrophoresis revealed that both the SH and SK isolates had a >96.6% genetic similarity when compared to the parent and inoculum strains. This level of similarity indicates that the isolates were progeny of the parent and/or inoculum strains.

In conclusion, the data presented here are not consistent with the recovery pattern of *Salmonella* serotypes in the poultry industry. SK is the most commonly isolated serotype from carcass rinsates analyzed by USDA, FSIS while we recovered more SH in this study. However, the level of initial exposure by the chicks to the various *Salmonella* serotypes has not been fully determined. The recovery rate of *Salmonella* from carcass

rinsates does support the findings that the most prevalent serotype on the farm the final week of production, tends to be the most prevalent serotype isolated from the carcass. However, the level recovered on the farm the day or week prior to processing may not be indicative of the level recovered from the carcass rinsates in the plant. The use of genotypic methods for determining that the isolates are progeny of the inoculum provides an ability to trace the *Salmonella* strains through a grow-out cycle and provides the ability to trace changes during this process. Careful consideration and screening is necessary to ensure that the phenotypic markers expressed and used for selection of strains matches the genotypic markers in the organisms. Bacteria are capable of multiple adaptations for survival and the changes need to be studied more to ensure a full understanding of how to protect humans from infections.

APPENDICES

APPENDIX A

ENVIRONMENTAL SAMPLING

Drag Swabs.

Drag swabs (three per room) were collected according to the stepped on method of Buhr et al., 2007. The swabs were then placed into sterile plastic collection bags and transported to the laboratory. The three swabs were combined into one sample in the laboratory and 50 mL of Buffered Peptone water (BPW, B-D) was added. The swabs were stomached for 60 s (Seward stomacher). Thirty mL of the swab solution was removed and 10 mL was placed into a sterile 15 mL centrifuge tube, 10 mL into a sterile 15 mL centrifuge tube containing enough stock solution of tetracycline (Tet, Sigma Chemical) for a final concentration of 200 ppm Tet and 10 mL into a sterile 15 mL centrifuge tube containing enough stock solution of streptomycin (Str, Sigma Chemical) for a final concentration of 125 ppm Str. All samples were pre-enriched overnight at 37°C and isolation continued according to the U.S. Food and Drug Administration Bacteriological Analytical Manual Online (2011). After pre-enrichment, 0.1 mL of broth was transferred to 10.0 mL of RV Broth and 0.5 mL of broth was transferred into 10.0 mL of TT and all tubes were incubated 18-24 h at 42°C. After selective enrichment tubes from pre-enrichments without either Tet or Str were streaked onto BGS agar, BGS agar with 125 ppm Str, BGS agar with 200 ppm Tet plates and dMLIA plates; tubes with Str in the pre-enrichment broth were streaked onto BGS agar, BGS agar with 125 ppm Str and dMLIA plates; and tubes with Tet in the pre-enrichment broth were streaked onto

BGS agar, BGS with 200 ppm Tet and dMLIA agar plates. All plates were incubated at 37°C for 24 h, observed and re-incubated for 24 h. Isolated colonies with typical growth patterns were picked using disposable inoculation loops/needles and inoculated onto TSI and LIA slants for screening. Tubes with typical reactions were further analyzed. One colony per plate for a maximum of 20 colonies per ceca sampled were further screened for serogrouping and serotyping.

