ECOLOGY OF SALMONELLA AND ITS ACQUISITION OF ANTIBIOTIC RESISTANCE IN AN INTEGRATED BROILER PRODUCTION SYSTEM POULTRY

by

KAREN A LILJEBJELKE

(Under the Direction of John J. Maurer)

ABSTRACT

In order to understand the ecology of *Salmonella* within an integrated commercial broiler production system, 289 *Salmonella enterica* were recovered from two integrated poultry farms during the production and processing of seven consecutive flocks. The variety and prevalence of *Salmonella* serotypes differed between farms, with fifteen serotypes identified. *Salmonella* Typhimurium and Enteritidis recovered from processed carcasses from Farm One were characterized using pulsed-field gel electrophoresis, and were indistinguishable from isolates recovered from the poultry house environment and mice trapped on this farm. Combining isolates from both farms, 61.9 percent were pan-sensitive to a panel of eighteen antimicrobials used in the National Antimicrobial Resistance Monitoring Service (NARMS) surveillance. Combinations of resistance against streptomycin, gentamicin, sulfamethoxazole, trimethoprim, and tetracycline, were observed for a variety of *S. enterica* serotypes and PFGE genetic types. Among all serotypes, 87.3 percent contained the class 1 integron marker, *intI1*. Resistance to streptomycin, gentamicin, sulfamethoxazole, trimethoprim, and ampicillin was positively correlated ($p < 0.05$) with presence of *intI1*, a marker for the class 1 integron. Results indicate that management practices at the breeder level may have a profound effect on the transmission and persistence of salmonellae within an integrated production system.

INDEX WORDS: *Salmonella, antibiotic resistance, broilers, integrated poultry production, Typhimurium, Enteritidis, class I integron, Tn21*

ECOLOGY OF SALMONELLA AND ITS ACQUISITION OF ANTIBIOTIC RESISTANCE IN AN INTEGRATED BROILER PRODUCTION SYSTEM

by

KAREN A LILJEBJELKE

B.S., Cornell University, 1985

M.S., Washington State University, 1988

DVM, The University of Georgia, 2000

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2004

ECOLOGY OF SALMONELLA AND ITS ACQUISITION OF ANTIBIOTIC RESISTANCE IN AN INTEGRATED BROILER PRODUCTION SYSTEM

by

KAREN A LILJEBJELKE

Approved:

Major Professor: John J. Maurer

Committee: Charles L. Hofacre
Margie D. Lee
David Stallknecht
Barry Harmon

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
December 2004

ACKNOWLEDGEMENTS

I want to give thanks and appreciation to everyone who has helped me in reaching this goal:

My committee:

Dr. John Maurer
Dr. Charles Hofacre
Dr. Margie D. Lee
Dr. Barry Harmon
Dr. David Stallknecht

My committee provided me with the resources and knowledge that made my graduate experience successful and rewarding.

Coworkers:

Marie Maier
Tameka Bythwood
Andrea Polachini de Oliveira
Dr. David Drum
Joseph Minicozzi
Dr. Yang Hong
Dr. Charlene Hudson
Dr. Tongrui Liu
Dr. Gloria Avellaneda
Dionna Rookey

These people are my friends and excellent resources in the laboratory. Thank you for your patience and support.

Those closest to me – my friends and family:

Rose Bjelke-Neary
Harold Bjelke
Audrey Bjelke
John Bjelke
Dr. Steve Leshin
Dr. Angela Dodd
My feline family

Thank you all so much for your love and support (and purrs).

TABLE OF CONTENTS

Page

CHAPTER 1

INTRODUCTION

In the United States alone there are over 38 million illnesses each year attributed to foodborne pathogens (Mead, Slutsker et al. 1999). It is estimated that foodborne infections account for 181,177 hospitalizations for acute gastroenteritis caused by known pathogens in the United States (Mead, Slutsker et al. 1999). Foodborne disease related deaths are estimated to be 2,718 (Mead, Slutsker et al. 1999). Most common bacterial pathogens associated with gastroenteritis are *Campylobacter* spp. at 2,453,926 cases per year, *Salmonella* spp. at 1,412,496 cases per year, and *Shigella* spp. at 448,240 cases per year (Mead, Slutsker et al. 1999). While *Campylobacter* is the most common cause of gastroenteritis, *Salmonella* has a higher death rate per year than *Campylobacter* (Mead, Slutsker et al. 1999). It has been estimated that approximately 1.4 million non-typhoidal salmonellosis cases occur in the U.S. each year (Mead, Slutsker et al. 1999), and consumption of poultry products has been implicated in 40% of these outbreaks (Olsen, Bishop et al. 2001).

It has been established that *Salmonella* can become endemic in many types of food animal production units (Sanchez 2002). Mice, rats, and insects have been conclusively shown to be vectors and reservoirs of *Salmonella* and other pathogens in food animal production (Henzler 1992.; Davies 1995.). To curtail the transmission of *Salmonella* to humans through the food chain, it is important to identify potential sources of contamination within the integrated poultry production system. Because of the linkage between colonization of live birds with *Salmonella* on farms with contamination of finished poultry products, further reductions in the

level of carcass contamination will require a variety of on-farm intervention strategies (Rigby, Petit et al. 1982; Sanchez 2002).

Due to public concern about food-borne illness and subsequent governmental pressure, the Food Safety and Inspection Service (FSIS) mandated in 1996 that meat and poultry processing plants implement a Hazard Analysis Critical Control Point (HACCP) program and meet set limits for *Salmonella* contamination of finished poultry products. Since that time, the poultry industry has made significant reductions in the levels of poultry carcass contamination with *Salmonella*, from 20% in 1998 to 10% in 2000 (Schlosser 2000). However, a recent survey (2002) of retail meats from supermarkets in the Washington D. C. area revealed that the levels of *Salmonella* contamination of beef, pork and poultry products still poses a threat to the consumer (White, Zhao et al. 2001). The continuing contamination of retail poultry products with *Salmonella* has important public health implications, especially considering the global increase in chicken consumption (Fuzihara, Fernandes et al. 2000).

The dissemination of drug-resistant *Salmonella* through the food chain has important public health implications considering the potential for treatment failure when cases of gastroenteritis require medical intervention, especially in children, the elderly, and the immuno-compromised (Cohen 1986). The emergence and rapid worldwide spread of the multiple drug resistant *S. enterica* Typhimurium phage-type DT104 clone, and the recent emergence of ceftriaxone-resistant *S. enterica* serotypes Typhimurium and Newport, has underscored the threat to both animal agriculture and human health that multiple drug resistant pathogens pose (Rankin SC 200; Glynn MK 1998; Hollinger K 1998; Fey PD 2000). Antibiotic resistance genes are widely disseminated in pathogenic, commensal and environmental bacteria (Goldstein C 2001; Nield, Holmes et al. 2001). Further, it has been shown that once antimicrobial resistance has

been introduced into an ecosystem, resistance can spread and persist without continuing selection pressure from antibiotics (Marshall 1990; Salyers and Amabile-Cuevas 1997). In addition, the reservoir of antimicrobial resistance genes is larger than previously thought (Nandi, Maurer et al. 2004). It is in this environment that the potential exists for *Salmonella* to acquire drug resistance genes from resident poultry microbiota due to selection pressure from therapeutic and non-therapeutic antibiotic usage. It follows then, that the longer *Salmonellae* persist in the environment of an animal production facility, the chance of acquiring resistance genes increases.

There were two specific aims for this study.

Specific Aim I: *To examine the ecology of Salmonella present on two integrated poultry farms in Northeast Georgia.*

Currently, the USDA HACCP inspection program places its emphasis on surveying bacteria isolated primarily from processing plant samples and carcasses at the time of slaughter. However, it is difficult for current post-harvest monitoring efforts to take into account the variability in *Salmonella* contamination that occurs not only between geographic regions, but within farms or levels of poultry production (breeder farms, hatchery, broiler farms, feed mill) that ultimately reflect the true level of *Salmonella* contamination within an integrated company. We therefore took advantage of the short production cycle, high population number, and vertically integrated system of poultry production to conduct an epidemiological examination of the movement of *Salmonella* within two integrated commercial poultry companies. In this study, we specifically focused on the epidemiology of *Salmonella* on two commercial, broiler chicken farms using molecular typing by pulsed field gel-electrophoresis (PFGE). Our objectives were to observe the ecology of the *Salmonella* serotypes and strains resident in two integrated poultry companies, and to collect detailed epizoological information which will help the poultry industry

develop practical intervention strategies towards reducing the levels of *Salmonella* contamination of finished poultry products.

By monitoring *Salmonella* contamination during the production of seven consecutive flocks on two commercial broiler farms, we identified a variety of *S. enterica* serotypes including those commonly associated with poultry in general (Poppe 1995; Bailey, Stern et al. 2001; Sarwari, Magder et al. 2001), other food animal species (Letellier 1999; Dargatz 2000; Anderson 2001), as well as human illnesses (Olsen, Bishop et al. 2001). Although we observed the same *Salmonella* serotypes, their prevalence varied between farms in this study, as compared to the results from other *Salmonella* regional and national studies (Anonymous 2001).

Indistinguishable PFGE types of *S.* Typhimurium, *S.* Enteritidis, and *S.* Heidelberg were isolated from processed chicken carcasses, the broiler chicken environment, and chick-box liners, implicating the hatchery as the source for these persistent serotypes on Farm One. Investigation of Company A's breeder farms, producing eggs for chicks to be placed on Farm One, yielded isolates of *S.* Typhimurium and *S.* Enteritidis with the same PFGE patterns as those *Salmonella* isolates resident on Farm One. Thus, vertical transmission from breeders to the broilers appeared to be the ultimate source of the resident *Salmonella* serotypes identified in this integrated production system.

Specific Aim II: *To identify and characterize the antibiotic resistance present in the Salmonella isolates from these farms.*

In this study, we analyzed the physical and temporal patterns of antibiotic resistance in *Salmonella* serotypes isolated from this same system. Preventing the development of antibiotic resistance will depend on our ability to interrupt horizontal and vertical transmission of endemic *Salmonella* strains within this production system.

Two hundred eighty-nine *Salmonella enterica,* representing fifteen serotypes were isolated from two poultry farms during the production and processing of seven consecutive flocks. The *Salmonella* serotypes recovered differed between the two companies, as did the levels of *Salmonella* contamination, and the antibiotic resistance phenotypes. Combining isolates from both farms, 61.9 percent were pan-sensitive to a panel of eighteen antimicrobials used in the national antimicrobial resistance monitoring service (NARMS) surveillance. Resistance to streptomycin, alone, and in combination with other resistances, was the most common (36.3%) antibiotic resistance phenotype observed. Combinations of resistance against streptomycin, gentamicin, sulfamethoxazole, trimethoprim, and tetracycline, were observed for a variety of *S. enterica* serotypes and PFGE genetic types. Among all serotypes, 87.3 percent contained the class 1 integron marker, *intI1*. Resistance to streptomycin, gentamicin, sulfamethoxazole, trimethoprim, and ampicillin was statistically positively associated with presence of *IntI1*, a marker for the class 1 integron. The prevalence of resistance to streptomycin was surprisingly high, considering that streptomycin is not used in poultry production, but can be explained by the high carriage of transposon Tn*21* observed among our isolates. The *Salmonella* present on these commercial broiler chicken farms possess the genetic potential, in their resident integrons, for acquiring multiple drug resistance. It is therefore surprising that our isolates do not have a higher prevalence and/or diversity of antibiotic resistance phenotypes, despite high antibiotic resistance gene load evident in this environment.

The uneven distribution of both *Salmonella* serotypes, and resistance phenotypes between the different areas of the integrated system examined (hatchery, house, carcass), can be explained by the hypothesis that we are observing two separate populations of *Salmonella* which have different degrees of interaction with the environmental microbiota depending on where in the

system they are located. Analysis of the temporal and physical distribution of the endemic serotypes previously identified with their antibiotic phenotypes, suggests that prevention of antimicrobial resistant *Salmonella* on poultry products is dependent on prevention of vertical transmission of *Salmonella* in the production system.

CHAPTER 2

LITERATURE REVIEW

I. Antibiotics and agriculture:

As the world's population has increased, the demand for food has increased, and this has in turn increased production pressures on agriculture that have resulted in the development of global agribusiness and systems for the mass production of food animals. Intensive farming and rearing practices have in turn resulted in the increased use of antibiotics in agriculture. It is estimated that of the approximately 50 tons of antibiotics used annually in human medicine, veterinary medicine, and agriculture in the United States, approximately half are used in agriculture, and use as animal growth promoters in feed accounts for approximately 15% of agricultural usage (Phillips 1999; Lipstitch, Singer et al. 2002). Antibiotics are used for a variety of purposes in agriculture. They are sprayed on fruit trees, added to animal feed, used for therapeutic treatment of individual sick animals, or added to the feed and drinking water of herds or flocks of animals where disease has been detected (metaphylactic use). Use of antibiotics in plant agriculture accounts for less than 0.5% of total use, with oxytetracycline and streptomycin the most frequently used antibiotics (McManus, Stockwell et al. 2002). Certain antibiotics are routinely added to animal feed as animal growth promoters (AGP). Animals raised for food purposes are commonly given antibiotics to treat and prevent disease, but also to improve growth rate and feed conversion (Anonymous 1999; Heilig, Lee et al. 2002). Research has shown that addition of AGP to feed increases feed efficiency and growth rate, and decreases prevalence of intestinal disorders such as necrotic enteritis which result from the intensive feeding regimens employed in modern animal production. Although the exact mechanism whereby continual sub-

therapeutic ingestion of certain antimicrobials enhances the feed efficiency and growth rate of animals is not known, there are several hypotheses regarding this effect (Stokstad 1949; Anonymous 1999). The use of antibiotics in feed has been shown to decrease the mass of normal intestinal flora, thereby theoretically decreasing competition between the intestine and flora for usage of nutrients in the feed, subsequently increasing the absorbtion of food metabolites (Anonymous 1999).

The poultry industry is an excellent model of the "factory farming" methods of food animal production that have been lambasted in the popular press as contributing to the development and spread of bacterial antibiotic resistance. The production cycle (hatch to catch) of broiler chickens is short, at five to seven weeks, and the birds are raised in dense populations (0.75 square feet per bird) (Mauldin 2002). Individual companies are both vertically and horizontally integrated, supplying chicks, feeds, and feed additives to the contracted producers. This closed system allows for company veterinarians to monitor production records for management problems and disease outbreaks, and control the use of antibiotics and feed and water additives by the individual producers. The all-in all-out flock rearing is designed to help break disease cycles, and should limit the development of antibiotic resistance among pathogens in the flocks (Hofacre 2001).

Broiler chicken feed typically contains coccidiostats which are arsenical compounds, ionophores, and a broad-spectrum antibiotic such as bacitracin, virginamycin, or flavomycin, to improve feed efficiency and body weight gains, and for reduced morbidity and mortality (Anonymous 1999; Mauldin 2002). The use of AGP in feed exposes the intestinal flora to a sub-lethal level of antimicrobials that serves to provide a continuous selection pressure in which

resistance to the antimicrobial agents can develop (Anonymous 1999; Heilig, Lee et al. 2002; Mauldin 2002).

Avoparcin, the veterinary analog of the human drug vancomycin, is used as a growth promoter in swine and poultry production in Europe, and its use in feed has been implicated as the selective force behind the emergence of vancomycin-resistant *Enterococci* (VRE) as a human pathogen (Bonten, Willems et al. 2001). Vancomycin-resistant *Enterococcus* has been increasingly reported to be associated with a variety of nosocomial and community-acquired infections in both Europe and in the United States (Anglim, Klym et al. 1997). It is in response to such reports that public concern has been raised concerning the use of veterinary antibiotics, particularly as feed additives, as contributing to the development and transmission of drug-resistant microbes through the food chain.

II. Food-borne Disease:

Food-borne diseases are one of the most common causes of illness in the world (Anonymous 1984). It is estimated that reported food-borne illness represents less than 10% of the real incidence, and some surveys indicate that the incidence of foodborne disease may be more than three hundred times more frequent than reported cases indicate (Todd 1989; Notermans and Hoogenboom-Verdegaal 1992). Although food production and handling in the United States is among the safest and most regulated in the world, there are over 38 million reports of illness in the United States each year that are attributed to food-borne pathogens (Mead, Slutsker et al. 1999). It is estimated that 181,177 hospitalizations for acute gastroenteritis caused by known food-borne pathogens occur each year in the United States (Mead, Slutsker et al. 1999). Each year in the United States food-borne disease related deaths are estimated to be 2,718 (Mead, Slutsker et al. 1999). The most common bacterial pathogens associated with

gastroenteritis in order of prevalence are; *Campylobacter* (2,453,926 cases per year), *Salmonella* (1,412,496 cases per year), and *Shigella* (448,240 cases per year) (Mead, Slutsker et al. 1999). While *Campylobacter* is the most common cause of gastroenteritis, *Salmonella* has a higher death rate per year than *Campylobacter* (Mead, Slutsker et al. 1999). *Salmonella* remains the leading cause of outbreak-associated gastroenteritis in the United States (Olsen, Bishop et al. 2001). It has been estimated that approximately 1.4 million non-typhoidal salmonellosis cases occur in the U.S. each year (Mead, Slutsker et al. 1999), and consumption of poultry products has been implicated in 40% of these outbreaks (Olsen, Bishop et al. 2001).

III. *Salmonella* and Poultry Production:

Chickens are the most abundant commercially farmed food animal in the world today (Lukefahr 1999). The *Salmonella enterica* serotypes commonly encountered in poultry include: *S.* Enteritidis, *S.* Typhimurium, *S*. Hadar, *S*. Newport, *S*. Seftenberg, *S*. Virchow, *S*. Infantis, and *S*. Schwarzengrund (Poppe, Irwin et al. 1991; Baggesen and Wegener 1994; Uyttendaele, Debevere et al. 1998; Beli, Duraku et al. 2001). In the United States, the most common *Salmonella* serotypes in commercially produced chickens, turkeys, quail and ducks include; *S*. Typhimurium, *S*. Enteritidis, *S*. Heidelberg, *S*. Newport, and *S*. Hadar (Olsen, MacKinnon et al. 2000). Similarly, the Centers for Disease Control (CDC) has reported that the most common *Salmonella* serotypes responsible for human salmonellosis in the United States are: *S*. Typhimurium, *S*. Enteritidis, *S*. Heidelberg, *S*. Newport, and *S*. Hadar (Olsen, MacKinnon et al. 2000). To curtail the transmission of *Salmonella* to humans through the food chain, it is important to identify potential sources of contamination within the integrated poultry production system.

Prior to 1970, *S.* Typhimurium was the most common serotype associated with foodborne disease outbreaks in the United States (Tauxe 1991; Olsen S.J. 2001.). Recently, *Salmonella* Enteritidis has supplanted Typhimurium as the dominant serotype associated with food-borne outbreaks of salmonellosis in the United States(Tauxe 1991). The majority of outbreaks caused by *Salmonella* serotype Enteritidis have been associated with the consumption of contaminated eggs and egg products (Angulo and Swerdlow 1998). In Europe, *S.* Enteritidis phage type 4 has been most often associated with human illness, while in the United States and Canada, *S.* Enteritidis phage-type 8 is most often associated with human morbidity and mortality, although phage type 4 has recently emerged in this country (Boyce T.G. 1996.). Although outbreaks of foodborne illness due to *S.* Enteritidis are most commonly associated with consumption of uncooked contaminated eggs, there are numerous reports in the literature from the United States and elsewhere, of sporadic cases of human illness traced to consumption of *S.* Enteritidis-contaminated poultry products (Rampling A. 1989.; Boonmar s. 1998.). The potential for causation of foodborne illness should therefore not be ignored, considering the presence of *S.* Enteritidis on poultry products.

It has long been established that *Salmonella* can become endemic in many types of food animal production units (Sanchez 2002). Mice, rats, and insects have been conclusively shown to be vectors and reservoirs of *Salmonella* and other pathogens in food animal production (Henzler 1992.; Davies 1995.). Control of mice in egg-layer facilities has been shown to be crucial to control of *S. enterica* Enteritidis infection of laying hens and subsequent contamination of table eggs (Henzler 1992.; Davies 1995.). In order to reduce or eradicate *Salmonella* from these environments, it is necessary to prevent its introduction or re-introduction onto the farm (Sanchez 2002). Because of the linkage between colonization of live birds with

Salmonella on farms with contamination of finished poultry products, further reductions in the level of carcass contamination will require a variety of on-farm intervention strategies (Rigby, Petit et al. 1982; Sanchez 2002). Because *Salmonella* can potentially enter at numerous points into the integrated poultry production system, the success of any intervention strategy will also require continuous monitoring of both the poultry production environment and the finished product for levels of *Salmonella* contamination. It follows then, that in order to reduce or eradicate *Salmonella* from the broiler farm environment and prevent it's introduction or re-introduction onto the farm requires detailed knowledge of the epizoology and ecology of *Salmonella* resident on farms, and in integrated production systems (Sanchez 2002).

Due to public concern about food-borne illness and subsequent governmental pressure, the Food Safety and Inspection Service (FSIS) mandated in 1996 that meat and poultry processing plants implement a Hazard Analysis Critical Control Point (HACCP) program and meet set limits for *Salmonella* contamination of finished poultry products. Since that time, the poultry industry has made significant reductions in the levels of poultry carcass contamination with *Salmonella*, from 20% in 1998 to 10% in 2000 (Schlosser 2000). However, a recent survey (2002) of retail meats from supermarkets in the Washington D. C. area revealed that the levels of *Salmonella* contamination of beef, pork and poultry products still poses a threat to the consumer (White, Zhao et al. 2001). The continuing contamination of retail poultry products with *Salmonella* has important public health implications, especially considering the global increase in chicken consumption (Fuzihara, Fernandes et al. 2000).

Currently, the USDA HACCP inspection program places its emphasis on surveying bacteria isolated primarily from processing plant samples and carcasses at the time of slaughter. However, it is difficult for current post-harvest monitoring efforts to take into account the

variability in *Salmonella* contamination that occurs not only between geographic regions, but within farms or levels of poultry production (breeder farms, hatchery, broiler farms, feed mill) that ultimately reflect the true level of *Salmonella* contamination within an integrated company.

IV. Antibiotic resistance and *Salmonella*:

The dissemination of drug-resistant *Salmonella* through the food chain has important public health implications considering the potential for treatment failure when cases of gastroenteritis require medical intervention, especially in children, the elderly, and the immuno-compromised (Cohen 1986). In addition, infections with antimicrobial resistant bacteria including *Salmonella*, have been associated with higher rates of morbidity and mortality, although this phenomenon has not been attributed to any particular genetic mechanism of increased virulence (Barza 1987; Helms M 2002; Martin J.L. 2004).

The emergence and rapid worldwide spread of the multiple drug resistant *S.* Typhimurium phage-type DT104 clone, and the recent emergence of ceftriaxone-resistant *S. enterica* serotypes Typhimurium and Newport, has underscored the threat to both animal agriculture and human health that multiple drug resistant pathogens pose (Rankin SC 200; Glynn MK 1998; Hollinger K 1998; Fey PD 2000). Antibiotic resistance genes are widely disseminated in pathogenic, commensal and environmental bacteria (Goldstein C 2001; Nield, Holmes et al. 2001). Further, it has been shown that once antimicrobial resistance has been introduced into an ecosystem, resistance can spread and persist without continuing selection pressure from antibiotics (Marshall 1990; Salyers and Amabile-Cuevas 1997). In addition, the reservoir of antimicrobial resistance genes is larger than previously thought (Nandi, Maurer et al. 2004). It is in this environment that the potential exists for *Salmonella* to acquire drug resistance genes from resident poultry microbiota due to selection pressure from therapeutic and non-

therapeutic antibiotic usage. It follows then, that the longer *Salmonellae* persist in the environment of an animal production facility, the chance of acquiring resistance genes increases.

V. New food-borne threats:

In the 1990s, a new multidrug-resistant strain of *S. enterica* serovar Typhimurium, definitive type 104 (DT104) emerged. It was first recognized in the United Kingdom, but in the following years the strain was isolated in other countries as well (Threlfall, Frost et al. 1994). *Salmonella typhimurium* DT104 was originally isolated from cattle, but has now been isolated from a wide range of animal host species, and the organism has become a common cause of human salmonellosis. While outbreaks of *Salmonella typhimurium* DT104 are commonly associated with ground beef (Zhao, Doyle et al. 2002), a number of studies have also examined the occurrence in swine and pork production (Sandvang, Aarestrup et al. 1998; Baggesen, Sandvang et al. 2000; Gebreyes and Altier 2002; White, Zhao et al. 2003). The emergence and rapid world wide spread of the multiple drug resistant *Salmonella typhimurium* clone DT104 has also increased awareness of the development of antibiotic resistance in human pathogens in general, and the possibility that antibiotic use in animal agriculture may contribute to antibiotic resistance in human pathogens and food-borne pathogens.

The emergence of ceftriaxone-resistant *Salmonella* is the latest antibiotic resistance threat to successful treatment of human *salmonellosis* faced by the medical community (56, 60). As reported by the CDC the prevalence of ceftriaxone-resistant *Salmonella* isolates from humans has increased from 0.07% in 1997 to 0.5% in 1998 (Dunne, Fey et al. 2000). Additionally, the 1999 National Antimicrobial Resistance Monitoring System (NARMS) Annual Report, states that the prevalence of ceftriaxone-resistant *Salmonella* isolates from food animals increased from 0.1% in 1996 to 2% in 1999 (Anonymous 2001). The increasing prevalence of multi-drug

resistant (MDR) *Salmonella enterica* serotype Newport isolates from cattle raises the question of whether the increasing level of resistance to the cephalosporin ceftriaxone, used in human medicine, is due to cross-resistance to ceftiofur, the cephalosporin currently approved for use in cattle and poultry (Rankin, Aceto et al. 200; Zhao, DeVillena et al. 2001; Zansky, Wallace et al. 2002).

VI. Regulation of antibiotic use in animals:

There is still debate over whether there is enough scientific evidence to make the statement that antibiotic use in animals has a deleterious effect on human health. Some authors believe that the evidence shows that the actual risk of transfer of antibiotic resistant organisms from animals to humans is very low. Evidence demonstrating an association between antimicrobial use in animals and antimicrobial resistance is humans is stronger in studies focusing on *Salmonella* and *Campylobacter* (Iovine and Blaser 2004). Sound policy decisions regarding limiting the use of antimicrobials in animals must be based on scientific risk assessments that address the fact that continued use of antibiotics in food animals is important to animal health and welfare, and food safety.

The issue of antibiotic resistance and public health has gotten the attention of consumers and governments in the United States and Europe. In the U.S., the medical community believes that limiting the use of certain antibiotics in veterinary medicine and agriculture which have analogs used in human medicine is necessary in order to control the development and spread of antibiotic resistance in human pathogens. In addition, attention has been brought to the judicious use of antibiotics in human medicine, and a national educational campaign by the CDC is focused on changing both the attitude of the medical establishment, and the public perspective on the judicious use of antibiotics.

The results of reviews on the effectiveness of prohibiting the use of antibiotic growth promoters in animal production on the prevalence of antibiotic resistance in isolates of food-borne disease-producing bacteria remain mixed. A recent (2001) study from Denmark examining vancomycin resistance in food-borne pathogens detected a surprisingly high prevalence (66%) of vancomycin- resistance in E*nterococci* in both isolates from swine, and from poultry products, despite the years-old ban on growth promoting antibiotics in that country (Gambarotto, Ploy et al. 2001). In contrast, another recent report (2001) from Denmark states that between 1995 and 2000, the prevalence of vancomycin reistance in *Enterococcus faecium* isolates from broiler chickens decreased significantly, and that this decrease was associated with an overall decrease in therapeutic antibiotic usage (Aarestrup, Seyfarth et al. 2001).

In addition to concerns over increases in cephalosporin resistance in food-borne pathogens, several studies have also high-lighted an increase in fluoroquinolone resistance among *Campylobacter* and *Salmonella* isolates from human food-borne disease (Hakanen, Kotilanen et al. 2001; Hakanen, Jousimies-Soumer et al. 2003). One recent (2003) study observed an increase in ciprofloxacin resistance among *Campylobacter jejuni* strains isolated between the years 1995 to 2000, from 40 to 60 percent (Hakanen, Jousimies-Soumer et al. 2003). A decrease in susceptibility to quinolones (fluoroquinolones) among *Salmonella enterica* serotypes isolated from food animals and humans worldwide has been recognized as a trend during the last several years of observation (Frost, Kelleher et al. 1996; Malorny, Schroeter et al. 1999; Hakanen, Kotilanen et al. 2001). After the recent bioterrorism events involving the use of Anthrax bacteria sent through the mail, the public responded with an increased demand for, and hoarding of, ciprofloxacin, a fluorquinolone antibiotic used in human medicine. Contemporaneously, the U.S. FDA began to consider a ban on the use of the fluorquinolone

enrofloxacin in veterinary medicine in order to preserve this class of antibiotic for use in human medicine. As a result of such pressure, the poultry industry enacted a voluntary ban on therapeutic use of enrofloxacin in broiler chickens for the treatment of airsacculitis, in order to allow time for scientific review of the benefits and risks of using fluorquinolone antibiotics in poultry production. These important regulatory decisions will affect the future of both agriculture and public health, and are being made on the support of a body of literature that gives only partial support to the hypothesis that antibiotic usage in animal agriculture is the prime force in development of antibiotic resistance in human pathogens.

VII. Genetic mechanisms of antibiotic resistance in the *Enterobacteriaceae*:

Bacteria possess a variety of mechanisms for the acquisition of new DNA and the genes thereby encoded. These mechanisms include mobile or mobilizable genetic elements such as transposons, conjugative plasmids, bacteriophages, and integrons. Integrons have been shown to be so widely distributed, and they can acquire multiple antibiotic resistance genes and retain them without selection pressure. In addition, it has been shown that bacteria of different species and classes can exchange genetic material. Therefore, integrons represent an important reservoir of antibiotic resistance genes in the environment (Salyers and Amabile-Cuevas 1997; Goldstein, Lee et al. 2001; White 2001).

VIII. Integrons:

Although only recently discovered in 1989, integrons are apparently quite ancient (Rowe-Magnus DA 2001). In the time since initial discovery, the study of integrons has provided many surprises; more integron classes have been described, more gene cassettes have been described, and more bacterial species containing integrons have been identified (Fluitt and Schmitz 1999; Nandi, Hofacre et al. 2004). To date, more than 60 different integron associated resistance gene

cassettes have been identified (Fluitt 1999). Eight classes of integrons have been identified to date (Nield, Holmes et al. 2001). The most recent classes discovered were found in environmental gram negative bacteria. These integron classes vary in the *int*1 gene sequence encoding the integrase recombinase enzyme (Goldstein, Lee et al. 2001; Nield, Holmes et al. 2001). The overall structure of integrons from the eight classes does not otherwise vary significantly. Thus far, up to nine gene cassettes have been identified in a single integron, and cells may contain more than one integron, each having a different complement of antibiotic resistance gene cassettes (Tosini, Visca et al. 1998). The majority of super-integron cassettes examined thus far appear to be unique to the gram negative genus *Vibrio* (Rowe-Magnus and Mazel 2002). But is thus predicted that super-integrons may be widespread throughout the bacterial kingdom (Rowe-Magnus, Guerout et al. 1999; Heidelberg, Eisen et al. 2000). Another recent discovery about the biology of the class 1 integrons is that they are not restricted to the gram negative bacteria, as previously thought, but are apparently also common in gram positive bacteria (Nandi, Maurer et al. 2004).

Although integrons are not by themselves mobile, they are found incorporated into transposons and plasmids, which are mobile (Liebert, Hall et al. 1999; Rowe-Magnus DA 2001). These elements acquire drug resistance genes through the recombination of novel gene cassettes into a specific integration site, *aat1*. Despite the differences in the integrases and *att1* sequences, the same gene cassettes are thought to be acquired by class 1, 2, and 3 integrons, as identical gene cassettes and been identified in these integron classes (Sundstrom and Skold 1990; Hall and Collis 1995). All integrons contain a 5' conserved sequence, which contains the integrase gene, two promoter sequences, and the aat1 recombination site (Hall and Collis 1995; Levesque, Piche et al. 1995). They also possess 3' conserved sequences, which include the genes *qacE*, *sul1*, and

a structure called the 59 base pair element. The qacE gene, whose product confers quaternary ammonium compound resistance, is non-functional in the class 1 integron due to a deletion mutation (Hall and Collis 1995). The sul1 gene is functional, and confers sulfonamide antibiotic resistance. These two conserved antimicrobial resistance gene sequences are believed to represent gene cassettes which have lost their 59 base pair elements, and thus have become "trapped" in the integron because they cannot be excised (Hall and Collis 1995).

Integron genes cassettes encode for resistance to many commonly used antibiotics such as aminoglycosides, cephalosporins, chloramphenicol, penicillins and trimethoprim (Fluitt and Schmitz 1999; Carattoli 2001). Gene cassettes consist of a single open reading frame encoding one gene sequence, and a 3'-end 59 base pair element which varies in sequence from cassette to cassette, but which contains two 7 base pair conserved core regions flanked by imperfect repeats (Hall and Collis 1995; Levesque, Piche et al. 1995; Fluitt and Schmitz 1999). The 59-base pair element acts as an integrase-specific recombination site. The sequence and length of the 59-base pair element is highly variable, with no two 59-base elements having the same sequence (Hall and Collis 1995). When integrated into the integron the 59-base pair elements, located between gene cassette pairs, have been shown to act as sites for intra- or inter- recombination of gene cassettes within or between integrons. The recombination cross-over point has been identified as the triplet sequence GTT within the conserved core site DNA sequence GTTRRRY (Hall, Brookes et al. 1991). The core site is found either alone at the boundary of the 5'-conserved segment within the integrated gene cassette, or as the last seven bases located at the 3' end of the 59-base pair element of a free gene cassette (Stokes and Hall 1989; Hall, Brookes et al. 1991).

Recombination of a gene cassette occurs at the 59 base pair element between the G and T residues of one of the core regions of the gene cassette, and the conserved *aat1* recombination

site located at the 3' end of the 5' conserved sequence of the integron (Hall and Collis 1995; Levesque, Piche et al. 1995). The *intI*1 protein, integrase, mediates this recombination, and has been shown to bind to both the aat1 site of the integron, and the conserved sequences of the 59 base pair elements of the gene cassettes (Fluitt and Schmitz 1999). The integrase IntI1 is an enzyme which is part of the tyrosine-specific recombinase family, which includes the λ-phage integrase. This family of enzymes catalyse the excision and integration of gene cassettes (Martinez and de la Cruz 1988; Collis and Hall 1995). The integrase enzyme binds strongly to four sites within the *attI* site, and weakly to the *attC* site on the gene cassette (Recchia, Stokes et al. 1994; Collis, Kim et al. 1998). Of the four binding sites on the *aatI1* sequence, two consist of a simple integrase recombination site made up of inversely oriented IntI1-binding sites, and two binding sites (DR1 and DR2), which enhance the efficiency of recombination (Collis, Kim et al. 1998; Gravel, Fournier et al. 1998).

The mobility of the gene cassettes is mediated by the class 1 integron recombinase enzyme integrase, which is encoded by the *int*1 gene found on the 5' end of the integron (Levesque, Piche et al. 1995; Bass, Liebert et al. 1999). During plasmid replication the *intI*1 gene must be expressed and translated in order for gene cassettes to be excised and incorporated into an integron (Collis, Grammoticopoulos et al. 1993). Once the gene cassettes are excised, they circularize via the 59 base pair sequence. Circularization of the gene cassette protects the DNA from nuclease activity, and has been shown to be essential for integration (Collis, Grammoticopoulos et al. 1993). Any gene which is able to become associated with a suitable 59-base pair element and become circularized, could in theory be inserted into an integron (Collis and Hall 1992).

Most gene cassettes do not contain a promoter sequence, but are expressed from one of two potential promoter sites located in the 5' conserved region of the integron (Collis and Hall 1995; Levesque, Piche et al. 1995; Fluitt and Schmitz 1999). The cmla, qacE, and qacG gene cassettes have been shown to have their own functioning promoter sequences (Fluitt and Schmitz 1999). The level of expression of a gene cassette varies with its position relative to the promoter. It has been demonstrated that the highest level of antibiotic resistance in *vivo* occurs when the gene cassette is directly behind the promoter region (Collis and Hall 1995; Fluitt and Schmitz 1999). It has been shown in *vitro* that gene cassettes can be excised and shuffled into new positions closer to the promoter when selection pressure from antibiotic exposure is applied, and thus expression of the gene would confer a survival advantage to the bacterium hosting the integron (Collis and Hall 1995). Antibiotic resistance operons are built by successive gene cassette integrations, with the most recent acquisition closest to the *aat1* recombination site (Collis, Grammoticopoulos et al. 1993).

Class 1 integrons have been shown to be present in a variety of veterinary *Enterobacteriaceae* isolated from a variety of animal species (Goldstein, Lee et al. 2001). In addition, there appears to be a positive correlation between multiple-antibiotic resistance and the presence of class 1 integrons among the *Enterobacteriaceae* (Martinez-Freijo, Fluit et al. 1998). There have been numerous surveys that have examined the incidence and characterization of integrons among gram-negative bacteria, including the foodborne pathogens *Salmonella, E. colli, and Campylobacter* (Sunde and Sorum 1999; Hudson, Quist et al. 2000; Keyes, Hudson et al. 2000; Sandvang and Aarestrup 2000; Soto, Guerra et al. 2001; Sunde and Sorum 2001; Lee and Sanchez 2002; Liebana, Garcia-Migura et al. 2002). These pathogens have been isolated from various host species including people and various food animals including cattle, swine, and

poultry (Martinez-Freijo, Fluit et al. 1999; Keyes, Hudson et al. 2000; Chanawong, M'Zali et al. 2001; Girlich, Poirel et al. 2001; Maguire, Brown et al. 2001; Sunde and Sorum 2001; Lee and Sanchez 2002; Leverstein-Van Hall, Paauw et al. 2002; Liebana, Garcia-Migura et al. 2002; Leverstein-van Hall, HE et al. 2003). Integrons containing gene cassettes have also been found in bacterial isolates from wild birds, raising interesting speculation about potential mechanisms of dissemination of antibiotic resistance genes between wild and domestic animals (Hudson, Quist et al. 2000).

The multi-resistant *Salmonella enterica* Typhimurium phagetype DT104 is an excellent example of the importance of integrons in the dissemination of antibiotic resistance genes. This *Salmonella* clone has a characterisitic multi-resistance phenotype of resistance against: ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline. This strain posses two integrons, one of which contains a gene cassette for a betalactamase (*pse*-1), and the other, genes for a chloramphenicol acetyltransferase (cml*A*), and sulfonamide resistance (*sul*1) (Carlson, Bolton et al. 1999). The gene for streptomycin resistance (*aadA*2), and a tetracycline efflux pump (*tetR*, *tetG*), are found in the second integron (Carlson, Bolton et al. 1999). Recently, some isolates of *Salmonella* DT104 has been discovered to have a gene (*floR*), encoding cross-resistance to florfenicol and chloramphenicol (Bolton, Kelley et al. 1999). The presence of integrons in DT104 portends that this strain can continue to acquire new resistance gene cassettes. *Salmonella typhimurium*, including strain DT104, has been found in the gastrointestinal tracts of pet birds of various species (Hudson, Quist et al. 2000). A large-scale survey of these isolates has shown a percentage to possess a variety of antibiotic resistance genes and integrons (Hudson, Quist et al. 2000). It has been speculated that the *S. typhimurium* clone DT104 has been spread around the world in the feces of migratory birds.

VIIII. Transposon Tn21:

Among the gram-negative bacteria within the *Enterobacteriaceae*, many of the transposons that encode for multiple antibiotic resistance belong to the Tn*21* subgroup of the Tn*3* family of transposable elements (Grinsted, de la Cruz et al. 1990). Tn*21* and many related transposons carry integrons in addition to the genes that allow for the transposon's own transposition (*tnp*). Transposons do not replicate autonomously and therefore must integrate into bacterial chromosome, plasmids, or phage for their survival and dissemination. They are capable of moving from one DNA molecule to another, independent of *recA*- dependent recombination (Olsen 1999). Tn*21* contains four discrete mobile elements: a class 1 integron, the *aadA1* gene cassette within the integron, and two insertion sequences IS*1326* and IS*1353* (Brown, Stokes et al. 1996; Liebert, Hall et al. 1999). The integron within Tn*21* is a class 1 integron designated In2. It has been theorized that this integron became integrated into an ancestral *tnp-mer* transposon which later evolved into the transposon Tn*21* (Stokes and Hall 1989). The transposition of Tn*21* is carried out by the transposase TnpA coded for by the transposon (Grinsted, de la Cruz et al. 1990). In addition to *tnpA* and the integron In2, transposon Tn*21* and other related Tn*21*-like mobile elements also possess the mercury resistance operon *mer* (Silver and Phung 1996; Liebert, Hall et al. 1999).

One study examined the incidence of Tn*21* among *E. coli* and *Salmonella* isolated from different avian species (Bass, Liebert et al. 1999). Among the *E. coli* isolated, 55% of the isolates were positive for the Tn*21* markers *merA* and *aadA*, and 62% of the serogroup B and E *Salmonella* isolates exhibited mercury and streptomycin resistance, commonly associated with the presence of the Tn*21* resistance phenotype.

Although streptomycin is not currently used in human medicine, resistance to streptomycin persists in isolates of human pathogens (Chiew, Yeo et al. 1998). Streptomycin is also not commonly used in veterinary medicine, but it has been used therapeutically in veterinary medicine for treating bacterial enteritis caused by *E. coli* and *Salmonella* spp. in claves and swine, and in poultry for the treatment of non-specific bacterial enteritis (Bayler 2003). Again, although streptomycin is no longer used in poultry, resistance to this antibiotic persists in veterinary isolates. In one study of pathogenic avian *E.* coli isolates sixty-percent of the streptomycin resistant isolates were positive for the genetic markers of class 1 integrons, *intI1,* and this resistance was attributed to the integron-associated, aminoglycoside resistance gene cassette, *aadA1*. Additionally, half of these isolates were positive for the transposon Tn*21* (Bass, Liebert et al. 1999). Class 1 integrons and Tn*21* have also been identified among multiple-antibiotic resistant *E. coli* isolated from healthy pigs on a survey of seven swine operations (Sunde and Sorum 2001). Hudson et al. (2000) found that 41% *Salmonella* isolates from diseased, non-domestic birds tested were positive for *intI1*, 10% were PCR-positive for the mercury resistance gene *merA*, and 18% were positive for the streptomycin resistance gene *aadA1*, both markers for the presence of transposon Tn21 (Bass, Liebert et al. 1999; Hudson, Quist et al. 2000). The evidence that resistance to antibiotics such as streptomycin whose usage has been discontinued still persists years later lends credence to the idea that as far as antibiotic resistance is concerned, we have let the genie out of the bottle.

X. The Future of Antibiotics:

The question of whether antibiotic resistance is here to stay is an important one to the future of mankind. The theory that the overall background level of antibiotic resistance genes has been increased by the imprudent use of antibiotics has been put forth by many scientists.

The question remains: can this background level of resistance be rehabilitated, and the background lowered to its pre-antibiotic era level? There are many factors affecting whether or not this is possible. Biochemical testing of surface water sources and water at sewage treatment facilities has revealed a surprisingly high level of contamination of water sources with many commonly prescribed drugs and their residues, including antibiotics. What effect this low level exposure to antibiotics has on the acquisition of antibiotic resistance genes by environmental bacteria is unknown, but may be another contributing factor in the increasing level of resistance found in environmental bacteria. The future of antibiotics in medicine will depend on elucidating the biology and ecology of resistance, exploring the linkages between the various uses of antibiotics and the emergence and maintenance of resistance, and on using this data to stablish prudent use guidelines for doctors, vets, and farmers, in order to preserve the precious resource of antibiotics for the future.

CHAPTER 3

VERTICAL AND HORIZONTAL TRANSMISSION OF SALMONELLA WITHIN AN INTEGRATED BROILER PRODUCTION SYSTEM[1]

---

[1]Liljebjelke, KA, Hofacre, CL, Liu, T, White, DG, Ayers, S, Young, S, Maurer, JJ. 2004. Submitted to Foodborne Pathogens and Disease.

ABSTRACT

Salmonella remains one of the leading causes of food-borne illness in the United States, many key questions regarding the introduction and persistence in animal production systems still remain. In order to understand the ecology of *Salmonella* within an integrated commercial broiler production system, two hundred eighty-nine *Salmonella enterica* were recovered from two integrated poultry farms during the production and processing of seven consecutive flocks. The variety and prevalence of *Salmonella* serotypes differed between farms. Overall, fifteen serotypes were identified, with the most common being: Typhimurium (55%), Montevideo (7.9%), Kentucky (9%), and Enteritidis (9.7%). *Salmonella* Typhimurium and Enteritidis isolates recovered from processed carcasses from Farm One were further characterized using pulsed-field gel electrophoresis (PFGE), and were shown to be indistinguishable from isolates recovered from the poultry house environment and mice trapped on this farm. Additionally, the same broiler *S.* Typhimurium and *S.* Enteritidis strains, identified by PFGE, were also isolated from samples taken at a company breeder farm, suggesting vertical transmission of these *Salmonella* serotypes in this poultry production system. Results indicate that management practices at the breeder level may have a profound effect on the transmission and persistence of salmonellae within an integrated production system as well as potential contamination of poultry derived products.

INTRODUCTION

Salmonella remains the leading cause of outbreak-associated gastroenteritis in the United States (Olsen, Bishop et al. 2001). It has been estimated that approximately 1.4 million non-typhoidal salmonellosis cases occur in the U.S. each year (Mead, Slutsker et al. 1999), and poultry has been implicated in 40% of these outbreaks (Olsen, Bishop et al. 2001). Due to public

concern and subsequent governmental pressure, the Food Safety and Inspection Service (FSIS) mandated in 1996 that meat and poultry processing plants implement a Hazard Analysis Critical Control Point (HACCP) program and meet set limits for *Salmonella* contamination of finished poultry products. Since that time, the poultry industry has made significant reductions in the levels of poultry carcass contamination with *Salmonella*, from 20% in 1998 to 10% in 2000 (Schlosser 2000). However, a recent survey of retail meats from supermarkets in the Washington D. C. area revealed that the levels of *Salmonella* contamination of beef, pork and poultry products still poses a threat to the consumer (White, Zhao et al. 2001). The continuing contamination of retail poultry products with *Salmonella* has important public health implications, especially considering the global increase in chicken consumption (Fuzihara, Fernandes et al. 2000).

It has been established that *Salmonella* can become endemic in many types of food animal production units (Sanchez 2002). In order to reduce or eradicate *Salmonella* from these environments, it is necessary to prevent its introduction or re-introduction onto the farm (Sanchez 2002). Because of the linkage between colonization of live birds with *Salmonella* on farms with contamination of finished poultry products, further reductions in the level of carcass contamination will require a variety of on-farm intervention strategies (Rigby, Petit et al. 1982; Sanchez 2002). Because *Salmonella* can potentially enter at numerous points into the integrated poultry production system, the success of any intervention strategy will also require continuous monitoring of both the poultry production environment and the finished product for levels of *Salmonella* contamination.

Currently, the USDA HACCP inspection program places its emphasis on surveying bacteria isolated primarily from processing plant samples and carcasses at the time of slaughter.

However, it is difficult for current post-harvest monitoring efforts to take into account the variability in *Salmonella* contamination that occurs not only between geographic regions, but within farms or levels of poultry production (breeder farms, hatchery, broiler farms, feed mill) that ultimately reflect the true level of *Salmonella* contamination within an integrated company. We therefore took advantage of the short production cycle, high population number, and vertically integrated system of poultry production to conduct an epidemiological examination of the movement of *Salmonella* within two integrated commercial poultry companies. In this study, we specifically focused on the epidemiology of *Salmonella* on two commercial, broiler chicken farms using molecular typing by pulsed field gel-electrophoresis (PFGE). Our objectives were to observe the ecology of the *Salmonella* serotypes and strains resident in two integrated poultry companies, and to collect detailed epizoological information which will help the poultry industry develop practical intervention strategies towards reducing the levels of *Salmonella* contamination of finished poultry products.