Litter Samples

Litter samples were collected according to the methods of Bailey et al., (2001) with the following modification. Only 4 litter grabs were collected per room, two near the feed pans and two from underneath the water line. The litter was placed into a single gallon zip-lock bag for each room, transported to the laboratory. After mixing the litter in the collection bag to create a homogeneous mixture, three 10 g samples were weighed into sterile 100 mL specimen cups for each sample. Ninety mL of BPW was added to each specimen cup. One cup was pre-enriched overnight at 37°C without antimicrobials, one was pre-enriched overnight with enough stock solution of Tet added for a final concentration of 200 ppm Tet, and one was pre-enriched overnight with enough stock solution of Str added for a final concentration of 125 ppm Str. After pre-enrichment, 0.1 mL of broth was transferred to 10.0 mL of RV Broth and 0.5 mL of broth was transferred into 10.0 mL of TT and all tubes were incubated 18-24 h at 42°C. After selective enrichment tubes from pre-enrichments without either Tet or Str were streaked onto BGS agar, BGS agar with 125 ppm Str, BGS agar with 200 ppm Tet plates and dMLIA plates; tubes with Str in the pre-enrichment broth were streaked onto BGS agar, BGS agar with 125 ppm Str and dMLIA plates; and tubes with Tet in the pre-enrichment broth were

streaked onto BGS agar, BGS with 200 ppm Tet and dMLIA agar plates. All plates were incubated at 37°C for 24 h, observed and re-incubated for 24 h. Isolated colonies with typical growth patterns were picked using disposable inoculation loops/needles and inoculated onto TSI and LIA slants for screening. Tubes with typical reactions were further analyzed. One colony per plate for a maximum of 20 colonies per ceca sampled were further screened for serogrouping and serotyping.

Serogrouping and Serotyping

Isolates exhibiting typical biochemical reactions on TSI and LIA slants were screened for somatic antigens using O-antiserum (B-D) and flagellar antigens using Microgen latex agglutination (Microbiology International, Frederick, Maryland).

Serotyping was conducted using the SMART Serotyping system as described by Leader et al., (2009) using an ABI31360xl Genetic Analyzer (Applied Biosystems, Foster City, California).

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Table A-1. Recovery of *Salmonella* Kentucky^{Tet^R}, *Salmonella* Heidelberg^{Str^R} or both serotypes from drag swab and litter samples at d 14 of grow-out

Sample	Serotype	Treatment Room			
		1	2	3	4
Drag Swab	SK ^{Tet^R}	-	+	+	+
	SH ^{Str^R}	-	+	+	+
	Both	-	+	+	+
Litter	SK ^{Tet^R}	-	+	+	+
	SH ^{Str^R}	-	+	+	+
	Both	-	+	+	+

APPENDIX B

PLASMID DETECTION

Plasmids were extracted from the 45 *Salmonella* strains using the Qiagen Miniprep kit (QIAGEN Inc., Valencia, CA) using the manufacturers QIAprep Spin protocol with modifications for purification of low-copy plasmids and cosmids (QIAGEN, 2006). Plasmid DNA was quantified using a NanoDrop ND1000 UV/Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA, v3.5.2). All plasmid preparations were diluted to approximate 100 ng/5 μ L in 1.0 M Tris + HCl, pH~8.0 containing 0.1 M EDTA (Sigma). The diluted plasmid preparation was mixed (5 μ L preparation) with 2 μ L of a sample loading buffer (Bio-Rad, Cat # 161-0767) before being loaded into the wells of the 0.8% Seakem[®] Gold (BioWhittaker Molecular Applications, Rockland, ME) agarose gel on a Bio-Rad Sub-Cell GT horizontal electrophoresis cell (Bio-Rad, Hercules, CA) utilizing the 15 x 15 cm tray and using the 20 well comb. The power supply for electrophoresis was an E-C 250-90 (E-C Apparatus Corp., Waltham, MA) set with the following parameters: 80 V, 65 min. Tris-Borate-Edta buffer (0.089 M Tris base, 0.089 M Borate, and 0.002 M EDTA (free acid) (TBE)) (Amresco Life Science Research Products and Biochemicals, Solon, OH, USA) was used as the running buffer and to prepare the gel. A 2.5 kb Molecular Ruler (Bio-Rad, Cat # 170-8205) and a Wide-Range DNA Molecular Marker (Amresco, Cat # E273) were used as molecular markers.

After electrophoresis all gels were stained in 1 L of de-ionized distilled H₂O with 0.5 μ g/mL ethidium bromide (50 μ L of a 10 mg/mL stock solution (Bio-Rad)) for one h and de-stained in de-ionized distilled H₂O for one h at room temperature with gentle

agitation. The agarose gels were documented on a Gel-Doc 1000 (Bio-Rad) and the base pair (bp) estimate of all plasmids was determined using a standard curve calculated from the molecular weight markers.

Table B.1. Plasmids detected

<i>Salmonella</i> Kentucky		<i>Salmonella</i> Heidelberg	
Identification	Plasmid Size (kbp ^a)	Identification	Plasmid Size (kbp)
71929P	21.5; 37.5	70309P	1.6
71929D0	37.5	70309D0	1.6; 37.5
1-2-2-BS	21.5	1-2-8-BS	1.6; 37.5
1-2-3-BT	ND ^b	1-3-6-BS	1.6; 2.4; 37.5
1-3-13-BT	ND	1-3-6-BT	ND
1-3-13-BS	21.5	1-4-14-BS	1.6; 37.5
1-4-11-BT	ND	1-4-14-BT	1.6; 37.5
1-4-11-BS	21.5	4-2-6-BS	1.6; 37.5
4.-2-6-BS	ND	4-2-10-BS	1.6
4-2-7-BT	ND	4-2-13-BT	1.6
4-3-7-BT	ND	4-3-14-BS	1.6
4-3-15-BS	2.4	6-2-14-BS	1.6; 2.4; 37.5
4-4-2-BT	ND	6-2-22-BT	1.6
4-4-6-BS	ND	6-3-5-BP	ND
6-3-3-BS	ND	6-3-7-BS	1.6; 2.4; 37.5
6-3-4-BS	ND	6-4-3-BS	1.6
6-3-16-BT	ND	6-4-19-BT	1.6
6-4-21-BT	21.5	CR-2-39-BP	1.6
6-4-29-BS	1.6	CR-3-11-BP	1.6
CR-2-19-BP	ND	CR-3-24-BP	1.6
CR-2-28-BP	ND	CR-4-9-BP	1.6; 37.5
CR-3-35-BP	ND	CR-4-30-BP	1.6
CR-4-23-BP	1.6; 21.5; 37.5		

^a kbp = kilo base pairs

^b None detected

APPENDIX C

PFGE USING THE RESTRICTION ENZYME *blnI*

PFGE was performed on each *Salmonella* isolate to assess their genotypic relatedness when compared to the parent and inoculum strains. PFGE was performed as previously described for the USDA VetNet program (1) which is a modification of the 23-24 h PFGE procedure described and used by PulseNet (2, 3). Restriction endonuclease *blnI* (Roche Diagnostics Corporation, Indianapolis, IN) was used for restriction digestion of the cDNA. The PFGE tagged image file format (TIFF) images were analyzed using BioNumerics version 5.10 (Applied Maths Scientific NV, Saint-Martens-Latem, Belgium). Strain relatedness was determined using the different bands algorithm for clustering and the un-weighted pair grouping for arithmetic means (UPGMA) tree building approach with a position tolerance of 1.7%. Patterns were assigned by placing isolates in a comparison and arranging them by decreasing similarity. PFGE profiles were compared against the both VetNet profiles and the CDC PulseNet profiles and assigned profile names according to the PulseNet database.