MATERIALS AND METHODS

Selection and description of poultry farms.

Two integrated poultry companies in Northeast Georgia participated in a yearlong study to examine the ecology of *Salmonella* on poultry farms. One poultry farm was chosen from each company.

Farm One was chosen from Company A based on our past experience of isolating *Salmonella* from this farm's complex of eight flock houses. Although several members of a family participate in management of the houses, according to ownership/management, they were designated as a single poultry farm unit for the purposes of this study. Of the eight houses surveyed, only one house was culture positive for *Salmonella* from environmental samples (litter

and drag swab) taken at the start of this study period. This house (A), and an adjacent house (B), were chosen for study. This farm also raises beef cattle in addition to poultry. The activity of mice and roof rats was evident on the premises, and no rodent control program was in place on this farm. There was no evidence of wild bird activity noted within the flock houses. The next closest poultry farm is located 0.4 miles from this poultry farm complex.

Approximately seventeen thousand chicks were placed in each house on Farm One. After six to seven weeks of rearing each flock to the market weight of 2.0 – 2.3 kg, the poultry litter was removed from the flock houses, and new litter was added to the houses before placement of the next flock. The litter consisted of dry pine shavings. No litter amendment was used on Farm One (Pope and Cherry 2000).

We surveyed four poultry farms contracted with Company B by drag-swab, and identified one farm (Farm Two) that was *Salmonella*-positive and subsequently chose this farm for participation in this study. There are four flock houses on this farm. One house tested positive for *Salmonella* and we chose this and the adjacent house for study. As on Farm One, there are other domestic animals on the premises of Farm Two including cattle, goats, geese, and a donkey. There are no other poultry farms in close proximity to this farm.

Approximately twenty thousand chicks were placed per house on Farm Two. Following the first study flock, the old litter was completely removed and replaced. After subsequent flocks the top 2.5 cm of old litter was removed from underneath the nipple-drinker water lines and feed lines in the houses, and a 2.5 cm layer of new litter was spread on the old litter before placement of the next flock. No litter amendments were used on Farm Two (Pope and Cherry 2000).

Sample collection and processing.

The two selected houses from each farm were sampled every 2 weeks during their six-to-seven week production cycles. At the time of chick placement (week 0), the following samples were collected from each house; chick-box liners (n = 30), litter drag-swab (n = 5), litter (n = 5), water (n = 1), feed (n = 2), dust drag-swab (n = 2), mice (variable). Exclusive of the chick-box liners, the same number and type of samples were collected during weeks 2, 4, and 6 of the grow-out period. Carcass rinses were obtained from 20 processed birds from each house at the time of processing (week 6-7). Excluding mice, cow feces, and insects, 102 samples were taken from each house during each flock grow-out.

Pooled litter samples were collected down to a depth of 7.5 cm from underneath the nipple-drinkers, feeders, and along the length of the flock house. Five pooled litter samples were collected from each house. Twenty-five grams of each sample was suspended in 100 ml of tetrathionate brilliant green broth (TBG) with 2 ml iodine (Difco, Division of Becton, Dickinson and Co., Sparks, MD). Cattle manure samples were processed in the same manner.

The environment of the broiler houses was sampled using drag swabs consisting of sterile gauze pads soaked with double-strength skim milk (Byrd, DeLoach et al. 1999). The swabs were dragged across the birds bedding material, or wiped along water lines or fan blades. Each swab was placed in 100 ml TBG with 2 ml iodine and incubated at 41.5°C for 18 hours (Blankenship, Bailey et al. 1993).

Twenty chicken carcasses were collected at the processing plant, ten prior to, and ten immediately after the 32^{o}C chlorinated water chill-tank. To reduce cross contamination between flocks, the study flocks were the first flocks processed the day of carcass sampling. Chicken carcasses were placed in individual sterile bags with 250 ml buffered-peptone water and agitated

vigorously using a mechanical shaking device designed for this purpose (Dickens 1985). The carcass rinses (50 ml) was used to inoculate 100 ml TGB plus 2 ml iodine, which was incubated as above.

Mice were captured in live traps (Victor Tin Cat, Woodstream Corp.; Lititz, PA) in the houses and transported to the laboratory. Following cervical dislocation, the mice were rinsed with 95% ethanol. The abdomen was aseptically opened with sterile scissors, and the gastrointestinal tract, liver, and spleen were aseptically removed. The organs were placed into 10 ml phosphate buffered saline pH 7.4, and macerated in a stomacher (Tekmar Co.; Cincinnati, OH) for five minutes, to which, 90 ml TBG and 2 ml iodine was added, and incubated as above.

In each house, feed samples were collected from the open hopper below the feed augur. Twenty-five grams of feed was placed in 225 ml of TBG with 4 ml iodine, and incubated as above. Water samples were collected from the incoming line where it was connected to the nipple drinker line. Before sample collection, the end of the water tap was disinfected with alcohol-based, antiseptic hand gel (Healthy Hands; NCH Corporation; Irving, TX) for 1 min. The water was allowed to run freely for 30 seconds before collecting sample. One hundred ml of water sample was added to 200 ml double strength TBG with 4 ml iodine, and incubated as above.

Chick-box liners were removed from the transport baskets at the time of flock placement, and transported to the laboratory in sterile bags at 4^{o}C. The surface of each chick-box liner was wiped with a drag-swab. Each swab was placed into 100 ml TBG with 2 ml iodine, and incubated as above.

Ten flock houses at a Company A-owned, broiler breeder farm were surveyed by drag-swab sampling during the period from September 11, 2003 - November 23, 2003. Three drag

swab samples were taken in each house, one from each of the slats, and one from the center scratch (litter) area. These samples were processed as described above. The *Salmonella* obtained from the processed samples were isolated and identified as described below.

Salmonella isolation and identification.

A 10-microliter loop of an overnight, TBG enrichment culture was streaked onto an XLT4 and BGN bi-plate, prepared as described by Hajna and Damon (1956), and incubated at 37°C overnight. The Media Lab, Department of Infectious Diseases, the University of Georgia prepared the bi-plates used in this study. Four to 5 isolated, H_2S positive colonies were stabbed and streaked onto Triple Sugar Iron (TSI) (Difco) slants, and incubated overnight at 37°C. Identity of suspect *Salmonella* was confirmed using poly O *Salmonella* specific antiserum (Difco). In addition, 1 ml of TBG enrichment broth was taken for DNA extraction. An *invA*-specific PCR was used to screen overnight TBG enrichments for the presence of *Salmonella* (Liu, Liljebjelke et al. 2002). To improve *Salmonella* recovery, a delayed secondary enrichment was done with TBG enrichments that were PCR-positive but culture-negative for *Salmonella* (Waltman, Horne et al. 1991; Waltman 1993). After 5-day incubation in TBG, one ml of TBG enrichment was transferred to 10 ml fresh TBG, incubated at 37°C overnight, and 10 microliters of the enrichment was plated onto XLT4/BGN bi-plates.

Molecular typing.

The O serogroups were identified for *Salmonella* isolates using standard serological typing procedures for *Salmonella* O antigens (Anonymous 1998). *Salmonella* isolates were then typed using restriction fragment length polymorphism (RFLP)-PCR (Hong 2003). The *fliC* and *fljB* RFLP DNA restriction patterns produced were matched against a database of RFLP patterns established for 52 known *S. enterica* serovars. Serotype was assigned to *Salmonella* isolate

based on information gained from O serotyping and match between RFLP DNA restriction pattern for our unknown and a pattern present in our database (Hong et al., 2003). Standard flagellar serotyping was performed on some isolates to confirm the validity of the RFLP-PCR, and for isolates in which no match was found in the RFLP database.

Salmonella isolates were typed genetically by PFGE according to the protocol by Barrett et al. (Barrett 1994). Improvement of PFGE restriction patterns was achieved with addition of 50 □M thiourea to the running buffer (Koort, Lukinmaa et al. 2002). Agarose-embedded *Salmonella* genomic DNA was digested with the restriction enzyme *Xba* I (30 units) for 16 to 18 hours at 37^{o}C. The DNA fragments were separated in a 1.2% agarose gel, using a pulsed-field gel electrophoresis apparatus (CHEF DR-II, Biorad; Hercules, CA). Electrophoretic conditions were: 25 hours, 200 V, and a linearly ramped pulse time of 2 to 40 seconds. A restricted *Saccharomyces cerevisiae* genomic DNA ladder was used as a molecular weight marker (Biorad). Interpretation of PFGE pattern and cluster relationships was determined using criteria described by Tenover et al (Tenover, Arbeit et al. 1995).

RESULTS

Epidemiology of Salmonella on two poultry farms in northeast Georgia during an entire production year.

During the sampling period, Farm One placed 251,133 birds, with an average of 17,938 birds per flock, per house. The average age to processing weight of 4.86 lbs was 45 days, with an average mortality rate per flock of 4.04%, and an average condemnation rate at the processing plant of 1.22%. Farm Two placed 613,087 birds, with an average of 43,791 birds per flock per house. The average age to processing weight of 4.39 lbs was 43 days, with an average mortality rate of 4.63%, and an average condemnation rate at the processing plant of 0.80%.

Salmonella enterica was isolated from 241, or 16.69% of 1,444 samples collected during the production and processing of seven consecutive broiler flocks in the two study houses on Farm One representing Company A (Table 1). Percentage of *Salmonella*-positive samples from Farm Two representing Company B, were fewer than Farm One during the study period (3.36% vs. 16.69% respectively, Table 1). With regards to carcass contamination, *Salmonella* was present on 10.4% and 1.8% of birds processed from Farms One and Two, respectively (Table 1).

Eleven different *Salmonella* serotypes were recovered from different samples from Farm One including; *S.* Typhimurium (64%), *S.* Enteritidis (12%), *S.* Montevideo (9%), and *S.* Kentucky (5.4%) (Table 2). Twelve *S. enterica* serotypes were isolated from Farm Two, with *S.* Kentucky (27%), *S.* Mbandaka (19%), *S.* Typhimurium (13%), and *S.* Ohio (10%) being the most prevalent. *Salmonella* Enteritidis, *S.* California, and *S.* Tennessee were isolated on Farm One but not from Farm Two, while serotypes *S.* Jerusalem, *S.* Mbandaka, *S.* Muenchen, and *S.* Ohio were isolated from Farm Two, but not Farm One.

Among the various environmental samples collected, drag swabs yielded the largest number of *Salmonella* isolates (48% of *Salmonella*-positive samples; n = 249), and the greatest variety of serotypes, (9 of 11 total) for Farm One. Litter samples also yielded a large number of *Salmonella* isolates (Farm One 25%, Farm Two 35%), second only to drag swabs taken in the environment. *Salmonella* Enteritidis, *S.* Heidelberg, *S.* Montevideo, *S.* Senftenberg, and *S.* Typhimurium were recovered from chick-box liners from Farm One as compared to serotypes *S.* Mbandaka, *S.* Montevideo, and *S.* Ohio from Farm Two.

Of carcass rinses positive for *Salmonella* from Farm One (n = 29), serotypes *S.* Typhimurium (83%), *S.* Enteritidis (10%), *S.* Heidelberg (3%), and *S.* Kentucky (3%) were isolated from carcass rinses from Farm One, whereas only *S.* Kentucky (100%, n = 5) was

recovered from carcass rinses for Farm Two. For both farms, all environmental sample types from the flock houses yielded *Salmonella* isolates at least once during the study period, and isolates were obtained from house environmental samples from all seven flocks (Table 3).

Although *Salmonella* was isolated from the house environment of Farm One during all four seasons, isolations were made from chick-box liners only during the summer and fall and from carcass rinses only during the fall and winter (Table 3). As on Farm One, the house environment of Farm Two yielded *Salmonella* during all four seasons with spring and summer yielding the majority of isolations, however, isolations were made from chick-box liners during spring and fall, while carcass rinses yielded *Salmonella* isolates only during the spring (Table 3).

Sampling of one Company A breeder farm resulted in isolation of *S.* Enteritidis from two houses, and *S.* Typhimurium from two of the ten houses surveyed during the fall of 2003. For both serotypes, isolations were made from drag-swabs taken from both the scratch area (litter) and the slats. *Salmonella* Kentucky was isolated from litter and slat drag-swabs from five of ten houses on the farm.

Genetic relatedness of S. enterica serotypes isolated from poultry farms in northeast Georgia.

Genetic typing by macrorestriction analysis of genomic DNA using PFGE revealed only two genetic types of *S.* Typhimurium present on Farm One. Restriction patterns were generated using the endorestriction enzyme *Xba* I and were designated PFGE types T1 and T2 (Fig. 1). Three genetic subtypes exhibiting two to five band differences were identified among the *S.* Typhimurium PFGE type T1 isolates from both Farms One and Two, and were designated T1.1, T1.2, and T1.3 (Fig. 1). The first two subtypes accounted for 95% (n = 131) of all *S.* Typhimurium isolations (Table 3). The molecular subtypes T1.1 and T1.2 are closely related, having only two bands difference in their macrorestriction patterns (Figure 1a). The *S.*

Typhimurium molecular subtype designated T1.3, is more distantly related to subtypes T1.1 and T1.2, having five bands difference from the subtype T1.1, and 6 bands difference from the subtype T1.2 (Tenover, Arbeit et al. 1995). The *S.* Typhimurium PFGE type T2 is unrelated to the other *S.* Typhimurium PFGE type isolated as it has seven bands difference in restriction pattern. The *S.* Typhimurium genetic type T2 was isolated only once from the environment of House B on Farm Two, and was not isolated on Farm One. The *S.* Typhimurium PFGE subtypes T1.1 and T1.2 were isolated from both poultry houses and from mice captured on Farm One. Throughout the study period, the *S.* Typhimurium PFGE subtype T1.1 was most prevalent in isolations made from House A (77%; n = 65), while subtype T1.2 was most prevalent in isolations made from House B on Farm One (90%; n = 68). On Farm One, the *S.* Typhimurium PFGE subtypes T1.1, T1.2, and T1.3 were isolated from mice captured in House A, and subtypes T1.1 and T1.2 were isolated from mice captured in House B (Fig. 2). The *S.* Typhimurium PFGE subtype T1.3, was the minor genetic type isolated from both houses in this study. Molecular typing also revealed that *S.* Typhimurium isolations from Farm Two were of the same PFGE subtype (T1.2) isolated on Farm One. The *S.* Typhimurium PFGE subtypes T1.1 and T1.3 were not isolated on Farm Two. Interestingly, the *S.* Typhimurium isolates from the Company A breeder farm had an indistinguishable X*ba1* PFGE type as the *S.* Typhimurium subtype T1.1 isolated from Farm One (Fig. 2).

Molecular typing of *S.* Enteritidis isolates from Farm One revealed two closely related genetic types designated E1.1 and E1.2 (Fig. 1). These genetic types had only one band difference in their restriction patterns. The *S.* Enteritidis genetic type E1.1 was isolated from both houses, a mouse captured in House A, and one post-chill carcass rinse from a bird reared in House A. *Salmonella* Enteritidis genetic type E1.2 was isolated from environmental samples

from House A and two pre-chill carcass rinses from birds from flocks 2, 4, and 7 reared in House A. The *S.* Enteritidis genetic type E1.2 was more prevalent in House A and from carcass rinses from birds raised in House A than genetic type E1.1. The *S.* Enteritidis isolates from the Company A breeder farm had the same *Xba1* PFGE type (E1.2) as isolates recovered from Farm One. PFGE analysis also demonstrated that *S.* Typhimurium and *S.* Enteritidis isolates recovered from carcasses were indistinguishable from those obtained from both the environment of the flock houses, and mice caught on Farm One.

Salmonella Anatum was isolated from both houses on Farm One, and from one house on Farm Two. There were two unrelated genetic types identified on Farm One designated A1 and A2, with only one type isolated from each house (Fig.1). The *S.* Anatum genetic type A3 was isolated only once, from cow feces collected outside of the flock houses on Farm Two, and was unrelated to either of the genetic types isolated from Farm One. The *S.* Montevideo isolates from Farm One were two PFGE types V1 and V2, which were unrelated, having more than seven band differences in their restriction patterns (Fig. 2). *Salmonella* Gaminara was isolated from both farms. One *S.* Gaminara PFGE type consisting of two closely related subtypes was isolated from feed on Farm One (Fig. 1). Two PFGE types were isolated from the environment of Farm Two which were unrelated either to the genetic type isolated from Farm One or to each other (Fig.1). There was only one PFGE genetic type identified for each of the following *S. enterica* serotypes: Heidelberg, Seftenberg, Tennessee, California, Lille, Jerusalem, Mbandaka, Muenchen, and Ohio. We were unable to use PFGE to type any of the *S.* Kentucky isolates recovered from either farm or the Company A breeder farm after repeated attempts using a variety of protocols. In addition some isolates of *S.* Senftenberg and *S.* Mbandaka were not typable with PFGE.

DISCUSSION

By monitoring *Salmonella* contamination during the production of seven consecutive flocks on two commercial broiler farms, we identified a variety of *S. enterica* serotypes including those commonly associated with poultry in general (Poppe 1995; Bailey, Stern et al. 2001; Sarwari, Magder et al. 2001), other food animal species (Letellier 1999; Dargatz 2000; Anderson 2001), as well as human illnesses (Olsen, Bishop et al. 2001). Although we observed the same *Salmonella* serotypes, their prevalence varied between farms in this study, as compared to the results from other *Salmonella* regional and national studies (Anonymous 2001). These inter- and intra-regional differences in *Salmonella* serotype distribution may reflect seasonal variations, regional differences with parent and grandparent lines, feed compositions, presence of disease, as well as other parameters (Cox, Bailey et al. 1991; Byrd, DeLoach et al. 1999; Bailey, Stern et al. 2001). Although serotyping has been useful to differentiate among salmonellae, it is not discriminatory enough to pin-point the source of a common *Salmonella* serotype like *S.* Typhimurium within a food-production system. While this approach has been useful in our understanding the flow of *Salmonella* within integrated poultry production system (Bailey et al., 2001), it cannot determine whether a *S. enterica* strain is transient or endemic, which is important to know in breaking the flow of *Salmonella* to the finished product. We therefore used PFGE to discern genetic differences among *S. enterica* serotypes, in order to trace-back *Salmonella* within this system and determine where in the poultry production pyramid, a strain might be endemic.

Despite the diversity *of Salmonella* genetic types that have been reported for some of the *S. enterica* serotypes identified in this study (Hoszowski 2001; Sander, Hudson et al. 2001), a single strain was observed for several of the *Salmonella* serotypes isolated on both broiler

chicken farms. Similar observations have been reported regarding the genetic diversity of *Salmonella* isolated from other food-animal production systems (Letellier 1999; Murase, Senjyu et al. 2001; Sander, Hudson et al. 2001). The *S. enterica* serotypes Typhimurium and Enteritidis isolated from *Salmonella*-contaminated poultry carcasses from Farm One shared the same, indistinguishable PFGE patterns as those isolated from the environment, in which the flocks were reared, as well as isolates from rodents caught in the same flock houses. The poorly controlled rodent population on this farm probably maintains *Salmonella* within the house environment, thereby enabling these serotypes to persist and colonize birds from flock-to-flock (Davies 2003). However, horizontal transmission of *Salmonella* from the environment to birds reared in these houses was not the only possible source for *Salmonella* contamination identified within this system.

Indistinguishable PFGE types of *S.* Typhimurium, *S.* Enteritidis, and *S.* Heidelberg were isolated from processed chicken carcasses, the broiler chicken environment, and chick-box liners, implicating the hatchery as the source for these persistent serotypes on Farm One. Investigation of Company A's breeder farms, producing eggs for chicks to be placed on Farm One, yielded isolates of *S.* Typhimurium and *S.* Enteritidis with the same PFGE patterns as those *Salmonella* isolates resident on Farm One. Thus, vertical transmission from breeders to the broilers appeared to be the ultimate source of the resident *Salmonella* serotypes identified in this integrated production system. In addition, we also identified one of the *S.* Typhimurium PFGE subtypes on both of the poultry farms participating in this study. This finding is interesting, considering the distance physically separating the two farms (> 50 miles), and that the integrators are contracted with two different poultry companies, which provide their own feed and placement birds. A possible explanation lies in the common industry practice of rearing male

birds of a different breed and source in separate houses at the same time as females on the same pullet farm, then mixing them in the same placement groups, along with "spike" males from other sources, thereby creating many opportunities to assemble *Salmonella* from various sources in a given hen house (Byrd, DeLoach et al. 1999). Thus, the source of the *S.* Typhimurium strain common to both farms is likely a common source of parent birds or primary breeders higher up the integrated production system (Byrd, DeLoach et al. 1999).

Vertical transmission of *Salmonella* from parent to progeny birds has been described for several of the *Salmonella* serotypes identified in this study (Bygrave and Gallagher 1989; Byrd, DeLoach et al. 1999). It has been shown experimentally that *Salmonella* infection of forming eggs in the ovary and shell glands of infected hens, or infection of naive chicks in the first few days of life with a particular strain or serotype will significantly reduce the ability of another strain or serotype to colonize the chicks (Bailey 1988; Iba A.M. 1992; G.C. 2000; Mead 2000; Lee Y.J. 2001). Persistent infection of birds lasting several weeks occurs with colonization of ceca, liver, spleen and reticuloendothelial system with low numbers of *Salmonella* (Barrow P.A. 1994). After the first weeks following initial infection, *Salmonella* are shed into the environment in decreasing numbers unless the birds are starved or stressed (Gast R.K. 1998; Corrier, Byrd et al. 1999). With feed withdrawal and the stress of catching and shipping, *Salmonella* shedding in feces increases, and crop contamination increases as hungry birds consume feces containing *Salmonella* (Hinton A. 1998; Corrier, Byrd et al. 1999; Courrier D.E. 1999). Crop contents have been shown to be a significant source of carcass contamination at processing, and the contamination increases with lack of uniformity of flock size at processing (Russell 2003). Uniformity of flock size can decrease when certain diseases are present, such as airsacculitis (Russell 2003). With this scenario in mind, it follows then, that prevention of *in ovo* or early

infection of broiler chicks with *Salmonella* strains should ultimately reduce carcass contamination with *Salmonella* (Lahellec, Colin et al. 1986). This underscores the need for *Salmonella* monitoring and control at all levels of the integrated production system.

Collectively, these data support previous conclusions that serotypes identified at the farm level are the same strains contaminating products sold at retail (Bhatia, McNabb et al. 1979; Bailey, Stern et al. 2001). However, understanding the ecology of *Salmonella* within an integrated poultry production system is crucial in knowing the point(s) within the poultry pyramid, where intervention is necessary to reduce vertical transmission and ultimately *Salmonella* contamination of processed birds. Company-wide, on-farm *Salmonella* monitoring, is also important to identify problem farm(s) in order to stem horizontal transmission of this organism to the finished product.

CONCLUSION

In summary, we were able to observe both horizontal and vertical transmission of two *Salmonella* serotypes on a commercial poultry farm in northeast Georgia. Since there are many possible sources of *Salmonella* contamination within the integrated poultry production system including the farm environment, feed, rodents, insects, hatchery, and parental line, intervention strategies must target these areas as well as monitor both the poultry production environment and the finished product for *Salmonella.* In addition, control of *Salmonella* contamination at all levels of the vertically integrated production system is essential to the ultimate control and eradication of this pathogen.

ACKNOWLEDGMENTS

This work was supported by a grant from the United States Department of Agriculture (1021RR188156). The State of Georgia Veterinary Medical Experimental Station supported Dr. Liljebjelke.

REFERENCES

Anderson, R.J., J.K. House, B.P. Smith, et al. 2001. Epidemiologic and biological characteristics of salmonellosis in three dairy herds. J. Am. Vet. Med. Assoc. 219:310-322.

Anonymous. 1998. Difco Manual, 11th Edition. Difco Laboratories, Division of Becton Dickinson and Co., Sparks, MD.

Bailey, J.S., N.J. Stern, P. Fedorka-Cray, et al. 2001. Sources and movement of *Salmonella* through integrated poultry operations: a multistate epidemiological investigation. J. Food Prot. 64:1690-1697.

Barrett, T.J., H. Lior, J.H. Green, et al. 1994. Laboratory investigation of a multistate food-borne outbreak of *Escherichia coli* O157:H7 by using pulsed-field gel electrophoresis and phage typing. J. Clin. Microbiol. 32: 3013-3017.

Barrow P.A., M.B. Huggins, and M.A. Lovell. 1994. Host specificity of *Salmonella* infection in chickens and mice is expressed in vivo primarily at the level of the reticuloendothelial system. Infect. Immun. 62:4602-5610.

Bhatia, T.R., G.D. McNabb, H. Wyman, et al. 1979. *Salmonella* isolation from litter as an indicator of flock infection and carcass contamination. Avian Dis. 23:838-847.

Blankenship, L., J. Bailey, N.A. Cox, et al. 1993. Two-step competitive exclusion flora treatment to diminish *Salmonellae* in commercial broiler chickens. Poult. Sci. 72:1667-1672.

Bygrave, A.C. and J. Gallagher. 1989. Transmission of *Salmonella enteritidis* in poultry. Vet. Rec. 124:571.

Byrd, J.A., D.E. Corrier, J.R. Deloach, et al. 1997. Comparison of drag-swab environmental protocols for the isolation of *Salmonella* in poultry houses. Avian Dis. 41:709-713.

Byrd, J.A., J.R. Deloach, D.E. Corrier, et al. 1999. Evaluation of *Salmonella* serotype distributions from commercial broiler hatcheries and grower houses. Avian Dis. 43:39-47.

Corrier, D.E., J.A. Byrd, B.M. Hargis, et al. 1999. Presence of *Salmonella* in the crop and ceca of broiler chickens before and after pre-slaughter feed withdrawal. Poult. Sci. 78:45-49.

Cox, N.A., J.S. Bailey, J.M. Mauldin, et al. 1991. Extent of salmonellae contamination in breeder hatcheries. Poult. Sci. 70:416-418.

Dargatz, D.A., P.J. Fedorka-Cray, S.R. Ladely, et al. 2000. Survey of *Salmonella* serotypes shed in feces of beef cows and their antimicrobial susceptibility patterns. J. Food Prot. 63:1648-1653.

Davies, R.H., and M. Breslin. 2003. Persistence of *Salmonella enteritidis* phage type 4 in the environment and arthropod vectors on an empty free-range chicken farm. Environ. Microbiol. 5:79-84.

Dickens, J.A., N.A. Cox, J.S. Bailey, et al. 1985. Automated microbiological sampling of broiler carcasses. Poult. Sci. 64:1116-1120.

Fuzihara, T.O., S.A. Fernandes, and B.D. Franco. 2000. Prevalence and dissemination of *Salmonella* serotypes along the slaughtering process in Brazilian small poultry slaughterhouses. J. Food Prot. 63:1749-1753.

Hajna, A., and S. Damon. 1956. New enrichment and plating media for the isolation of *Salmonella* and *Shigella* organisms. Appl. Microbiol. 4:341-345.

Hong, Y., T. Liu, C. Hofacre, et al. 2003. A restriction fragment length polymorphism based polymerase chain reaction as an alternative to serotyping for identifying *Salmonella* serotypes. Avian Dis. 47:387-395.

Hoszowski, A., and D. Wasyl. 2001. Typing of *Salmonella enterica* subsp. *enterica* serovar Mbandaka isolates. Vet. Microbiol. 80:139-148.

Iba, A.M., A. Berchieri Junior, and P.A. Barrow. 1995. Interference between *Salmonella* serotypes in intestinal colonization of chickens: correlation with in vitro behavior. FEMS Microbiol. Lett. 131:153-159.

Koort, J., S. Lukinmaa, M. Rantala, et al. 2002. Technical improvement to prevent DNA degradation of enteric pathogens in pulsed-field gel electrophoresis. J. Clin. Microbiol. 40:3497-3498.

Lahellec, C., P. Colin, G. Bennejean, et al. 1986. Influence of resident *Salmonella* on contamination of broiler flocks. Poult. Sci. 65: 2034-2039.

Lee Y.J., M.S. Kang, Y.K. Woo, et al. 2001. Competitive exclusion against *Salmonella gallinarum* of *Salmonella enteritidis* infected chickens. J. Vet. Sci. 2:33-36.

Letellier, A., S. Messier, J. Pare, et al. 1999. Distribution of *Salmonella* in swine herds in Quebec. Vet. Microbiol. 67:299-306.

Liebana, E., D. Guuns, L.Garcia-Migura, et al. 2001. Molecular typing of *Salmonella* serotypes prevalent in animals in England: assessment of methodology. J. Clin. Microbiol. 39:3609–3616.

Liu, T., K. Liljebjelke, E. Bartlett, et al. 2002. Application of nested polymerase chain reaction to detection of *Salmonella* in poultry environment. J. Food Prot. 65:1227-1232.

Mead, G.C. 2000. Prospects for competitive exclusion treatment to control *Salmonellas* and other foodborne pathogens in poultry. Vet. J. 159: 107-108.

Mead, P.S., L. Slutsker, V. Dietz, et al. 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. 5:607-625.

Murase, T., K. Senjyu, T. Maeda, et al. 2001. Monitoring of chicken houses and an attached egg-processing facility in a laying farm for *Salmonella* contamination between 1994 and 1998. J. Food Prot. 64:1912-196.

Olsen, S. J., R. Bishop, F.W. Brenner, et al. 2001. The changing epidemiology of *Salmonella*: trends in serotypes isolated from humans in the United States, 1987-1997. J. Infect. Dis. 183:753-761.

Pope, M.J., and T.E. Cherry. 2000. An evaluation of the presence of pathogens on broilers raised on poultry litter treatment-treated litter. Poult. Sci. 79: 1351-1355.

Rigby, C., J.R. Pettit, A.H. Bentley, et al. 1982. The relationships of *Salmonellae* from infected broiler flocks, transport crates or processing plants to contamination of eviscerated carcasses. Can. J. Comp. Med. 46:272-278.

Russell, S.M. 2003. The effect of airsacculitis on bird weights, uniformity, fecal contamination, processing errors and populations of *Campylobacter* spp. and *Escherichia coli*. Poult. Sci. 82: 1326-1331.

Sanchez, S., C.L. Hofacre, M.D. Lee, et al. 2002. Animal sources of human salmonellosis. J. Am. Vet. Med. Assoc. 221:492-497.

Sander, J., C. R. Hudson, L. Dufour-Zavala et al. 2001. Dynamics of *Salmonella* contamination in a commercial quail operation. Avian Dis. 45:1044-1049.

Sarwari, A.R., L.S. Magder, P. Levine, et al. 2001. Serotype distribution of *Salmonella* isolates from food animals after slaughter differs from that of isolates found in humans. J. Infect. Dis. 183:1295-1299.

Schlosser, W., A. Hogue, E. Ebel, et al. 2000. Analysis of *Salmonella* serotypes from selected carcasses and raw ground products sampled prior to implementation of the Pathogen Reduction; Hazard Analysis and Critical Control Point Final Rule in the US. Int. J. Food Microbiol. 58:107-111.

Tenover, F.C., R.D. Arbeit, R.V. Goering, et al. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. 33:2233-2239.

Waltman, W.D., A.M. Horne, C. Pirkle, et al. 1991. Use of delayed secondary enrichment for the isolation of *Salmonella* in poultry and poultry environments. Avian Dis. 35:88-92.

Waltman, W.D., A.M. Horne, and C. Pirkle. 1993. Influence of enrichment incubation time on the isolation of *Salmonella*. Avian Dis. 37:884-887.

White, D. G., A. Datta, P. McDermott, et al. 2003. *Antimicrobial susceptibility and genetic relatedness of* Salmonella *serovars isolated from animal-derived dog treats in the USA. J. Antimicrob. Chemother. 52:860-863.*

White, D.G, S. Zhao, R. Sudler, et al. 2001. The isolation of antibiotic-resistant *Salmonella* from retail ground meats. New Engl. J. Med. 345:1147-1154.

Figure 1.

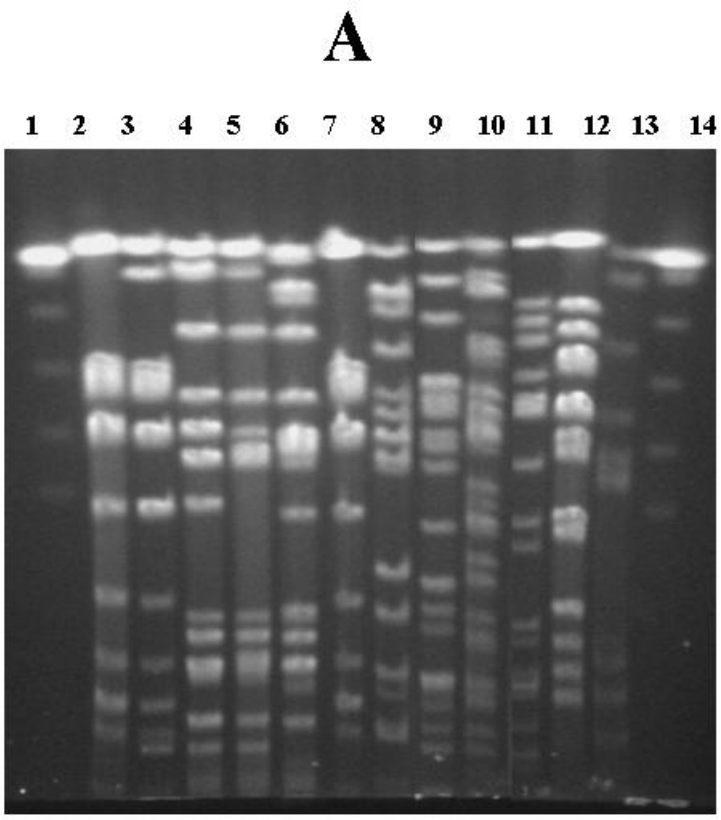

Figure 1.

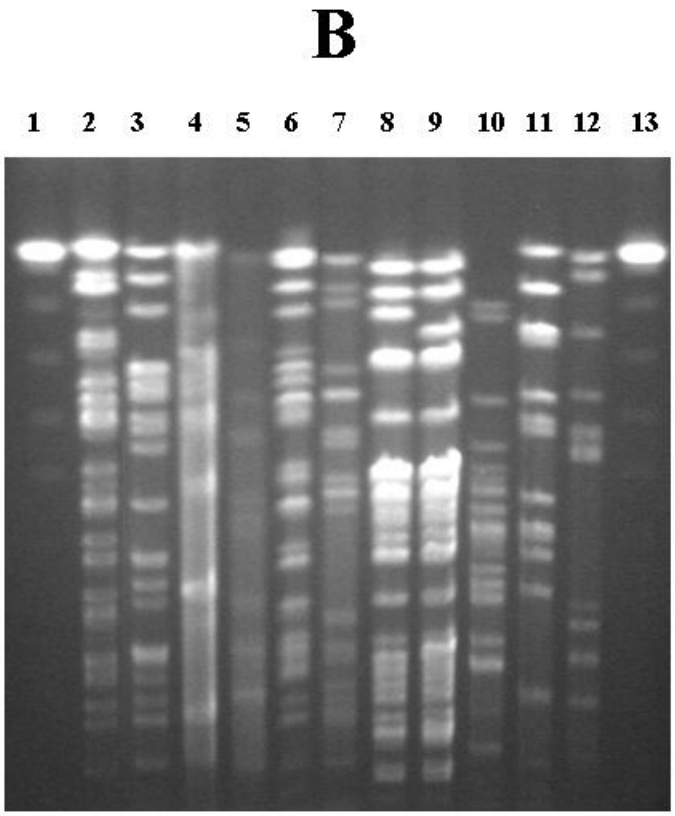

Figure 2.

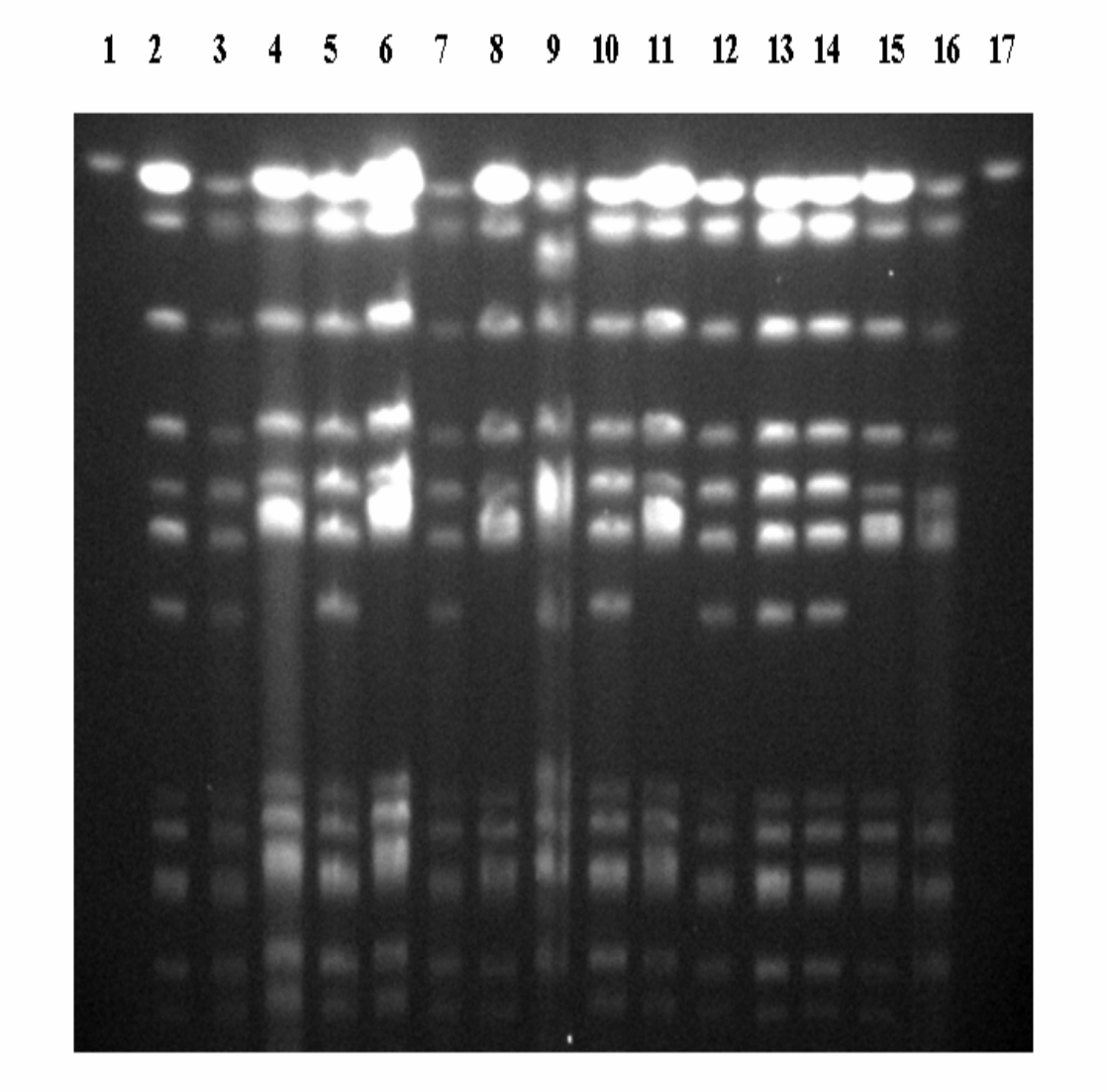

FIGURE LEGENDS

FIG. 1. *Salmonella enterica* PFGE types present on study farms. (A). *Salmonella* Enteritidis PFGE types E1.1 and E1.2 (lanes 2,3); *S.* Typhimurium PFGE types T1.1, T1.2, T1.3 and T2 (lanes 4-7); *S.* Heidelberg PFGE type H1 (lane 8); *S.* Montevideo PFGE type V1 (lane 9); *S.* Mbandaka PFGE type M1 (lane 10); *S.* Muenchen PFGE type U1 (lane 11); *S.* Tennessee PFGE type N1 (lane 12), *S.* Typhimurium SR11 control (Lane 13), *Saccharomyces cerevisiae* molecular weight standard (Lane 1, 14). (B). *Salmonella* Montevideo PFGE type V2 (lane 2); *S.* Montevideo PFGE type V1 (lane 3); *S.* Ohio PFGE type O1 (lane 4); *S.* Anatum PFGE types A1.1, A2.1 (lanes 5, 6); *S.* Gaminara PFGE type G2 (lane 7); *S.* Gaminara PFGE types G1.1, G1.2 (lanes 8, 9); *S.* Gaminara PFGE type G3 (lane10), *S.* Jerusalem PFGE type J1 (lane 11); *S.* Typhimurium SR11 control (Lane 12); *Saccharomyces cerivisiae* molecular weight standard (Lanes 1, 13).

FIG. 2. Molecular epidemiology of *Salmonella* Typhimurium PFGE subtypes T1.1 and T1.2 resident in Company A. *Salmonella* Typhimurium PFGE subtype T1.1 isolates recovered from; broiler breeder environment (lane 2), chick-box liners (lane 3), broiler house litter (lane5), dust (lane 7), mouse (lane 10), pre- and post chill tank carcasses (lanes 12–14). S*almonella* Typhimurium PFGE subtype T1.2 isolates recovered from; chick-box liner (lane 4), litter (lane 6), dust (lane 8), mouse (lane 11), and post chill tank carcass (lane 15). *Salmonella* Typhimurium PFGE subtype T1.3 isolate recovered from water (lane 9), *S.* Typhimurium SR11 control (lane 16), and *Saccharomyces cerevisiae* molecular weight standard (lanes 1, 17).

TABLES

TABLE 1. *SALMONELLA* CONTAMINATION DURING THE PRODUCTION AND PROCESSING OF SEVEN CONSECUTIVE BROILER FLOCKS IN TWO INTEGRATED BROILER COMPANIES.

System Location Represented by Sample Type	*Sample Type (Number of Samples)*	*Company A/Farm One Number Positive (% Positive)*	*Company B/Farm Two Number Positive (% Positive)*
Hatchery	Chick-Box Liner (n = 420)	25 (5.95)	5 (1.19)
Broiler House	Drag Swab (n = 280)	110 (39.28)	19 (6.78)
	Feed (n = 56)	2 (3.57)	0 (0.00)
	Water (n = 56)	1 (1.78)	0 (0.00)
	Litter Sample (n = 280)	61 (21.78)	17 (6.07)
	Dust (n = 56)	6 (10.71)	2 (3.57)
	Mice (n = 12)	7 (58.33)	*
Processing Plant	Carcass Rinse (n = 280)	29 (10.36)	5 (1.78)
	Total Samples	241/1442 (16.69)	48/1430 (3.36)

* No mice were caught.

TABLE 2. *SALMONELLA ENTERICA* SEROTYPES RECOVERED FROM SAMPLES REPRESENTING STAGES IN THE INTEGRATED BROILER PRODUCTION.

System Location Represented by Sample Type

	Hatchery	*Broiler House Environment*						*Plant*	
Salmonella Serotype	*Chick-Box Liner*	*Drag Swab*	*Feed*	*Water*	*Litter*	*Dust*	*Mice*	*Carcass Rinse*	*Total Number Isolates*
FARM ONE									
Anatum		2				1			3
California							1		1
Enteritidis	1	10			13		1	3	28
Gaminara		2	2						4
Heidelberg	1	1			2		1	1	6
Kentucky		8			3	1		1	13
Lille		1							1
Montevideo	8	10			4				22
Seftenberg	8								8
Tennessee		1			1				2
Typhimurium	7	75		1	38	4	4	24	153
Total	25	110	2	1	61	6	7	29	241
FARM TWO									
Anatum*									1
Gaminara					4				4
Heidelberg		1							1
Jerusalem					1				1
Kentucky		7			1			5	13
Lille		2							2
Mbandaka	3	2			3	1			9
Montevideo	1								1
Muenchen					1				1
Ohio	1	2			2				5
Senftenberg		2			2				4
Typhimurium		3			2	1			6
Total	5	19	0	0	17	2	0	5	48

**Salmonella* isolate from cow feces obtained outside broiler flock house.

Table 3. Temporal distribution of *Salmonella.enterica* serotypes and strains collected during the production and processing of seven consecutive flocks.

Serotype	Flock 1 (Apr.28 – Jun.6)			Flock 2 (Jun.22-Jul. 31)			Flock 3 (Aug. 18 – Oct. 3)			Flock 4 (Oct. 19– Dec. 6)			Flock 5 (Jan. 11– Feb. 9)			Flock 6 (Mar. 22. – May. 1)			Flock 7 (Jun. 4-Jul. 10)			Total
	H	E	C	H	E	C	H	E	C	H	E	C	H	E	C	H	E	C	H	E	C	
FARM ONE																						
Anatum														A1.2 (1) A1.3 (1)						A1.2 (1)		3
California														C1.1 (1)								1
Enteritidis	E1.1 (1)	E1.1 (2)			E1.2 (3)							E1.1 (1) E1.2 (2)		E1.2 (2)			E1.1 (4) E1.2 (1)			E1.1 (11) E1.2 (1)		28
Gaminara											G1.1 (1)			G1.1 (3)								4
Heidelberg	H1.1 (1)	H1.1 (3)			H1.1 (1)				H1.1 (1)													6
Kentucky												NT(1)					NT(11)			NT(1)		13
Lille		L1.1 (1)																				1
Montevideo				V1.1 (5) NT(3)	NT(1)												V1.2 (3) V1.3 (1) V1.5 (9)						22
Seftenberg							NT(1)			S1.1 (7)												8
Tennessee																	N1.1 (1)			N1.1 (1)		2
Typhimurium		T1.1 (7)		T1.2 (5)	T1.1 (5) T1.2 (20) T1.3 (5)	T1.2 (1)	T1.1 (2)	T1.1 (8) T1.2 (5) T1.3 (1)	T1.1 (6)		T1.1 (3)	T1.1 (15)		T1.1 (10) T1.2 (9)	T1.1 (1) T1.2 (1)		T1.1 (4) T1.2 (13)			T1.1 (10) T1.2 (22)		153
Total	2	13	0	13	35	1	3	14	7	7	4	19	0	27	2	0	47	0	0	47	0	241

Table 3. (Continued)

	Flock 1 (May 1– Jun. 9)			Flock 2 (Jun. 20– Jul. 25)			Flock 3 (Aug. 11– Oct. 1)			Flock 4 (Oct. 5 - Nov. 20)			Flock 5 (Dec. 20 – Jan. 31)			Flock 6 (Feb. 10– Mar. 19)			Flock 7 (Apr. 2– May 16)			Total
	H	E	C	H	E	C	H	E	C	H	E	C	H	E	C	H	E	C	H	E	C	
FARM TWO																						
Anatum		A1.4 (1)																				1
Gaminara																	G1.2 (2) G2.1 (1) G3.1 (1)					4
Heidelberg		H1.1 (1)																				1
Jerusalem					J1.1(1)																	1
Kentucky											NT(5)									NT(3)	NT(5)	13
Lille		L1.1 (2)																				2
Mbandaka	N T(1)			M1. 1(1) NT(1)				M1. 1(1)						M1. 1(5)								9
Montevideo				V1.1 (1)																		1
Muenchen								U1.1 (1)														1
Ohio	O 1. 1(1)							NT(4)														5
Seftenberg					NT(4)																	4
Typhimurium		T1.2 (5)															T2.1 (1)					6
Total	2	9	0	3	5	0	0	6	0	0	5	0	0	5	0	0	5	0	0	3	5	48

Table 3 Footnotes- H: hatchery; E: broiler house environment; and C: poultry carcass. (n): number of *S. enterica* isolates. Alphabetical letters represent PFGE for *S. enterica* serotypes listed in left most column (e.g. Typhimurium: T). First number following letter represents the distinctive genetic types identified by PFGE. Different numbers represent distinctive genetic types where there were >7 band differences in PFGE pattern. Of the *S. enterica* isolates with similar PFGE patterns, subtypes were identified with 1-7 band differences in their DNA profile and given a second number to distinguish PFGE sub-types. NT: Not typable by PFGE.

CHAPTER 4

ECOLOGY OF SALMONELLA AND ACQUISITION OF ANTIBIOTIC RESISTANCE IN INTEGRATED BROILER PRODUCTION[1]

[1]Liljebjelke, KA, Hofacre, CL, White, DG, Ayers, S, Maier, SM, Dai, Y, Lee, MD, Maurer, JJ. 2004. Prepared for submission to Foodborne Pathogens and Disease.