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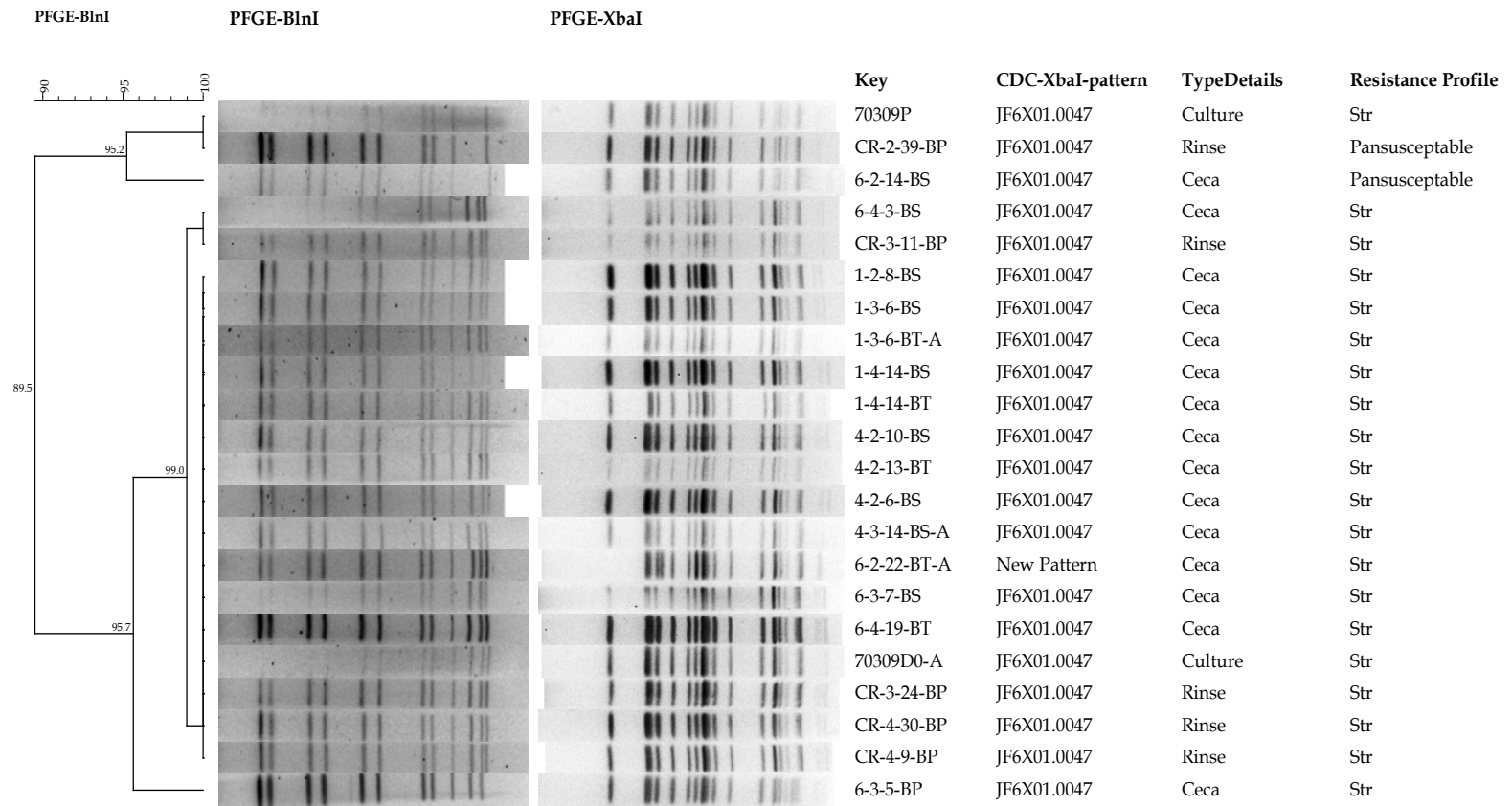


Figure C.1. PFGE profile and dendrogram for the *Salmonella* Heidelberg^{StrR} strains 70309P, 70309D0 and the recovered *Salmonella* Heidelberg^{StrR} isolates using *blnI* as the second restriction enzyme