ABSTRACT

Salmonella remains the leading cause of food-borne illness in the United States, and the dissemination of drug-resistant *Salmonellae* through the food chain has important implications for treatment failure of salmonellosis. We investigated the ecology of *Salmonella* in integrated broiler production in order to understand the flow of antibiotic susceptible and resistant strains within this system. Two hundred eighty-nine *Salmonella enterica,* representing fifteen serotypes were isolated from two poultry farms during the production and processing of seven consecutive flocks. The *Salmonella* serotypes recovered differed between the two companies, as did the levels of *Salmonella* contamination, and their antibiotic resistance phenotypes. Combining isolates from both farms, 61.9 percent were pan-sensitive to a panel of eighteen antimicrobials used in the National Antimicrobial Resistance Monitoring Service (NARMS) surveillance. Resistance to streptomycin, alone, and in combination with other resistances, was the most common (36.3%) antibiotic resistance phenotype observed. Combinations of resistance against streptomycin, gentamicin, sulfamethoxazole, trimethoprim, and tetracycline, were observed for a variety of *S. enterica* serotypes and PFGE genetic types. Among all serotypes, 87.3 percent contained the class 1 integron marker, *intI1*. Resistance to streptomycin, gentamicin, sulfamethoxazole, trimethoprim, and ampicillin was positively correlated ($p < 0.05$), with the presence of *intI1*, a marker for the class 1 integron. There was statistically significant difference and ranking among *S. enterica* serotypes from a given poultry farm, with regards to antibiotic susceptibility to gentamicin, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim. Analysis of the temporal and physical distribution of the endemic serotypes previously identified with their antibiotic phenotypes, suggests that prevention of antimicrobial resistant *Salmonella*

on poultry products is dependent on prevention of vertical transmission of *Salmonella* in the production system. (269 Words)

INTRODUCTION

Salmonella remains the leading cause of outbreak-associated gastroenteritis in the United States, and consumption of poultry products has been implicated in 40% of these outbreaks (Mead 1999.; Olsen, Bishop et al. 2001). Since implementation of the HACCP program improvement has been made in the level of *Salmonella* contamination of processed chicken carcasses (Schlosser W. 2000). However, a recent study of contamination of retail meat from the Washington D. C. area revealed a surprising level of contamination of beef, pork and poultry products with drug-resistant *Salmonella* (Schlosser W. 2000; White DG 2001). The dissemination of drug-resistant *Salmonella* through the food chain has important public health implications considering the potential for treatment failure when cases of gastroenteritis require medical intervention, especially in children, the elderly, and the immunocompromised (Cohen 1986). In addition, infections with antimicrobial resistant bacteria including *Salmonella*, have been associated with higher rates of morbidity and mortality (Barza 1987; Helms M 2002; Martin J.L. 2004).

The use of antibiotics in food animal production has been implicated as contributing to the emergence of drug resistance in food-borne human pathogens (Cohen 1986; Smith KE 1999). The emergence and rapid worldwide spread of the multiple drug resistant *S. enterica* Typhimurium phage-type DT104 clone, and the recent emergence of ceftriaxone-resistant *S. enterica* serotypes Typhimurium and Newport, has underscored the threat to both animal agriculture and human health that multiple drug resistant pathogens pose (Rankin SC 200; Glynn MK 1998; Hollinger K 1998; Fey PD 2000). Antibiotic resistance genes are widely disseminated in pathogenic, commensal and environmental bacteria (Goldstein C 2001; 44.

Nield 2001.; Nield 2001.). Further, it has been shown that once antimicrobial resistance has been introduced into an ecosystem, resistance can spread and persist without continuing selection pressure from antibiotics (Marshall 1990). In addition, the reservoir of antimicrobial resistance genes is larger than previously thought (Nandi S. 2004). It is in this environment that the potential exists for *Salmonella* to acquire drug resistance genes from resident poultry microbiota due to selection pressure from therapeutic and non-therapeutic antibiotic usage. It follows then, that the longer *Salmonellae* persist in the environment of an animal production facility, the chance of acquiring resistance genes increases.

We took advantage of the integrated nature of poultry production to observe the antibiotic resistance phenotypes acquired by salmonellae during broiler chicken production in order to identify potential critical control points for *Salmonella* contamination and antibiotic resistance development, ultimately in order to provide information relevant to reducing the level of carcass contamination with antibiotic resistant *Salmonella*.

MATERIALS AND METHODS

Selection and description of study farms.

Selection and description of study farms was as previously described (Liljebjelke et al., 2004).

History of antibiotic usage on broiler chicken farms.

Approximately seventeen thousand chicks were placed in each house on Farm One. After six to seven weeks of rearing each flock to the market weight of 2.0 – 2.3 kg, the poultry litter was removed from the flock houses, and new litter was added to the houses before placement of the next flock. The litter consisted of dry pine shavings. No litter amendment was used on Farm One (Pope and Cherry 2000). Gentamicin was administered *in ovo* (0.1 mg/egg) on day 17 of development, at the company-owned hatchery supplying chicks for all placements on Farm One.

During the course of this study, no antibiotics were used therapeutically to treat birds on this farm. Chicks were reared on starter feed containing virginiamycin (10g/ton) (25g/ton) for the first two weeks. The starter feed contained coccidiostat rotated in the following order: Flock 1; diclazuril (1g/ton), Flock 2; narasin (72g/ton), Flock 3; monensin (100g/ton), Flocks 4, 5; nicarbazin (82g/ton), Flocks 6, 7; salinomycin (60g/ton). Flocks were then fed grower feed containing bacitracin (25g/ton) for the subsequent two weeks. The grower feed contained coccidiostat rotated in the following order: Flock 1; salinomycin (60g/ton), Flocks 2, 3; narasin (72g/ton), Flocks 4, 5; lasalocid (82g/ton), Flocks 6, 7; diclazuril (1g/ton). Finisher feed containing virginiamycin (15g/ton), without coccidiostat was fed for one to two weeks as birds approached market weight. Withdrawal feed containing neither antibiotics nor coccidiostats was fed for the last week of grow-out. Feed was withdrawn for 16 hours prior to catch.

Approximately twenty thousand chicks were placed per house on Farm Two. Following the first study flock, the old litter was completely removed and replaced. After subsequent flocks the top 2.5 cm of old litter was removed from underneath the nipple-drinker water lines and feed lines in the houses, and a 2.5 cm layer of new litter was spread on the old litter before placement of the next flock. No litter amendments were used on Farm Two (Pope and Cherry 2000). At the company-owned hatchery which supplied chicks for all broiler chicken flocks, gentamicin (0.2 mg/chick) was injected subcutaneously into day-old chicks. Chicks were reared on starter feed containing bacitracin (25g/ton), and salinomycin (50g/ton) for the first two weeks. Flocks were reared on grower feed containing bacitracin (25g/ton) and salinomycin (50g/ton) for two weeks, then finisher feed without growth promotant or coccidiostat for one to two weeks as the flock approached market weight. Withdrawal feed without antibiotic or coccidostat was fed for the last week of flock grow-out. Feed was withdrawn for 16 hours prior to catch and

shipment to the company-owned processing facility. Airsacculitis caused by *Escherichia coli* was diagnosed in house B during the sixth week of the third study flock on Farm Two, and oxytetracycline was prescribed by the company veterinarian for treatment between 9/15/2000 – 9/19/2000. The antibiotic was administered to the birds through drinking water on the first day at the dosage of 10.4 mg/kg bird-weight, and subsequently at the dosage of 5.1 mg/kg bird-weight for four additional days.

Salmonella isolation and analysis.

The 289 *Salmonellae* analyzed in this study were isolated, serotyped, phage-typed, and genetic-typed, by pulsed-field gel electrophoresis (PFGE), as previously described (Liljebjelke et al., 2004).

Antimicrobial resistance profiles.

The minimum inhibitory concentrations (MIC) of the antimicrobial agents tested was determined with the Sensititre® automated antimicrobial susceptibility system (Trek Diagnostic Systems, Westlake, OH), and interpreted according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines for micro-broth dilution methods (2000; 2000). Sensititre® susceptibility testing was performed according to the manufacturer's instructions. Resistance was reported as minimum inhibitory concentration (MIC) in micrograms per ml. The three-letter abbreviation used and resistance breakpoint concentration in microgram/ml are given in parentheses. The following antimicrobials were assayed: amikacin (AMI >64 µg/ml), amoxicillin/clavulanic acid (AUG >32/16 µg/ml), ampicillin (AMP >32 µg/ml), apramycin (APR 32 µg/ml), ceftriaxone (AXO >64 µg/ml), cefazolin (CEF 32µg/ml), cefoxitin (FOX >32 µg/ml), ceftiofur (TIO >8 µg/ml), cephalothin (CEP >32 µg/ml), chloramphenicol (CHL >32 µg/ml), ciprofloxacin (CIP >4 µg/ml), kanamycin (KAN 64 µg/ml), gentamicin (GEN >16

μg/ml), imipenem (IMP >4 μg/ml), naladixic acid (NAL >32 μg/ml), streptomycin (STR >64 μg/ml), sulfadimethoxine (SMX >512 μg/ml), tetracycline (TET >16 μg/ml), trimethoprim/sulfamethoxazole (TMS >4/76 μg/ml).

Polymerase chain reaction (PCR) screen for antibiotic resistance-associated genes intI1 and Tn21.

The presence of the class 1 integron was determined using the PCR method and DNA primers as previously described (Bass et al., 1999). Transposon Tn*21* was analyzed using primers GATAGCACTCCAGCCCGCAGAA and AGGATCTGCTCGGCCATTCC anchored in *tnpR* and *intI1*, respectively. This PCR primer pair amplifies a 595 bp PCR product. Template was prepared according to procedure previously described (Hinton 1983). The 10μl reaction mixture contained 2mM $MgCl_2$, 0.1mM primer, 0.2mM nucleotide, and 1.0 unit Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN). The thermocycler (Idaho Technology Rapidcycler, Idaho Falls, ID) program parameters were as follows: 1) 94^{o}C for 0 seconds; 2) 55^{o}C for 0 seconds; 3) 72^{o}C for 15 seconds, repeated for 30 cycles. All DNA products were separated by 1% agarose gel electrophoresis, using Tris-Acetate-EDTA buffer, at 80V for one hour. A 100bp DNA ladder (Promega, Madison, WI) served as the molecular weight standard. PCR products were purified by using a WIZARD Magic PCR column (Promega; Madison, WI). DNA was sent to the Molecular Genetics Instrumentation Facility for double-stranded DNA sequencing. Oligonucleotides serving as forward and reverse PCR primers were also used as primers for sequencing double-stranded DNA. Identity of the amplicon was confirmed by sequencing PCR products and searching NCBI Genbank database for matches, using the BLAST search algorithm.

Statistical analysis.

Unless otherwise indicated, the Fisher Exact test with $\alpha = 0.05$ was used to test for random vs. non-random associations between specific data values using SAS/STAT 8.1 software (SAS Institute Inc., Cary, N.C.). We determined, statistically, if there was a random vs. non-random association, with null hypothesis that there was a non-random association between the following variables: 1) farm vs. resistance to the antibiotics, gentamicin, streptomycin, and sulfamethoxazole, for *Salmonella* serotypes: Kentucky, Senftenberg, and Typhimurium; 2) resistance to the antibiotics: ampicillin, gentamicin, streptomycin, tetracycline, sulfamethoxazole, and trimethoprim vs. *S.* Enteritidis, *S.* Kentucky, *S.* Montevideo, and *S.* Typhimurium, Farm One only; 3) resistance to the antibiotics: ampicillin, gentamicin, streptomycin, tetracycline, sulfamethoxazole, and trimethoprim, vs. the *S.* Typhimurium PFGE types 1.1, 1.2, and 1.3, Farm One only; and 4) antibiotic resistance vs. oxytetracycline treatment for Farm Two only. Certain serotypes, phages types and antibiotic resistances were excluded from analysis due to small sample size or low frequency of resistance to specific antibiotic(s). A ranking of *Salmonella* serotypes: Enteritidis, Kentucky, Typhimurium, and Montevideo, or *S.* Typhimurium PFGE types 1.1, 1.2, and 1.3 with regards to multiple drug resistance, was determined by fitting the linear model: $\text{Log}(\mu_i) = \beta_o = \beta_1 * \text{serotype}_i$ or PFGE type_i, where μ_i = mean number of antibiotic resistances, assuming that the data conformed to a Poisson distribution. Using the non-parametric Mantel-Haenszel Chi-Square analysis of the frequency distribution of MIC values between integron-positive and integron-negative groups treated as independent samples, we tested the hypothesis that presence of the class 1 integron is associated with higher MIC values (resistance) for antibiotics currently or historically used in poultry production ($p < 0.05$).

RESULTS AND DISCUSSION

Antibiotic susceptibility of poultry Salmonella.

Sixty one point nine percent of the 282 *S. enterica* isolates examined were sensitive to all eighteen antimicrobials tested. Of the drug-resistant isolates, 30.5% had resistance to streptomycin, 12.8% resistance to gentamicin, 20.2% resistance to sulfamethoxazole, 13.5% resistance to tetracycline, and 7.8% had resistance to trimethoprim/sulfamethoxazole (Table 1). Resistance against streptomycin alone was the most prevalent resistance phenotype (30.5%). Twenty point seven percent of 289 *Salmonella* isolates had resistance to three or more antibiotics (Table 1).

Fifty three point eight percent of *S. enterica* Kentucky isolates (n = 13), from Farm One, were resistant to three or more antibiotics, with resistance to gentamicin (61.5%), streptomycin (61.5%), and sulfamethoxazole (76.9%), most prevalent. Fifty three point eight percent of S. *enterica* Montevideo isolates (n = 22) from Farm One were resistant to three or more antibiotics, with 18.2% resistant to streptomycin, 54.5% resistant to sulfamethoxazole, and 59.1% resistant to tetracycline. Fifty nine point one percent of *S.* Montevideo isolates from Farm One were resistant to trimethoprim, and one of 22 isolates (4.5%) exhibited resistance to the antibiotic chloramphenicol. A diversity of antibiotic resistance phenotypes was also observed for *S.* Typhimurium (Table 2), with combinations of resistance to the antibiotics streptomycin, gentamicin, tetracycline, and sulfamethoxazole accounting for twenty seven point nine percent of the phenotypes observed (Table 2). Trimethoprim resistance was observed for *S.* Gaminara, *S.* Anatum, *S.* Tennessee, *S.* Montevideo, and *S.* Kentucky. We observed chloramphenicol resistance for *S.* Lille, *S.* Montevideo, and *S.* Gaminara.

Similar to our results, the 2002 NARMS retail meat survey reported that *Salmonella* isolated from chicken were largely pan-susceptible (66.6%), or resistant to the antibiotics:

sulfamethoxazole (18.7%), streptomycin (32.3%), gentamicin (3.4%), ampicillin (5.1%), trimethoprim (1.7%), and tetracycline (34.3%) (White DG 2001). In addition, the majority of the 1,526 NARMS 2001 *Salmonella* isolates were sensitive to all antimicrobials tested (51.6%), with resistance to: tetracycline (26.7%), streptomycin (23.7%), sulfamethoxazole (9.1%), gentamicin (6.3%), and ampicillin (15.1%), the most prevalent antibiotic resistances (Anonymous 2001). The antibiotic resistance phenotypes most frequently observed for 2001 NARMS poultry *Salmonella* isolates are also very similar to those observed in the poultry isolates from this study: GEN, STR, SMX (1.1%), TET, SMX (1.5%), STR, SMX, TET (3.2%), STR, TET (3.8%), and TET alone (5.5%) (Anonymous 2001).

Using the Fisher Exact Test, there was a statistically significant difference ($p < 0.05$) between the two farms in the antibiotic susceptibility of *S. enterica* Kentucky to the antibiotics gentamicin, streptomycin and sulfamethoxazole, (Farm One > Farm Two), and *S. enterica* Seftenberg to the antibiotics gentamicin and streptomycin, (Farm One > Farm Two). There was no statistically significant difference, at $p < 0.05$, between the two farms in antibiotic susceptibility of *S. enterica* Typhimurium to any of the antibiotics tested, including streptomycin and sulfamethoxazole. Following tetracycline treatment on Farm Two, *Salmonella* isolates were less likely to be resistant to tetracycline, as determined using one-sided, Fisher Exact test at $\alpha = 0.05$ ($p = 0.0046$), or other antibiotics (Cochran-Mantel-Haenszel method, $p = 0.0046$). The therapeutic treatment of flock four on Farm Two with tetracycline for *E. coli* airsacculitis did not seem to have a significant impact on development of antibiotic resistance in *Salmonella* isolated from subsequent flocks. This result is not surprising, considering that the all-in, all-out production method used in the commercial poultry industry is designed to break disease cycles

and should minimize antibiotic resistance development, as long as pathogen persistence from flock-to-flock is prevented (Hofacre 2002).

There was a statistically significant ($p < 0.05$) ranking in favor of resistance to the following antibiotics among *S. enterica* serotypes from Farm One: gentamicin (*S.* Kentucky > *S.* Montevideo, *S.* Typhimurium, and *S.* Enteritidis), streptomycin (*S.* Kentucky > *S.* Typhimurium > *S.* Montevideo > *S.* Enteritidis), sulfamethoxazole (*S.* Kentucky > *S.* Montevideo > *S.* Typhimurium > *S.* Enteritidis), tetracycline (*S.* Montevideo > *S.* Kentucky, *S.* Typhimurium, and *S.* Enteritidis), and trimethoprim (*S.* Montevideo > *S.* Kentucky, *S.* Typhimurium, and *S.* Enteritidis). Fitting of resistance data to the generalized linear model revealed a statistically significant ranking of antibiotic resistance ($p < 0.05$) among Farm 1, *S. enterica* serotypes with regards to the total level of antibiotic resistance, from least-to-most susceptible: *S.* Enteritidis ($\mu_i = 0.15$) > *S.* Typhimurium ($\mu_i = 0.68$) > *S.* Montevideo ($\mu_i = 1.75$) > *S.* Kentucky ($\mu_i = 2.15$). Overall, the *S.* Kentucky and *S.* Montevideo isolates had a statistically significant increased level of antibiotic resistance compared to the *S.* Typhimurium and *S.* Enteritidis isolates, although the *S.* Kentucky isolates obtained from carcass rinses from flock 7 on Farm Two were pan-susceptible.

Antibiotic resistance of S. Typhimurium strains endemic to Farm One.

The one hundred fifty-nine isolates of *S.* Typhimurium were largely pan-sensitive (66.6%) (Table 2). The most prevalent resistances identified were against: streptomycin (36.6%), sulfamethoxazole (12.4%), gentamicin (9.4%), and tetracycline (6.4%). Resistance against the other 14 antimicrobials was minor (≤5%). Eleven point one percent of *S. enterica* Typhimurium isolates were resistant to three or more antibiotics. The most prevalent *S. enterica* Typhimurium resistance phenotypes observed were: STR alone (23.7%), and the multidrug resistant phenotype:

STR, GEN, SMX, and TET (5.3%). A diversity of antibiotic resistance phenotypes (n = 9) was observed among the few *S.* Typhimurium genetic types identified by PFGE (Table 3). Combinations of resistance against streptomycin, gentamicin, sulfamethoxazole, and tetracycline accounted for 85.3% of *S.* Typhimurium resistance phenotypes observed (Table 3).

The *S.* Typhimurium PFGE subtype T1.1 from Farm One was identified as phage type (PT) 193, a phage type commonly associated with illnesses in humans (van Leeuwen 1982; Robins-Browne 1983; Thorton 1993; Asensi 1995; Baggesen 1998; Pontello 1998; Ang-Kucuker 2000; Nastasi 2000; Threlfall 2000; Cruchaga 2001; Hannu 2002). This *Salmonella* phage type has also been isolated from cattle (Hinton 1983; Daly 2000; Threlfall 2000) poultry (Baggesen 1998; Rajashekara 2000), pigs (Baggesen 1998; Daly 2000) and dogs (Daly 2000). Like *S.* Typhimurium DT104, PT 193 isolates generally exhibit resistance to three or more antibiotics, but the resistance phenotypes observed have been variable (Daly 2000; Gebreyes 2002). In contrast, the majority (68.0%) of our *S.* Typhimurium phage type DT193 isolates from Farm One were sensitive to all 18 antibiotics tested, while thirty-two percent had the following resistance phenotypes: STR alone; STR, SMX, TET, TMS; and GEN, STR, SMX, TET, TMS. The other *S.* Typhimurium PFGE types, T1.2 and T1.3 were identified as phage types DT107 and U302, respectively. Our *S.* Typhimurium phage types DT107 and DT193 appear to be genetically-related as determined by PFGE (Tenover FC 1995). Close genetic-relatedness as determined by PFGE among different *S.* Typhimurium and *S.* Enteritidis phage types has been reported by others (Hudson CR 2000; Olsen, Bishop et al. 2001). *S. enterica* Typhimurium U302 is a phage-type that has been associated with multidrug resistance, having the same complement of antibiotic resistance genes present in DT104 (Cruchaga 2001; Walker 2001), however, we identified only one isolate from this study, having similar multi-resistance pattern: AMP, GEN,

STR, SMX but CHL-sensitive, with the remainder of these isolates pan-susceptible. The *S.* Typhimurium DT107 isolates were similar to the *S.* Typhimurium DT193 isolates, in that the majority were pan-susceptible (59.6%), with the most prevalent antibiotic resistance phenotype streptomycin resistance alone (25.4%), however, the DT107 isolates differed with respect to additional multiple drug resistance phenotypes identified: GEN, STR, SMX, TET; GEN, STR, SMX; SMX, TET, TMS; and AXO, FOX, TIO, NAL, STR, SMX. We identified one *S.* Typhimurium DT107 with the multidrug resistance profile: AMP, AUG, FOX, TIO, AXO, CEP, APR, AMI, and NAL.

There was a statistically significant association, at $\alpha = 0.05$, between *S.* Typhimurium genetic types and antibiotic resistance for the antibiotic ampicillin (T1.3> T1.1, T1.2). There was no significant difference in resistance phenotypes between the three *S.* Typhimurium genetic types isolated from Farm One with the exception that PFGE type T1.3 was significantly more likely to be resistant to ampicillin. Fitting the generalized linear model for the three *S.* Typhimurium genetic types, there was a significant difference with regards to the total level of antibiotic resistance, from most-to-least susceptible: T1.3 ($\mu_i = 0.80$); T1.2 ($\mu_i = 0.74$); and T1.1 ($\mu_i = 0.62$). We could not perform similar statistical analyses for *S.* Typhimurium isolated from Farm Two due to the small sample size.

Class 1 integrons in antibiotic resistance of poultry Salmonella.

Differences in the antibiotic susceptibility profiles observed among certain *Salmonella* serovars or strains may reflect variation in mobile genetic elements that ferry these antibiotic resistance genes within this environment. Integrons are genetic elements that "capture" antibiotic resistance gene(s) and integrate them into their integration site *attC* via its recombinase, or integrase, IntI (Bass, Liebert et al. 1999). These genetic elements are responsible for multi-drug

resistance associated with many gram-negative pathogens (Goldstein, Lee et al. 2001). Class 1 integrons are especially prevalent among *Salmonella* and *Escherichia coli* isolated from poultry (Bass et al., 1999; Goldstein et al., 2001). This integron was present in 83.1% of salmonellae isolated from poultry environment (Table 4); prevalence similar to earlier observations for *Salmonella* isolated from multiple poultry companies (Goldstein et al., 2001). However, there was disparity in the distribution of this genetic element among *Salmonella* serovars, in part explaining higher antibiotic susceptibility observed for *S.* Enteritidis compared to the other *Salmonella* serotypes.

The presence of the class 1 integrase *intI1*, a marker of class 1 integrons, was positively associated with presence of resistance to the following antibiotics: gentamicin ($p = 0.001$), streptomycin ($p = 0.020$), sulfamethoxazole ($p = 0.013$), trimethoprim/sulfamethoxazole ($p = 0.015$), and ampicillin ($p = 0.020$). The association of these particular resistance phenotypes with the presence of the class 1 integron is in agreement with antibiotic resistance gene cassette(s) present within this element (Fluitt and Schmitz, 1999). The most prevalent resistances observed in this study, STR, GEN, and SMX, were statistically positively associated with the presence of *intI1* ($p < 0.05$), and consistent with the most common class 1 integron antimicrobial resistance gene cassettes found in the *Enterobacteriaceae* (Fluitt 1999; White 2001).

The prevalence of resistance to streptomycin was surprisingly high, considering that streptomycin is not used in poultry production, but can be explained by the high carriage (62.0%) of transposon Tn*21* observed among our streptomycin-resistant isolates. This ubiquitous mobile genetic element often contain the streptomycin-resistance gene *aadA1* present within its resident class 1 integron (Liebert, Hall et al. 1999). The antibiotic resistance gene, *aadA1* does not confer

cross resistance to another aminoglycoside, gentamicin, an antibiotic currently used in poultry production in the United States, and resistance to which we did observe among our isolates. The *Salmonella* present on these commercial broiler chicken farms possess the genetic potential, in their resident integrons, for acquiring multiple drug resistance, as evident its high prevalence of this element even among antibiotic-susceptible poultry salmonellae (76.3%). It is therefore surprising that our isolates do not have a higher prevalence and/or diversity of antibiotic resistance phenotypes, despite high antibiotic resistance gene load evident in this environment (Nandi et al., 2004).

Model of transmission to and maintenance of antibiotic resistance in Salmonella resident within integrated poultry production system.

Examination of the distribution of resistance phenotypes among the endemic strain of *S.* Typhimurium isolated from different parts of the integrated production system over time revealed a non-random distribution of phenotypes (Table 3). *Salmonella* Typhimurium strains from chick-box liners were either streptomycin resistant (69.2%), or pan-sensitive (30.8 %). Similar observation was made for Typhimurium strains isolated from processed carcasses (STR = 50%), while these same strains isolated from the house environment showed a greater diversity of antibiotic resistance phenotypes over time (Table 3). If the population of *Salmonella* we are examining has the potential for resistance gene acquisition, the question arises as to why we do not see the same distribution of antibiotic resistance phenotypes in this poultry production system. This phenomenon could be explained by the following discussion of what is already known about *Salmonella* transmission and maintenance within integrated poultry production systems.

The uneven distribution of both *Salmonella* serotypes, and resistance phenotypes between the different areas of the integrated system examined (hatchery, house, carcass), can be explained by the presence of two separate *Salmonella* populations which have different degrees of interaction with the environmental microbiota depending on where in the system they are located. Population A represents the endemic *Salmonella* Typhimurium strain acquired by either vertical transmission or early infection in the hatchery or house, as described previously (Liljebjelke et al., 2004). Once the chick is colonized, the endemic *Salmonella* strain becomes sequestered in the liver, spleen, ceca, and reticuloendothelial system (RES) of the broiler chicken, resulting in persistent infection by low numbers of bacteria (Beal, Wigley et al. 2004). Early infection with a particular *Salmonella* strain precludes subsequent infections with other *Salmonella* strains and serovars (Beal et al., 2004). Population B represents the environmental *Salmonella* strains contributed to the system by repeated introduction from the hatchery, environmental contamination, the mouse reservoir, and fecal shedding of population A into the environment. Shedding of the sequestered strain (population A) into the environment should decrease over the age of the broiler chicken, until the dual stresses of food withdrawal and shipping at market age (Corrier, Byrd et al. 1999; Beal, Wigley et al. 2004). During shipping *Salmonella* shedding in feces increases and contamination of the crops of birds occurs when hungry birds consume feces containing *Salmonella* (Corrier et al., 1999). The percentage of *Salmonella*-positive birds can increase at this time by internal exposure of *Salmonell*a-negative birds to feces containing *Salmonella*. Crop contamination is recognized as a significant source of carcass contamination at the processing plant (Russell 2003). Crop contamination of processed carcasses would therefore lead to carcass contamination with population A (endemic strain), and cross-contamination in the chiller could increase the percentage of *Salmonella*-

positive carcasses. This hypothesis is supported by the data collected in this study, which found the antibiotic susceptible, *Salmonella* endemic strain (population A) on contaminated carcasses (Liljebjelke et al 2004). The sequestered *Salmonella* population (A) present in the deep tissue of the broiler would be physiologically prevented from interacting with, and thus acquiring, antibiotic resistance genes from the environmental microbiota. This hypothesis is also supported by the phenotypic data collected, in that both the hatchery and carcass isolates were largely sensitive, with only Tn*21*-mediated streptomycin resistance observed. In contrast, the environmental *Salmonella* population (B) would be free to participate in genetic exchange with both the gram-positive and gram-negative microbial flora in the litter environment (Nandi S. 2004). The hypothesis is again supported by the increased variety of resistance phenotypes observed among isolates from the broiler house environment as compared to those with the same PFGE genetic types isolated from hatchery and carcass rinse samples (Table 3). In addition, *Salmonella* isolates from both *Salmonella* populations have similar prevalence of *intI1* and Tn*21* carriage, yet acquisition of new resistance genes appeared to occur only in the *Salmonella* isolated from the environment (Tables 2, 3).

It is also interesting to consider that the two populations would have different selection pressures: the sequestered population (A), for cell invasion and colonization, and the environmental population (B), would be selected for persistence and survival in the house environment. It is perhaps fortuitous that there was only one incidence of therapeutic antibiotic usage on one farm during this year long study, as were able to observe the natural ecology of *Salmonella* and its associated antibiotic resistance within this integrated poultry system without the effects of selective pressure from antibiotic usage.

CONCLUDING STATEMENT

We have identified the origins of *Salmonella* contamination within the poultry production system, and identified *Salmonella* strains endemic within this system. In this study, we analyzed the physical and temporal patterns of antibiotic resistance in *Salmonella* serotypes isolated from this same system. Preventing the development of antibiotic resistance will depend on our ability to interrupt horizontal and vertical transmission of endemic *Salmonella* strains within this production system.

Genetic transfer of antimicrobial resistance genes within bacterial communities is now widely recognized as contributing to the evolution of antibiotic resistance on a global scale. The significant role of transmissible genetic elements such as integrons and transposons in the maintenance and dissemination of antibiotic resistance genes is particularly important in modern animal agriculture, where use of both therapeutic and non-therapeutic antimicrobial usage confers strong selection pressure on the microbial ecology to maintain and disseminate antibiotic resistance. Further characterization of the molecular genetic basis of the resistance phenotypes seen in the *Salmonella* isolates from this study, and examination of the microbial gene pool will help to further elucidate the genetic mechanisms of maintenance and dissemination of antibiotic resistance to food-borne pathogens in integrated production systems (Nandi S. 2004).

ACKNOWLEDGMENTS

This work was supported by a grant from the United States Department of Agriculture (99-35212-8680). The State of Georgia Veterinary Medical Experimental Station supported Dr. Liljebjelke.

REFERENCES

Ang-Kucuker, M., V. Tolun, R. Helmuth, et al. 2000. Phage types, antibiotic susceptibilities and plasmid profiles of *Salmonella typhimurium* and *Salmonella enteritidis* strains isolated in Istanbul, Turkey. Clin. Microbiol. Infect. 6:593-599.

Anonymous. 2000a. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. National Committee for Clinical Laboratory Standards. Villanova, PA.

Anonymous. 2000b. Performance standard for antimicrobial disk susceptibility tests - seventh edition. National Committee for Clinical Laboratory Standards. Villanova, PA.

Anonymous. 2001. National antimicrobial susceptibility monitoring program for veterinary and human isolates. United States Food and Drug Administration, Laurel, MD.

Asensi, M.D., A.P. Costa, E. Moura, et al. 1995. Lysotypes and plasmidial profile of *Salmonella* serovar typhimurium isolated from children with enteric processes in the cities of Rio de Janeiro, RJ, and Salvador, BA-Brazil. Rev. Inst. Med. Trop. Sao Paulo. 37:297-302.

Baggesen, D.L., and F.M. Aarestrup. 1998. Characterization of recently emerged multiple antibiotic-resistant *Salmonella* enterica serovar Typhimurium DT104 and other multiresistant phage types from Danish pig herds. Vet. Rec. 143:95-97.

Barza, M. 1987. Potential mechanisms of increased disease in humans from antimicrobial resistance in food animals. Clin. Infect. Dis. 34(S3):123-125.

Bass, L., C. Liebert, M.D. Lee, et al. 1999. Incidence and characterization of integrons, genetic elements mediating multiple-drug resistance, in avian *Escherichia coli*. Antimicrob. Agents Chemother. 43:2925-2929.

Beal, R., P. Wigley, C. Powers, et al. 2004. Age at primary infection with *Salmonella enterica* serovar Typhimurium in the chicken influences persistence of infection and subsequent immunity to re-challenge. Vet. Immunol. Immunopathol. 100:151-164.

Cohen, M., and R.V. Tauxe. 1986. Drug-resistant *Salmonella* in the United States: an epidemiological perspective. Science 234:964-969.

Corrier, D.E., J.A. Byrd, B.M. Hargis, et al. 1999. Presence of *Salmonella* in the crop and ceca of broiler chickens before and after pre-slaughter feed withdrawal. Poult. Sci. 78:45-49.

Cruchaga, S., A. Echeita, A. Aladuena, et al. 2001. Antimicrobial resistance in salmonellae from humans, food and animals in Spain in 1998. J. Antimicrob. Chemother. 47:315-321.

Daly, M., J. Buckley, E. Power, et al. 2000. Molecular characterization of Irish *Salmonella enterica* serotype Typhimurium: detection of class I integrons and assessment of genetic relationships by DNA amplification fingerprinting. Appl. Environ. Microbiol. 66:614-619.

Dancer, S.J. 2004. How antibiotics can make us sick: the less obvious adverse effects of antimicrobial chemotherapy. Lancet Infect. Dis. 4:611-619.

Fey P.D., S.T. Safranek, M.E. Rupp, et al. 2000. Ceftriaxone-resistant *Salmonella* infection acquired by a child from cattle. New Engl. J. Med. 342:1242-1249.

Fluitt, A., and F.J. Schmitz. 1999. Class 1 integrons, gene cassettes, mobility, and epidemiology. Eur. J. Clin. Microbiol. Infect. Dis. 18:761-770.

Gebreyes, W.A., and C. Altier. 2002. Molecular characterization of multidrug-resistant *Salmonella enterica* subsp. enterica serovar Typhimurium isolates from swine. J. Clin. Microbiol. 40:2813-2822.

Glynn, M.K., C. Bopp, W. Dewitt, et al. 1998. Emergence of multi-drug resistant *Salmonella enterica* serotype Typhimurium DT104 infections in the United States. New Engl. J. Med. 338:1333-1338.

Goldstein, C, M.D. Lee, S. Sanchez, et al. 2001. Incidence of class 1 and class 2 integrases in clinical and commensal bacteria from livestock, companion animals, and exotics. Antimicrob. Agents Chemother. 45:723-726.

Hannu, T., L. Mattila, A. Siitonen, et al. 2002. Reactive arthritis following an outbreak of *Salmonella typhimurium* phage type 193 infection. Ann. Rheum. Dis. 61:264-266.

Helms, M., P. Vastrup, P. Gerner-Smidt, et al. 2002. Excess mortality associated with antimicrobial drug-resistant *Salmonella typhimurium* DT104. Emerg. Infect. Dis. 8:490-495.

Hinton, M., E.A. Ali, and A.H. Linton. 1983. The excretion of *Salmonella typhimurium* in the faeces of calves fed milk substitute. J. Hyg. 91:33-45.

Hofacre, C. 2002. The health and management of poultry production. Int. J. Infect. Dis. 6:353-357.

Hollinger, K, C. Wray, S. Evans, et al. 1998. *Salmonella typhimurium* DT104 in cattle in Great Britain. J. Amer. Vet. Med. Assoc. 213:1732-1733.

Hudson, C.R., C. Quist, M.D. Lee, et al. 2000. The genetic-relatedness of *Salmonella* from non-domestic birds in the Southeastern United States. J. Clin. Microbiol. 38:1860-1865.

Liebert, C., R.M. Hall, and A.O. Summers. 1999. Transposon Tn*21*, flagship of the floating genome. Microbiol. Mol. Biol. Rev. 63:507-522.

Liljebjelke, K.A., C. Hofacre, T. Liu, et al. 2004. Vertical and horizontal transmission of *Salmonella* within integrated broiler production system. Foodborne Path. Dis. (Submitted).

Marshall, B., D. Petrowski, and S.B. Levy. 1990. Inter-and intraspecies spread of *Escherichia coli* in a farm environment in the absence of antibiotic usage. Proc. Natl. Acad. Sci. USA 87:6609-6613.

Martin, J.L., M. Fyfe, K. Dore, et al. 2004. Increased burden of illness associated with antimicrobial resistant *Salmonella enterica* serotype Typhimurium infections. J. Infect. Dis. 189:377-384.

Mead, P. S., L. Slutsker, V. Deitz, et al. 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. 5:607-625.

Nandi S., J.J. Maurer, C. Hofacre, et al. 2004. Gram-positive bacteria are a major reservoir of class 1 antibiotic resistance integrons in poultry litter. Proc. Natl. Acad. Sci. USA 101: 7118-7122.

Nastasi, A., and C. Mammina. 2000. Surveillance of multidrug-resistant strains of *Salmonella enterica* serotype Typhimurium in southern Italy in the years 1992-1997. Eur. J. Epidemiol. 16:135-139.

Nield, B.S., A.J. Holmes, M.R. Gillings, et al. 2001. Recovery of new integron classes from environmental DNA. FEMS Microbiol. Lett. 195:59-65.

Olsen, S.J., R. Bishop, F.W. Brenner, et al. 2001. The changing epidemiology of *Salmonella*: trends in serotypes isolated from humans in the United States, 1987-1997. J. Infect. Dis. 183:753-761.

Pontello, M., L. Sodano, A. Nastasi, et al. 1998. A community-based outbreak of *Salmonella enterica* serotype Typhimurium associated with salami consumption in Northern Italy. Epidemiol. Infect. 120: 209-214.

Pope, M.J., and T.E. Cherry. 2000. An evaluation of the presence of pathogens on broilers raised on poultry litter treatment-treated litter. Poult. Sci. 79: 1351-1355.

Rajashekara, G., E. Haverly, D.A. Halvorson, et al. 2000. Multidrug-resistant *Salmonella typhimurium* DT104 in poultry. J. Food Prot. 63:155-161.

Rankin, S.C., H. Aceto, J. Cassidy, et al. 2002. Molecular characterization of cephalosporin-resistant *Salmonella enterica* serotype Newport isolates from animals in Pennsylvania. J. Clin. Microbiol. 40:4670-4684.

Robins-Browne, R. M., B. Rowe, R. Ramsaroop, et al. 1983. A hospital outbreak of multiresistant *Salmonella typhimurium* belonging to phage type 193. J. Infect. Dis. 147:210-216.

Schlosser, W., A. Hogue, E. Ebel, et al. 2000. Analysis of *Salmonella* serotypes from selected carcasses and raw ground products sampled prior to implementation of the Pathogen Reduction; Hazard Analysis and Critical Control Point Final Rule in the US. Int. J. Food Microbiol. 58:107-111.

Smith, K.E., J. M. Besser, C.W. Hedberg, et al. 1999. Quinolone-resistant *Campylobacter jejuni* infections in Minnesota, 1992 - 1998. New Engl. J. Med. 340:1525-1531.

Tenover, F.C., R.D. Arbeit, R.V. Goering, et al. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. 33:2233-2239.

Thorton, L., S. Gray, P. Gingham, et al. 1993. The problems of tracing a geographically widespread outbreak of salmonellosis from a commonly eaten food: *Salmonella typhimurium* DT193 in northwest England and north Wales in 1991. Epidemiol. Infect. 111:465-471.

Threlfall, E.J., L.R. Ward, J.A. Skinner, et al. 2000. Antimicrobial drug resistance in non-typhoidal *Salmonellas* from humans in England and Wales in 1999: decrease in multiple resistance in *Salmonella enterica* serotypes Typhimurium, Virchow, and Hadar. Microb. Drug Resist. 6:319-325.

van Leeuwen, W.J., C.E. Voogd, P.A. Guinee, et al. 1982. Incidence of resistance to ampicillin, chloramphenicol, kanamycin, tetracycline and trimethoprim of *Salmonella* strains isolated in the Netherlands during 1975-1980. Antoine Van Leeuwenhoek 48:85-96.

Walker, R.A., E. Lindsay, M.J. Woodward, et al. 2001. Variation in clonality and antibiotic-resistance genes among multiresistant *Salmonella enterica* serotype typhimurium phage-type U302 (MR U302) from humans, animals, and foods. Microb. Drug Resist. 7:13-21.

White, D.G., S. Zhoa, R. Sudler, et al. 2001. The isolation of antibiotic-resistant *Salmonella* from retail ground meats. New Engl. J. Med. 345:1147-1154.

White, P., C.J. McIver, and W. D. Rawlinson. 2001. Integrons and gene cassettes in the *Enterobacteriaceae*. Antimicrob. Agents Chemother. 45: 2658-2661.

TABLES

Table 1. Most prevalent antibiotic resistances observed in the *Salmonella enterica* serotypes isolated from production and processing of seven consecutive commercial broiler flocks.

Salmonella enterica Serotype (Number Isolates)	*% Sensitive*	*% STR*	*% GEN*	*% SMX*	*% TET*	*% TMS*	*% AMP*	% Multidrug Resistant[1]
Farm One								
Typhimurium (153)	66.6	36.6	9.8	12.4	5.9	1.9	0.6	11.1
Enteritidis (28)	92.8	1	1	1	0	0	1	1
Montevideo (22)	40.9	18.2	0	54.5	59.1	59.1	0	53.8
Kentucky (13)	23.1	61.5	61.5	76.9	7.7	7.7	0	53.8
Heidelberg (6)	33.3	50.0	33.3	33.3	16.7	16.7	16.7	66.7
All Serotypes (241)	60.7	35.4	13.8	23.4	13.3	10.4	0.87	22.7
Farm Two								
Kentucky (13)	100	0	0	0	0	0	0	0
Mbandaka (9)	55.6	11.1	11.1	11.1	33.3	0	0	11.1
Typhimurium (6)	66.6	33.3	16.7	16.7	16.7	0	0	16.7
Ohio (5)	80.0	20.0	0	0	0	0	0	0
Senftenberg (4)	75.0	0	25.0	25.0	25.0	0	25.0	25.0
All Serotypes (48)	72.3	8.5	6.4	8.5	17.0	0	4.3	10.6

[1]Resistance to three or more antibiotics

Table 2. Antibiotic resistance phenotypes among *S. enterica* serotypes and PFGE genetic types isolated from poultry farms One and Two during the production of seven consecutive flocks.

Salmonella Serotype	*PFG Type*	*AntibioticResistance Phenotype*[1]	*Total Number of Isolates*
Enteritidis PT8	E1.1	Sensitive	18
		AMP	1
		GEN STR SMX	1
	E1.2	Sensitive	6
Typhimurium DT193	T1.1	Sensitive	50
		STR	16
		STR SMX	1
		GEN STR SMX TET	3
		STR SMX TET TMS	1
		GEN STR SMX TET TMS	2
	T1.2	Sensitive	47
DT107		STR	20
		STR CEP	1
		STR SMX	1
		GEN STR SMX	5
		GEN STR SMX TET	4
		STR SMX AXO FOX TIO AMI APR NAL	1
Typhimurium U302	T1.3	Sensitive	4
		GEN STR SMX AMP	1
UT	T2	Sensitive	1
	T3	Sensitive	1
Montevideo	V1	Sensitive	4
	V2	SMX TET TMS	8
		STR SMX TET TMS	2
		CHL SMX TET TMS	2
	NT	Sensitive	4
		STR	1
		CHL	1
		SMX TET TMS	2
Kentucky	NT	Sensitive	16

Salmonella Serotype	*PFG Type*	*Antibiotic Resistance Phenotype*[1]	*Total Number of Isolates*
		STR SMX	2
		GEN SMX	1
		GEN STR SMX	6
		GEN STR TET TMS	1
Senftenberg	S1	GEN STR	1
		GEN STR SMX	3
		GEN STR SMX CEP	1
		SMX TET TMS CHL	1
	G1.2	STR SMX TET TMS CHL	1
	G2	STR SMX TET TMS CHL KAN	1
	G3	AMP CEP	1
Mbandaka	M1	Sensitive	4
		GEN STR TET	1
		SMX	1
	NT	Sensitive	1
		TET	1
Anatum	A1	STR SMX TET TMS	1
	A2	STR SMX TET TMS	1
		SMX TET TMS	1
	A3	Sensitive	1
		STR SMX TET TMS	1
Ohio	O1	STR	1
	NT	Sensitive	4
Tennessee	T1	SMX TMS	1
		TET TMS	1
California	C1	Sensitive	1
Heidelberg	H1	Sensitive	4
		STR	2
		GEN STR SMX	1
		AMP CEP	1

		AUG FOX		
Jerusalem	J1		TET	1
Lille	L1		TET	1
		CEP CHL		
	NT		TET	1
		CEP CHL		
		CHL		1
Muenchen	U1	SMX		1

[1]Antibiotics: AMP, ampicillin; AUG, augmentin; FOX, cefoxitin; CEP, cephalothin; GEN, gentamicin; KAN, kanamycin, STR, streptomycin; AMI, amikacin; NAL, naladixic acid; TET, tetracycline; SMX, sulfamethoxazole; TMS, trimethoprim; and CHL, chloramphenicol. NT = not typeable by PFGE, UT = untypable by phage typing.

Table 3. Temporal and physical distribution of antibiotic resistance phenotypes of the related S. typhimurium PFGE types resident on Farm One during the production of seven consecutive flocks. [1]Multidrug resistance (MDR) to antibiotics: STR, SMX, FOX, AMI, AXO, NAL, TIO,AND APR.

Flock	*S. typhimurium PFGE Type*	*Antibiotic Resistance Phenotype*	*Location*		
			Hatchery (No. Isolates)	*House (No. Isolates)*	*Carcass (No. Isolates)*
1	T1.1	Sensitive		**2**	
		STR		**2**	
		STR SMX TET TMS		**1**	
		STR GEN SMX TET TMS		**2**	
2	T1.1	Sensitive		**5**	
	T1.2	Sensitive	**3**	**17**	
		STR	**2**	**1**	**1**
		STR GEN SMX		**2**	
		STR GEN SMX TET		**1**	
	T1.3	Sensitive		**3**	
		STR GEN SMX AMP		**1**	
3	T1.1	Sensitive	**1**	**4**	**1**
		STR	**7**	**3**	**5**
		STR SMX		**1**	
	T1.2	None		**4**	
		STR		**2**	
	T1.3	Sensitive		**1**	
4	T1.1	Sensitive		**3**	**14**
		STR			**1**

Table 3. Contd.

Flock	*S. typhimurium PFGE Type*	*Antibiotic Resistance Phenotype*	*Location*		
			Hatchery (No. Isolates)	*House (No. Isolates)*	*Carcass (No. Isolates)*
		STR			**1**
5	T1.1	Sensitive		**8**	**1**
		STR		**1**	
	T1.2	Sensitive		**5**	**1**
		STR		**5**	
	T1.3	Sensitive		**1**	
6	T1.1	Sensitive		**3**	
	T1.2	Sensitive		**2**	
		STR		**9**	
		STR SMX		**1**	
		CEP		**1**	
		MDR[1]		**1**	
7	T1.1	Sensitive		**7**	
		STR		**1**	
	T1.2	STR GEN SMX TET		**3**	
		Sensitive		**14**	
		STR		**2**	
		STR GEN SMX		**3**	
		STR GEN SMX TET		**2**	

TABLE 4. DISTRIBUTION OF CLASS 1 INTEGRONS AMONG POULTRY *SALMONELLA*

Serotype	*Distribution of Class 1 Integron: Antibiotic Susceptible (%)*	*Distribution of Class 1 Integron: Resistant to ≥1 Antibiotic (%)*	*Total Carriage of Class 1 Integron (%)*	*Presence of Tn21 in Streptomycin-Resistant, Integron-Positive Salmonella (%)*
All Serovars (n = 279)	129 (76.3)	103 (93.6)	232 (83.1)	44 (62.0)
Enteritidis (n = 26)	13 (54.2)	2 (100.0)	15 (57.7)	1 (100.0)
Typhimurium (n = 161)	83 (80.8)	54 (93.1)	137 (85.1)	27 (62.8)
T1.1 (n = 73)	45 (90.0)	22 (95.6)	67 (91.8)	15 (71.4)
T1.2 (n = 82)	36 (75.0)	31 (91.2)	67 (81.7)	11 (52.4)
Kentucky (n = 26)	12 (75.0)	10 (100.0)	22 (84.6)	9 (100.0)
Montevideo (n = 16)	8 (100.0)	8 (100.0)	16 (100.0)	3 (75.0)
Other Serovars (n = 50)	13 (72.2)	27 (84.4)	40 (80.0)	4 (28.6)

CHAPTER 5

SYSTEMIC SALMONELLA ENTERITIDIS CONTAMINATION ON A COMMERCIAL BROILER FARM IN NORTHEAST GEORGIA[1]

[1]Liljebjelke, KA, Hofacre, CL, Liu, T, White, DG, Ayers, S, Young, SE, Maurer, JJ. 2004. Prepared for submission to Journal of Avian Disease.