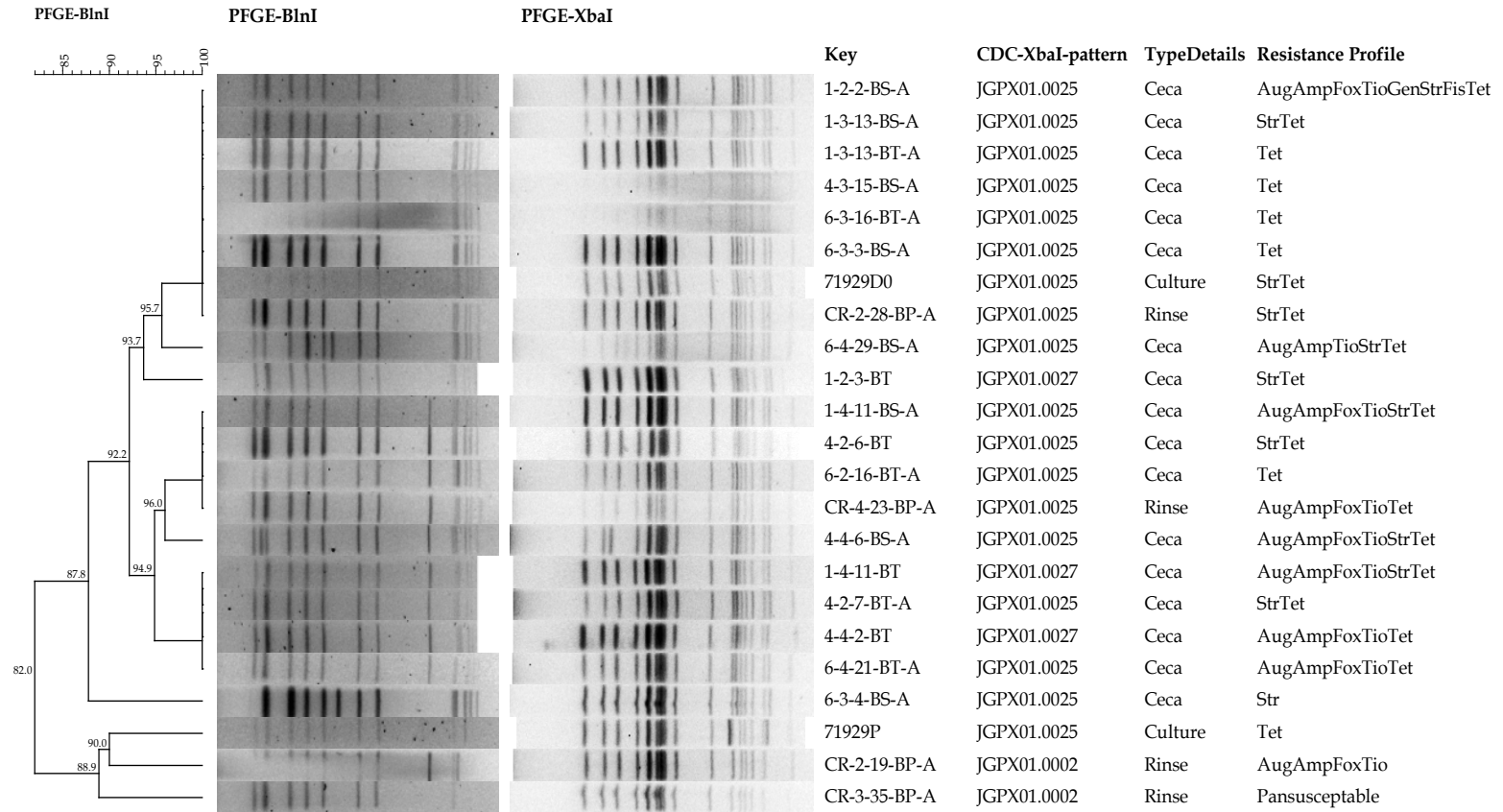


Figure C.2. PFGE profile and dendrogram for the *Salmonella* Kentucky^{TetR} strains 71929P, 71929D0 and the recovered *Salmonella* Kentucky^{TetR} isolates using *blnI* as the second restriction enzyme