SUMMARY

Salmonella is a significant cause of food-borne illness in the United States. The control of carcass contamination requires detailed knowledge of the sources and reservoirs of *Salmonella* within an integrated poultry production system. We examined the ecology of *S. enterica* Enteritidis on a commercial broiler farm during seven consecutive flocks in order to understand the flow of *Salmonellae* within this integrated production system. *S. enterica* Enteritidis was consistently isolated (9.2% of samples) during the one year sampling period. Pulsed field gel electrophoresis (PFGE), phage typing, and random amplified polymorphic DNA (RAPD) was used to type *S.* Enteritidis isolated in this study. A cladogram was generated from PFGE and RAPD patterns to determine genetic-relatedness among *Salmonella* isolated from broiler carcasses, poultry house environment of broiler chickens and broiler-breeder flocks, which supplied chicks for poultry farm examined in this study. A resident clone was identified persisting on this farm from flock-to-flock. Other *S.* Enteritidis strains were identified that were genetically related to this endemic *Salmonella* clone. In addition, one closely-related *S.* Enteritidis genetic sub-type was isolated from two different broiler-breeder flocks. The *S.* Enteritidis clone was isolated from both the poultry house environment, mouse trapped in this poultry house, and processed broiler carcass originating from this flock. Using molecular sub-typing, we documented the maintenance and transmission of a *S.* Enteritidis clone, and persistence of other closely-related *S.* Enteritidis strains on this broiler chicken farm, and contamination of broiler chickens processed from this farm with these persistent *S.* Enteritidis strains.

INTRODUCTION

Salmonella enterica remains a leading cause of outbreak-associated gastroenteritis in the United States (Olsen, Bishop et al. 2001). It has been estimated that approximately 1.4 million

cases of non-typhoidal salmonellosis occur each year in the United States, with consumption of poultry products implicated in 40 % of these outbreaks (Olsen, Bishop et al. 2001). Prior to the 1970s, *S. enterica* Typhimurium was the most common serotype associated with foodborne disease outbreaks in the United States (R. 1991.; Olsen S.J. 2001.). Since then, *S.* Enteritidis has supplanted *S.* Typhimurium as the dominant serotype associated with food-borne outbreaks of salmonellosis in the United States (R. 1991.). The majority of outbreaks caused by *Salmonella* serotype Enteritidis have been associated with the consumption of contaminated eggs and egg products (Angulo and Swerdlow 1998). In Europe, *S. enterica* Enteritidis phage type 4 has been most often associated with human illness, while in the United States and Canada, *S. enterica* Enteritidis phage-type 8 is most often associated with human morbidity and mortality, although phage type 4 has recently emerged in this country (Boyce T.G. 1996.).

In response to increasing concern over foodborne illness, the Food Safety and Inspection Service (FSIS) mandated in 1996 that meat and poultry processing plants implement a Hazard Analysis Critical Control Point (HACCP) program and meet USDA-set limits for *Salmonella* contamination of finished poultry products. Since that time, the poultry industry has made significant reductions in the level of *Salmonella* contamination of poultry carcasses, from 20 % in 1998 to 10 % in 2000 (Schlosser W. 2000). Although outbreaks of foodborne illness due to *S. enterica* Enteritidis are most commonly associated with consumption of uncooked contaminated eggs, there are reports in the literature from the United States and elsewhere, of sporadic cases of human illness traced to consumption of *S. enterica* Enteritidis-contaminated poultry products (Rampling A. 1989.; Boonmar s. 1998.). The potential for causation of foodborne illness should therefore not be ignored, considering the presence of *S. enterica* Enteritidis on poultry carcasses, and the emergence of *S.* Enteritidis phage type 4 in the United States.

It is well known that *Salmonella* can become endemic in many kinds of food animal production units. Mice, rats, and insects have been conclusively shown to be vectors and reservoirs of *Salmonella* and other pathogens in food animal production (Henzler 1992.; Davies 1995.). Control of mice in egg-layer facilities has been shown to be crucial to control of *S. enterica* Enteritidis infection of laying hens (Henzler 1992.; Davies 1995.). Because of the linkage shown between the colonization of live birds on the farm with *Salmonella* and the contamination of finished poultry products with the same *S. enterica* serotypes, any further reductions in the level of carcass contamination will no doubt require on-farm intervention strategies (LaHellec C. 1986.). It follows then, that in order to reduce or eradicate *Salmonella* from the broiler farm environment and prevent it's introduction or re-introduction onto the farm requires detailed knowledge of the epizoology and ecology of *Salmonella* resident on farms, and in integrated production systems (Sanchez 2002).

The combination of phage typing, PFGE typing, and RAPD typing provides a powerful discriminatory tool for the epidemiological analysis of related strains of *S. enterica* Enteritidis (Lin A. 1996; Lopes VC 2004). In addition, the use of delayed culture enrichment, coupled with PCR screen, maximizes recovery of *Salmonella* from environmental samples (Waltman WD 1991.). The use of molecular genetic techniques to type bacterial isolates, along with the extended period and scope of sampling, expands upon data collected in previous farm surveys, and provides valuable insight into the ecology of *Salmonella enterica* Enteritidis strains resident in this commercial broiler operation (Bailey, Stern et al. 2001).

METHODS AND MATERIALS

Selection and description of poultry farms. The selection and description of the study farm was described previously (Liljebjelke et al 2004). In addition to the broiler farm, one

company-owned broiler breeder farm complex which supplies eggs to the company hatchery, supplying the study farm with day-old chicks, was sampled for *Salmonella* contamination from August 2003 to December 2003.

Sample collection scheme. At the time of chick placement (day 0), the following samples were collected from each of two houses; chick-box liners (30), drag-swab (5), litter (5), water (1), feed (2), dust (2), mice (variable). The two study houses were subsequently sampled every 2 weeks during the six-to-seven week production cycles until slaughter. Exclusive of the chick-box liners, the same number and type of samples were collected during weeks 2, 4, and 6 of the grow-out period. Carcass rinses were obtained from 20 processed birds from each house at the time of processing (week 6-7). Excluding mice, cow feces, and insects, 102 samples were taken from each house during the grow-out period of seven consecutive flocks. A total of 1,540 samples were collected during the study. Pooled litter samples were collected down to a depth of 7.5 cm from underneath the nipple-drinkers, feeders, and along the length of the flock house. Five pooled litter samples were collected from each house. The environment of the broiler houses was sampled using drag swabs consisting of sterile gauze pads soaked with double-strength skim milk (Byrd, DeLoach et al. 1999). The swabs were dragged across the birds bedding material for the length of the house, or wiped along water lines or fan blades to collect dust. Swabs were placed in individual sterile bags for transportation to the laboratory at 4^0C. Twenty chicken carcasses were collected at the processing plant, ten prior to, and ten immediately after the 32^oC chlorinated water chill-tank. Chicken carcasses were placed in individual sterile bags with 250 ml buffered-peptone water and agitated vigorously using a mechanical shaking device designed for standardizing carcass rinse sampling (Dickens 1985). In order to avoid cross-contamination between flocks, the study flock was the first flock of the day

processed at the plant. These samples were treated as described previously (Liljebjelke et al 2004). Mice were captured in live traps in the study houses and transported to the laboratory (Victor Tin Cat, Woodstream Corp.; Lititz, PA). Sample collection from mice was described previously (Liljebjelke et al 32004).

Feed samples were collected from the open hopper below the feed augur. Water samples were collected from the incoming line where it was connected to the nipple drinker line. Before sample collection, the end of the water tap was disinfected with antibacterial soap and the water was allowed to run freely for 30 seconds. At the time of flock placement the chick-box liners were removed from the transport baskets, and transported to the laboratory in sterile bags at 4^{o}C. The surface of each chick-box liner was wiped with a drag-swab.

Sampling of the broiler breeder farm consisted of three drag swab samples taken in each of ten houses. One swab each was used to sample each of the two slat surfaces in the layer house, and one swab was dragged across the bedding in the scratch area in the center of the house.

Sample processing, *Salmonella* isolation and identification. Samples were processed as previously described (Liljebjelke 2004). *Salmonella* was isolated and identified using a Kauffman-White scheme as previously described (Liljebjelke 2004). The flagellar antigens were typed using a RFLP-PCR procedure previously described (Hong 2003).

Molecular typing. *Salmonella* isolates were genetically typed by pulsed-field gel electrophoresis (PFGE) according to protocol previously described (Barrett 1994). Improvement of PFGE restriction patterns was achieved with addition of 50 μM thiourea to the running buffer (Koort JM 2002). The genetic relatedness of bacterial isolates by PFGE pattern was determined using the criteria of Tenover et al 1995 (Tenover FC 1995). *S. enterica* Enteritidis isolates were

sub-typed using the random amplified polymorphic DNA (RAPD) method as previously described (Hudson, Quist et al. 2000). The PCR primers and control strains were those described in earlier work (20). The designations given to the resulting patterns were assigned such that a RAPD pattern was considered unique if there was at least one band difference in the RAPD pattern (20).

Construction of cladogram. The characteristics PFGE pattern, phage type, 1283 RAPD pattern, and 1247 RAPD pattern were treated as discrete unrelated characteristics, and analyzed with the unweighted pair group method analysis (UPGMA) in the sequence type analysis and recombinational tests (START) program (Jolley KA 2001). These data were then used to construct a cladogram of the most likely relationship between discrete characters using nearest neighbor matching (Jolley KA 2001).

RESULTS

S. enterica isolated from poultry farms in northeast Georgia. *Salmonella* Enteritidis was isolated at least once from all flocks except flock number three. A total of one hundred eighty-seven drag-swab samples from seven flocks yielded nine *S.* Enteritidis isolates (4.8%). Thirteen of 280 pooled litter samples (4.6%) yielded *S.* Enteritidis isolates. Two hundred eighty carcass rinses yielded 3 isolates of *S.* Enteritidis (1.1%). One isolate was obtained from chick box-liners (0.002%). Of the drag-swab samples taken in each of the ten houses comprising the broiler breeder farm, *S.* Enteritidis was isolated from two houses (3/30 swabs, 10%). The ages of the flocks in the two *S.* Enteritidis positive houses were 38 and 47 weeks.

Genetic relatedness of *S.* Enteritidis isolated from poultry farms in northeast Georgia. All but one of 27 *S.* Enteritidis isolates was identified as phage type 8. The remaining isolate was classified as reaction-does-not-conform (RDNC) (Table 1.). Two PFGE patterns were obtained

with *Xba*1 endonuclease enzyme restriction of the isolates (Fig. 1.). The patterns designated E1.1 and E1.2, are closely related, having only two bands difference in their *Xba*1 macro-restriction patterns (Tenover, Arbeit et al. 1995). The *S.* Enteritidis PFGE type E1.1 was isolated from litter and drag swab samples from both houses, a mouse caught in house A, and from one post-chill carcass from a bird reared in house A (Table 1). The PFGE type E1.1 was isolated from flocks 1, 4, 5, 6, and 7. Molecular sub-typing of *S. enterica* Enteritidis PFGE type E1.1 by RAPD PCR using the primers designated 1283 and 1247, revealed nine related genetic sub-types, with one sub-type comprising 53% (9/17) of the Enteritidis E1.1 PFGE type isolates from this farm (Table 1). This dominant *S.* Enteritidis genetic sub-type had the typing combination: PFGE type E1.1 / Phage type 8 / 1283 RAPD type A / 1247 RAPD type CC, while the nine other PFGE-type E1.1 sub-types obtained were related or closely related (Table 1, Fig. 3).

S. enterica Enteritidis PFGE type E1.2 was isolated from litter and drag swab samples from house A during flocks 2 and 7, and from two pre-chill carcasses from flock 4. *Salmonella* Enteritidis PFGE type E1.2 was more prevalent in house A than PFGE type E1.1 (Table 1). Overall, PFGE type E1.1 was more prevalent than PFGE type E1.2 (65 % of *S.* Enteritidis isolations). Sub-typing of *S.* Enteritidis PFGE type E1.2 isolates with RAPD PCR using the primers designated 1283 and 1247 generated seven related genetic sub-types (Fig. 2, Table 1). Of the seven *S.* Enteritidis PFGE type E1.2 isolates obtained, two (25%), were of the typing combination: *S.* Enteritidis PFGE type E1.2 / Phage type 8 / 1283 RAPD type B / 1247 RAPD type AA, while the six other sub-typing combinations were related or closely related (Table 1, Fig. 3).

Our phage type 8 *S.* Enteritidis isolates shared a common PFGE type with two of three phage type 8 *S.* Enteritidis isolates (Hudson, Quist et al. 2000) from the *Salmonella* reference

collection which were used in this study for genetic comparisons and served as internal controls for reproducibility. Of these two *Salmonella* reference strains sharing the same PFGE type, neither produced RAPD patterns in common with our *S.* Enteritidis isolates (Table 1).

Overall, the 26 *S.* Enteritidis isolates were found to be of 16 related genetic sub-types using this combination of DNA typing methods. On phylogenetic analysis, the non-clonal isolates separated into two closely related major clades, each of which contained two sub-clades of related isolates (Fig. 3). The isolate obtained from the broiler breeder facility was determined to be only distantly related to the resident sub-type persisting on the broiler farm (Fig. 3). The broiler breeder farm isolate had distinct RAPD patterns when compared to the broiler farm isolates.

DISCUSSION

S. enterica Enteritidis was isolated from feces of day-old chicks entering the farm, the flock house environment, a mouse trapped in the broiler house, finished poultry carcasses from this farm, and from the environment of one of the broiler breeder farms supplying chicks to this farm. As demonstrated by molecular typing, *S.* Enteritidis colonized the poorly controlled rodent population on this farm, maintained itself within the house environment, and persisted from flock-to-flock. Twice we identified *S.* Enteritidis isolates from chicken carcasses and the poultry house environment with the same PFGE and RAPD-PCR typing patterns, confirming the origin of the carcass contamination with *S.* Enteritidis. Molecular typing of *S.* Enteritidis isolates from mice trapped in the broiler houses demonstrated that the resident strain is being maintained flock-to-flock in the mouse population on this farm. In addition, molecular typing of *S.* Enteritidis isolated from the feces of day-old chicks suggest that the *S.* Enteritidis strains on this

farm could have been introduced onto the farm from the breeder hens to the chicks supplying the farm.

Two closely related genetic types were identified among all of the *S.* Enteritidis isolates using pulsed field gel electrophoresis (PFGE). A disadvantage of this typing method is that it can only identify large changes in chromosomal DNA such as those produced by phage insertion, or large-scale insertions or deletions of DNA in the chromosome. In addition, it is known that *S.* Enteritidis is a clonal organism (20), as evidenced by the comparatively few unique PFGE patterns archived in the PFGE database PULSENET (REFS). Discrimination of smaller scale, DNA changes in *Salmonella* can be identified with the use of random amplification of polymorphic DNA, or RAPD-PCR method (Lin A. 1996; Laconcha I. 1998.). The ability to distinguish *Salmonella* strains within a PFGE type is crucial for understanding the ecology of *S.* Enteritidis in this system. By combining the molecular typing results from PFGE and RAPD, we subsequently increased the number of *S.* Enteritidis genetic sub-types identified to sixteen. Analysis of these *Salmonella* sub-types revealed a clonal, *S. Enteritidis* sub-population resident on this poultry farm. This result agrees with those from a previous study, which found that *S.* Enteritidis PT 8 isolates, from human illness in the United States, were closely related by PFGE profiles and hypothesized that this was attributed to the efficient spread genetically-related clusters of *S.* Enteritidis clones (Buchrieser C. 1997).

We identified a single *S.* Enteritidis clone which persisted unchanged through four of the seven flocks in the study period. The *S.* Enteritidis clone was also isolated from a mouse trapped in the broiler house, and from a carcass from a bird reared in this house. Analysis of the cladogram showed a group of related *S.* Enteritidis strains isolated contemporaneously with the resident *S.* Enteritidis clone. The heterogeneity of this cluster of related *S.* Enteritidis strains may

represent minor genetic changes occurring naturally over time in the resident *S.* Enteritidis population. Similarly, temporal genetic changes have been observed in closely-related *S.* Pullorum population (Dodson et al., 1999). Overall, the moderate diversity of RAPD types displayed among our *S.* Enteritidis isolates may represent minor genetic changes occurring over time in a single strain being maintained on this farm, as well as introduction of new strains from other sources. There are many opportunities for introduction of *Salmonella* into various levels of the commercial poultry production system, and if strains are persisting in various levels of the system, there will be genetic changes occurring over time in each of these resident *Salmonella* strains (Mauldin 2002). In addition, the *S.* Enteritidis strains present in different levels of the production system may ultimately be related to each other, having been derived from *Salmonella* strains coming into each level of the system from above.

The *S.* Enteritidis isolate obtained from the broiler breeder environment was a genetic type more distantly related to the *S.* Enteritidis population present in the broiler houses (Figure 3). This is not surprising, considering that the broiler breeder flock from which this *S.* Enteritidis strain was isolated could not have contributed to the make-up of any of the flocks sampled during the study period, as this flock was sampled more than a year after sampling of the broiler flock had ended, and broiler breeder flocks are turned over at 47 weeks of age. This *S.* Enteritidis strain may represent a progenitor *Salmonella* genetic type, or a *S.* Enteritidis strain that co-evolved with our resident *Salmonella* strain from a common source, or may be unrelated. Isolation of *S. enterica* Enteritidis from the broiler breeder facility and from chick-box liners does indicate systemic contamination with *S. enterica* Enteritidis in this integrated system.

Phage typing showed all of the *S. enterica* Enteritidis isolates as being phage-type 8, a phage type common to layer operations in the eastern United States (Ebel 1992). Although

generally associated with layer operations (Ebel 1993), *S. enterica* Enteritidis phage types 4, 8, and 13 have been isolated from broiler chicken carcasses in the United States, Canada, and other countries (Boonmar s. 1998.; Limawongpranee, Hayashidani et al. 1999; Kusunoki J. 1999.; White, Zhao et al. 2001; Gradel 2003). Although *S. enterica* Enteritidis phage types 4 and 8 are commonly associated with salmonellosis from consuming contaminated eggs, their potential for causing human illness from consumption of contaminated poultry-based foods is documented in reports of both sporadic cases and outbreaks of food-borne illness associated with consumption of poultry (Rampling, Anderson et al. 1989; Rampling A. 1989.; Mead, Slutsker et al. 1999; Olsen, Bishop et al. 2001; Gradel K 2003). Outbreaks of salmonellosis are linked to consumption of undercooked contaminated foods, cross-contaminated foods, and foods stored with inadequate refrigeration. It follows then, that whole chicken carcasses contaminated with *S. enterica* Enteritidis should pose an equal threat of foodborne illness if handled incorrectly as chicken carcasses contaminated with other salmonella serotypes (Rampling A. 1989.).

There are many possible sources for *Salmonella* contamination in poultry production, including the farm environment, feed, rodents, insects, the hatchery, and the parental line (Davies 2003). Sub-typing of *Salmonella* isolates obtained from the broiler breeder farm supplying chicks to this broiler farm, we concluded that the *S.* Enteritidis contamination of poultry carcasses is a reflection of the systemic contamination of this integrated production company. We documented transmission and maintenance of *S.* Enteritidis through integrated poultry production by using the combination of PFGE typing, phage typing, and RAPD typing to identify and track individual *Salmonella* strains. Using molecular typing, we were able to observe both horizontal and vertical transmission of *S.* Enteritidis in integrated broiler production in northeast Georgia. We identified isolates of the same *S.* Enteritidis PFGE genetic types from

the house environment (litter, drag swabs, mice), and carcass rinses throughout the year-long study period. One of the strains was isolated from environmental samples from the broiler breeder farm supplying chicks for placement on this farm, suggesting that these *S.* Enteritidis strains could have been introduced into the farm via vertically infected chicks from the hatchery. We demonstrated that these related strains were present in the mice in the flock houses, and that they persisted from flock-to-flock, and were carried through processing to the finished product.

Salmonella Enteritidis was consistently isolated from this farm during the course of one year, in contrast to the dynamic and volatile changes in *S. enterica* serotypes observed over time on other broiler farms (Caldwell 1995; Baiely J.S. 2002.). This may be due to the level of *Salmonella* contamination and degree of mouse infestation on this farm compared to other farms which have been studied. We have shown that the resident *S.* Enteritidis strain on this study farm can be isolated from the mouse reservoir. Mice have been established as reservoirs of *Salmonella* in previous studies (Henzler 1992.; Davies 1995.). This suggests that mice could be trapped and used as environmental sentinels by monitoring the *Salmonella* serotypes isolated. The level of environmental contamination might also be reflected in the frequency of S*almonella* isolation from mice trapped in flock houses. If mice in this environment are maintaining *Salmonella* serotypes or strains from flock to flock, then it is therefore crucial to control this vector in both the broiler breeder houses and the broiler houses.

To be able to effectively reduce contamination of poultry products with *Salmonella*, we must be able to pinpoint the origins of *Salmonella* contamination within the integrated production system. The success of any intervention strategy will require continuous monitoring of both the poultry production environment and the finished product for levels of *Salmonella* contamination, because *Salmonella* can potentially enter at numerous points into the integrated poultry

production system. The key to control of carcass contamination may ultimately rest at the level of controlling the level of S*almonella* infection of the breeder broiler flocks.

ACKNOWLEDGEMENTS

This work was supported by a grant from the United States Department of Agriculture (99-35212-8680). The State of Georgia Veterinary Medical Experimental Station supported KAL.

REFERENCES

Angulo, F., and D. Swerdlow. 1998. *Salmonella enteritidis* infections in the United States. JAVMA 213:1729-1731.

Baiely J.S., N. A. C., S.E. Craven, D.E. Cosby. 2002. Serotype tracking of *Salmonella* through integrated broiler chicken operations. J Food Prot 65:742-745.

Bailey, J. S., N. J. Stern, P. Fedorka-Cray, S. E. Craven, N. A. Cox, D. E. Cosby, S. Ladely, and M. T. Musgrove. 2001. Sources and movement of *Salmonella* through integrated poultry operations: a multistate epidemiological investigation. J Food Prot 64:1690-7.

Barrett, T. J., H. Lior, J. H. Green, R. Khakhria, J. G. Wells, B. P. Bell, K. D. Greene, J. Lewis, and P. M. Griffin. 1994. Laboratory investigation of a multistate food-borne outbreak of *Escherichia coli* O157:H7 by using pulsed-field gel electrophoresis and phage typing. J. Clin. Microbiol. 32:3013-3017.

Bhatia T.R., G. D. M., H. Wyman, G.P. Nayar. 1979. Salmonella isolation from litter as an indicator of flock infection and carcass contamination. Avian Dis 24:838-847.

Boonmar s., A. B., s. Pornrunangwong, N. Marnrim, K. Kaneko, M. Ogawa. 1998. *Salmonella* in broiler chickens in Thailand with special reference to contamination of retail meat with *Salmonella enteritidis*. J Vet Med Sci 60:1233-1236.

Boyce T.G., D. K., D.L. Swerdlow, T.M. Gomez, B. Serrano, L.N. Nickey,F.W. Hickman-Brenner, G.B. Malcolm, P.M. Griffin. 1996. Recurrent outbreaks of *Salmonella enteritidis* infections in a Texas restaurant: Phage type 4 arrives in the United States. Epidemiol Infect 117:29-34.

Buchrieser C., R. B., O. Buchrieser, A. Kristl, J.B. Luchansky, C.W. Kaspar. 1997. Genomic analyses of Salmonella enteritidis phage type 4 strains from Austria and phage type 8 strains from the United States. Zentralbl Bakteriol 285:379-388.

Byrd, J. A., J. R. DeLoach, D. E. Corrier, D. J. Nisbet, and L. H. Stanker. 1999. Evaluation of *Salmonella* serotype distributions from commercial broiler hatcheries and grower houses. Avian Dis 43:39-47.

Caldwell, D., BM Hargis, DE Corrier, L Vidal, JR DeLoach. 1995. Evaluation of persistence and distribution of *Salmonella* serotype isolation from poultry farms using drag-swab sampling. Avian Dis 39:617-621.

Davies, R. H., and M. Breslin. 2003. Persistence of *Salmonella enteritidis* phage type 4 in the environment and arthropod vectors on an empty free-range chicken farm. Environ. Microbiol. 5:79-84.

Davies, R. H., C. Wray. 1995. Mice as carriers of *Salmonella enteritidis* on persistently infected poultry units. Vet Rec 137:337-341.

Dickens, J. A., N.A. Cox, J.S. Bailey, J.E. Thomson. 1985. Automated microbiological sampling of broiler carcasses. Poult Sci 64:1116 - 1120.

Ebel, E. D., J. Mason, L. A. Thomas, K. E. Ferris, M. G. Beckman, D. R. Cummins, L. Scroeder-Tucker, W. D. Sutherlin, R. L. Glasshoff, and N. M. Smithhisler. 1993. Occurrence of

Salmonella enteritidis in unpasteurized liquid egg in the United States. Avian Dis. 37:135-142.

Ebel, E. D., M. J. David, and J. Mason. 1992. Occurrence of *Salmonella enteritidis* in the U. S. commercial egg industry: report on a national spent hen survey. Avian Dis. 36:646-654.

Gradel K, R. E. 2003. A questionnaire-based retrospective field study of persistence of *Salmonella entertidis* and *Salmonella typhimurium* in Danish broiler houses. Preventative Vet Med 56:267-284.

Gradel, K. O., E. Rattenborg. 2003. A questionnaire-based, retrospective field study of persistence of *Salmonella enteritidis* and *Salmonella typhimurium* in Danish broiler houses. Prev Vet Med 56:267-284.

Henzler, D. J., H.M. Opitz. 1992. The role of mice in the episootiology of *Salmonella enteritidis* infection on chicken layer farms. Avian Dis 36:625-631.

Hong, Y., T. Liu, C. Hofacre, M. Maier, S. Ayers, D. G. White, L. Wang, and J. J. Maurer. 2003. A restriction fragment length polymorphism based polymerase chain reaction as an alternative to serotyping for identifying *Salmonella* serotypes. Avian Dis 47:387-395.

Hudson, C., C. Quist, M. Lee, K. Keyes, S. Dodson, C. Morales, S. Sanchez, D. White, and J. Maurer. 2000. The genetic-relatedness of *Salmonella* from non-domestic birds in the Southeastern United States. J Clin Microbiol 38:1860 - 1865.

Jolley KA, F. E., Chan MS, Maiden MC. 2001. Sequence type analysis and recombinational tests (START). Bioinformatics 17:1230-1231.

Koort JM, L. S., Rantala M, Unikila E, Siitonen A. 2002. Technical improvement to prevent DNA degradation of enteric pathogens in pulsed-field gel electrophoresis. J Clin Microbiol 40:3497-3498.

Kusunoki J., A. K., Y. Yanagawa, C. Monma, M. Shingaki, H. Obata, T. Itoh, K. Ohta, Y. Kudoh, A. Nakamura. 1999. Biochemical and molecular characterization of *Salmonella* ser. *enteritidis* phage type 1 isolated from food poisoning outbreaks in Tokyo. Kansenshogaku Zasshi 73:437-444.

Laconcha I., N. L.-M., A. Rementeria, A. Audicana, I. Perales, J. Garaizar. 1998. Phage typing combined with pulsed-field gel electrophoresis and random amplified polymorphic DNA increases discrimination in the epidemiological analysis of *Salmonella enteritidis* strains. Int J Food Microbiol 40:27-34.

LaHellec C., P. c., G. Bennejean. 1986. Influence of resident *Salmonella* on contamination of broiler flocks. Poult Sci 65:2034-2039.

Limawongpranee, S., H. Hayashidani, A. T. Okatani, K. Ono, C. Hirota, K. Kaneko, and M. Ogawa. 1999. Prevalence and persistence of *Salmonella* in broiler chicken flocks. J Vet Med Sci 61:255-9.

Lin A., M. A. U., T.J. Barrett, R.A. Goldsby. 1996. Application of random amplified polymorphic DNA analysis to differentiate strains of Salmonella enteritidis. J Clin Microbiol 34:870-876.

Lopes VC, V. B., Halvorson DA, Lauer DC, Gast R, Nagaraja KV. 2004. Comparison of methods for differentiation of *Salmonella enterica* serovar Enteritidis phage type 4 isolates. Am J Vet Res 65:538-543.

Mauldin, J. M. 2002. The breeder and hatchery industries, p. 623-775. *In* W. W. Bell DD (ed.), Commercial Chicken Meat and Egg Production, 5 ed. Kluwer Academic Publishers, Norwell Park.

Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the United States. Emerg Infect Dis 5:607-25.

Olsen S.J., R. B., F.W. Brenner, T.H. Roels, N. Bean, R.V. Tauxe, L. Slutsker. 2001. The changing epidemiology of Salmonella: Trends in serotypes isolated from humans in the United States, 1987-1997. J Infect Dis 183:753-761.

Olsen, S. J., R. Bishop, F. W. Brenner, T. H. Roels, N. Bean, R. V. Tauxe, and L. Slutsker. 2001. The changing epidemiology of *Salmonella*: trends in serotypes isolated from humans in the United States, 1987-1997. J Infect Dis 183:753-61.

Tauxe, R. 1991. Salmonella: A postmodern pathogen. J Food Prot 54:563-568.

Rampling, A., J. R. Anderson, R. Upson, E. Peters, L. R. Ward, and B. Rowe. 1989. *Salmonella enteritidis* phage type 4 infection of broiler chickens: a hazard to public health. Lancet 2:436-8.

Rampling A., J. R. A., R. Upson, E. Peters, L.R. Ward, B. Rowe. 1989. Salmonella enteritidis phage type 4 infection of broiler chickens: A hazard to public health. The Lancet August:436-438.

Sanchez, S., C. L. Hofacre, M. D. Lee, J. J. Maurer, and M. P. Doyle. 2002.J. Am. Vet. Assoc. 221:492-497. 2002. Animals sources of human salmonellosis. J. Am. Vet. Assoc. 221.

Schlosser W., A. H., E. Ebel, B. Rose, R. Umholtz, K. Ferris, W. James. 2000. Analysis of Salmonella serotypes from selected carcasses and raw ground products sampled prior to implementation of the pathogen reduction; Hazard analysis and critical control point final rule in the US. Int J Food Microbiol 58:107-111.

Tenover, F., R. Arbeit, R. Goering, P. Mickelson, B. Murray, D. Persing, and B. Swaminathan. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: Criteria for bacterial strain typing. J Clin Microbiol 33:2233 - 2239.

Tenover FC, A. R., Goering RV, Mickelson PA, Murray BE, Persing DH, Swaminathan B. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: Criteria for bacterial strain typing. J Clin Microbiol 33:2233 - 2239.

Waltman WD, C. P., T. Dickson. 1991. Use of delayed secondary enrichment for the isolation of Salmonella in poultry and poultry environments. Avian Dis 35:88-92.

White, D. G., S. Zhao, R. Sudler, S. Ayers, S. Friedman, S. Chen, P. F. McDermott, S. McDermott, D. D. Wagner, and J. Meng. 2001. The isolation of antibiotic-resistant salmonella from retail ground meats. N Engl J Med 345:1147-54.

Table 1.

Table 1. Molecular sub-typing scheme for *S.* Enteritidis isolates using phage typing, RAPD PCR, and PFGE.

Isolate Number	Collection Date	Flock Number	House Number	Sample Type	Phage Type	1283 RAPD[3]	1247 RAPD[3]	*Xba*I PFGE[4]
298	4/28/00	1	A	chick box liner	8	E	AA	E1.1
679	6/22/00	2	A	drag swab	8	C	AA	E1.2
680	6/22/00	2	A	drag swab	8	B	AA	E1.2
928	7/17/00	2	A	litter	8	B	BB	E1.2
1876	12/6/00	4	A	prechill carcass	RDNC[1]	B	AA	E1.2
1877	12/6/00	4	A	prechill carcass	8	A	CC	E1.2
1908	12/6/00	4	B	postchill carcass	8	A	CC	E1.1
2132	1/11/01	5	A	drag swab	8	A	CC	E1.1
2133	1/11/01	5	A	drag swab	8	D	DD	E1.1
2403	3/22/01	6	B	litter	8	B	AA	E1.1
2404	3/22/01	6	B	litter	8	B	BB	E1.1
2604	3/22/01	6	B	litter	8	B	II	E1.1
2624	4/19/01	6	A	drag swab	8	A	CC	E1.1
2822	6/4/01	7	A	drag swab	8	A	CC	E1.1
2824	6/4/01	7	A	drag swab	8	A	CC	E1.1
2830	6/4/01	7	A	drag swab	8	A	FF	E1.2

Table 1. Continued.

Isolate Number	Collection Date	Flock Number	House Number	Sample Type	Phage Type	1283 RAPD[3]	1247 RAPD[3]	*Xba*I PFGE[4]
2833	6/4/01	7	B	litter	8	A	EE	E1.1
2837	6/4/01	7	A	litter	8	A	GG	E1.2
3003	6/20/01	7	A	drag swab	8	A	CC	E1.1
3067	7/2/01	7	A	drag swab	8	A	CC	E1.1
3074	7/2/01	7	A	litter	8	A	CC	E1.1
3076	7/2/01	7	A	litter	8	A	GG	E1.1
3078	7/2/01	7	A	litter	8	A	HH	E1.1
3115	7/2/01	7	A	mouse	8	A	CC	E1.1
707sc[5]	9/26/03	NA	5	drag swab	8	L	PP	E1.2
SE5[6]				egg yolk	8	J	JJ	E5.1
SE15[6]				poultry	8	K	JJ	E1.1
SARB17[6]				poultry	RDNC	G	KK	F1.1
SARB18[6]				poultry	4	H	LL	E3.1
SARB19[6]				egg yolk	RDNC	I	MM	E4.1
SR11[6,7]				NA	U302	F	OO	G1.1
χ3227[6]				poultry	8	A	NN	E1.1

Table 1. Continued: Footnotes.

[1]Reaction Does Not Conform.

[2]Not applicable.

[3]1283 and 1247 RAPD patterns were assigned a unique alphabetical designation if there was a single DNA band difference in the electrophoresis profile (20).

[4]PFGE patterns were assigned alphabetical and numerical designations that correspond to their genetic relatedness, as defined by Tenover et al (39) and as described by Hudson et al (20).

[5]Sample taken from broiler-breeder chicken farm.

[6]*S.* Enteritidis, control strains for RAPD typing (20).

[7]*S.* Typhimurium, control for gel-to-gel reproducibility of RAPD and PFGE patterns.

Figure 1.

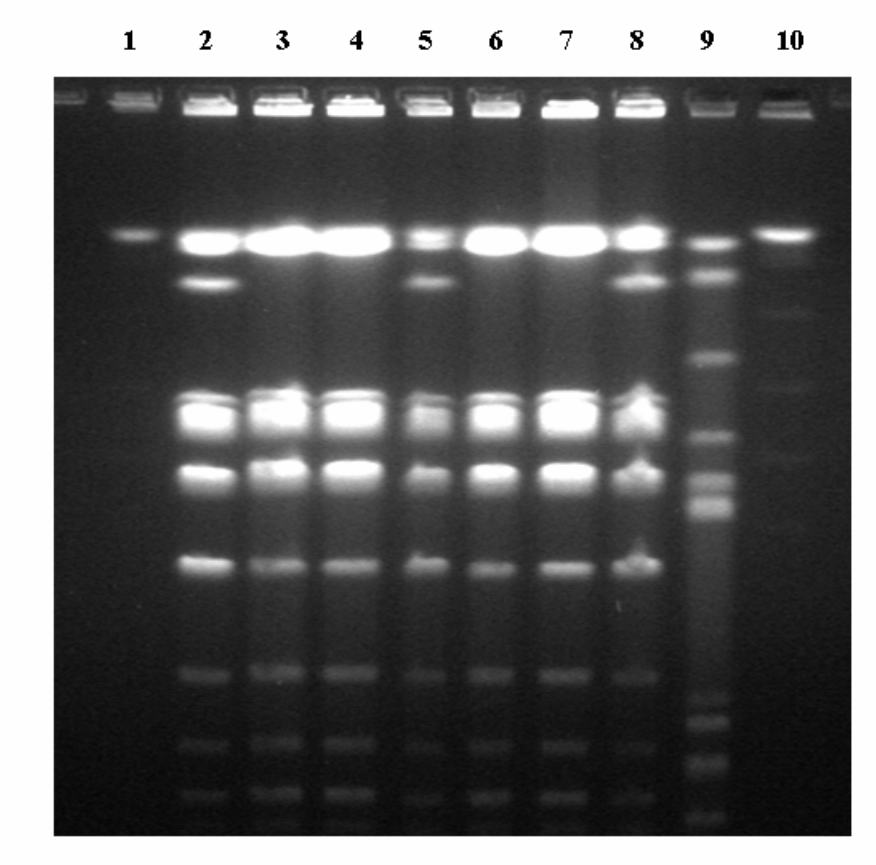

Figure 2.

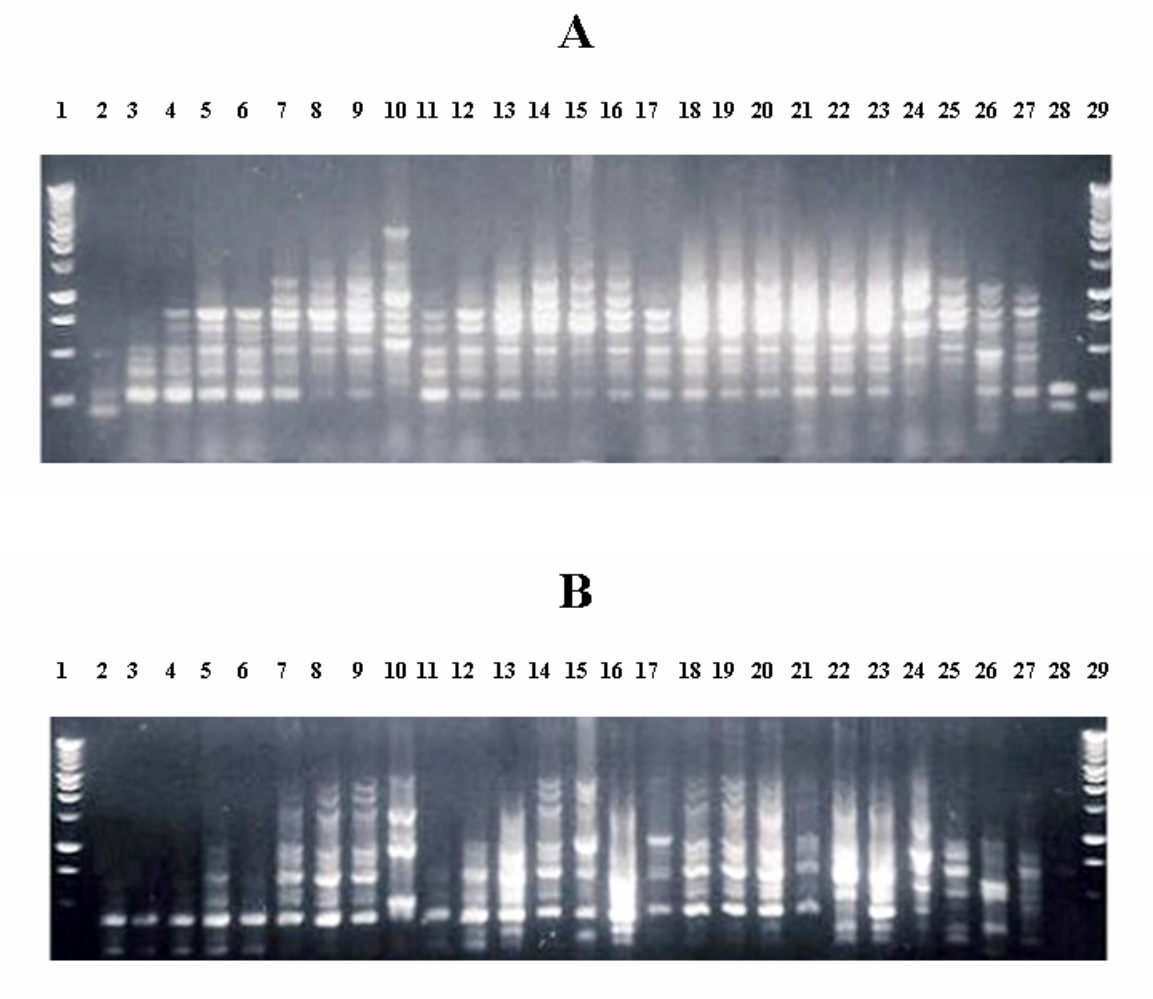
A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29

Figure 3.

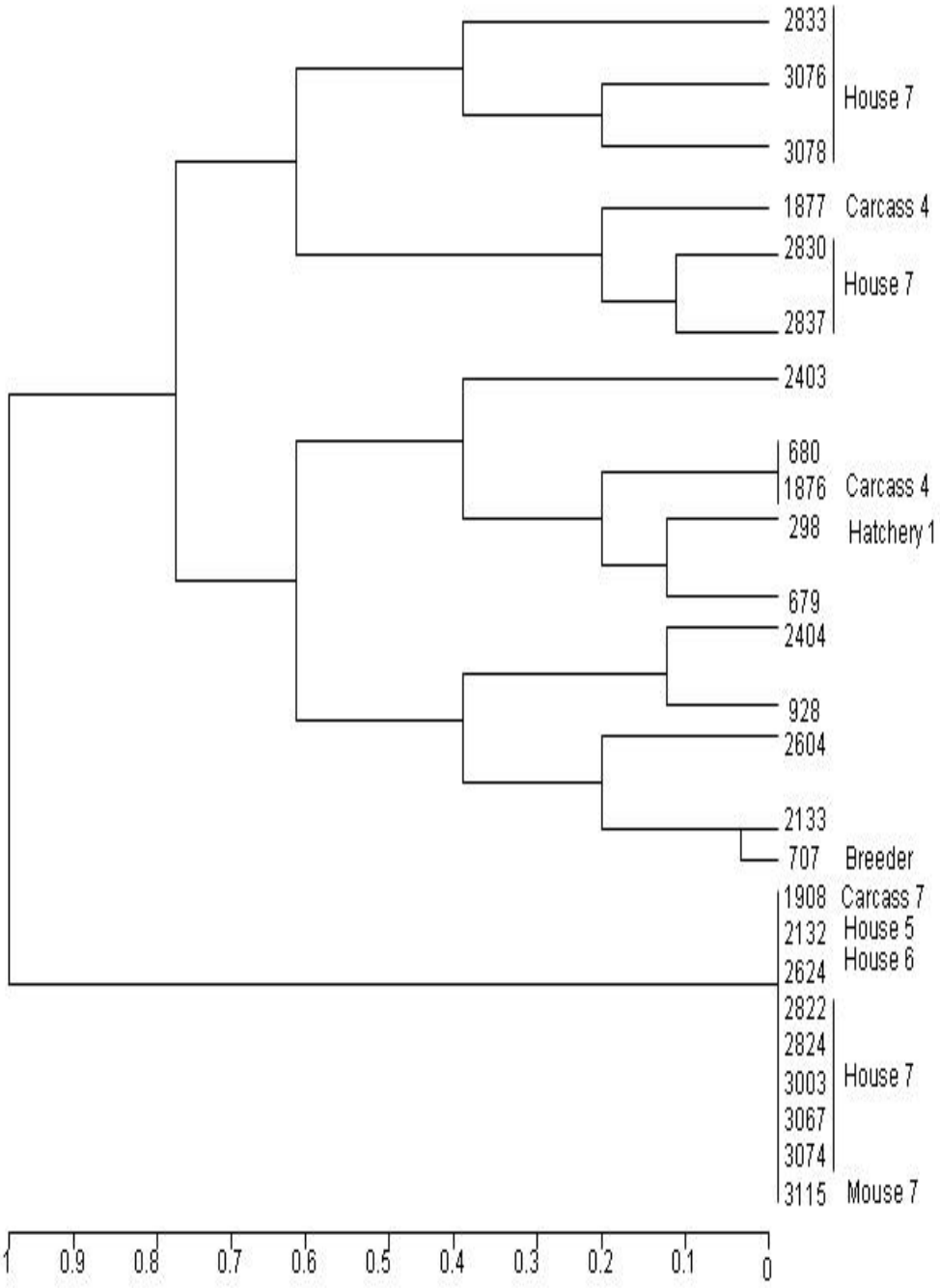
2833
3076
House 7
3078
1877 Carcass 4
2830
House 7
2837
2403
680
1876 Carcass 4
298 Hatchery 1
679
2404
928
2604
2133
707 Breeder
1908 Carcass 7
2132 House 5
2624 House 6
2822
2824
3003 House 7
3067
3074
3115 Mouse 7
1 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0

FIGURE LEGENDS

Figure 1. *Salmonella* Enteritidis genetic types identified by PFGE using restriction endonuclease *Xba* I. *S. cerivisiae* molecular weight standard (lanes 1, 10), *S.* Enteritidis: broiler-breeder flock isolate 707 (lane 2); hatchery isolate 298 (lane 3); broiler chicken flock isolates 2833 (lane 4) and 2837 (lane 5); mouse isolate 3115 (lane 6), broiler chicken carcass isolates 1908 (lane 7) and 1877 (lane 8); and *S.* Typhimurium SR11 control (lane 9). *S.* Enteritidis PFGE types E1.1 (lanes 3, 4, 6, and 7) and E1.2 (lanes 2, 5, and 8).

Figure 2. *Salmonella* Enteritidis genetic sub-typing using RAPD PCR. *Salmonella* isolates representative of the different patterns obtained are shown. **(A)** 1283 primer. *S.* Enteritidis isolates; 298,679, 680, 928, 1876, 1877, 1908, 2132, 2133, 2403, 2404, 2822, 2824, 2830, 2833, 2837, 3003, 3067, 3074, 3076, 3078, 3115, (lanes 2–23), *S.* Typhimurium SR11 control (lane 24), *S.* Enteritidis SARB control strains 17, 18, 19 (lanes 25–27), sterile water control (lane 28), and 1 kb molecular weight ladder (lanes 1, 29). **(B)** 1247 primer. *S.* Enteritidis isolates; 298,679, 680, 928, 1876, 1877, 1908, 2132, 2133, 2403, 2404, 2822, 2824, 2830, 2833, 2837, 3003, 3067, 3074, 3076, 3078, 3115, (lanes 2–23), *S.* Typhimurium SR11 control (lane 24), *S.* Enteritidis SARB control strains 17, 18, 19 (lanes 25–27), sterile water control (lane 28), and 1 kb molecular weight ladder (lanes 1, 29).

Figure 3. Cladogram illustrating genetic-relatedness of *S.* Enteritidis poultry isolates determined using the DICE coefficient of similarity and UPGMA. The phage type, PFGE type, 1247 RAPD type, and 1283 RAPD type were used as non-weighted discrete characteristics in determining the degree of relatedness of study isolates. The degree of relatedness between groups of *S.* Enteritidis isolates is indicated on the horizontal scale. Sample type and flock

number are indicated for selected isolates on the right hand side of the isolate number on the cladogram.

CHAPTER 6

DISCUSSION

Ecology of *Salmonella* in Integrated Broiler Production:

By monitoring *Salmonella* contamination during the production of seven consecutive flocks on two commercial broiler farms, we identified a variety of *S. enterica* serotypes including those commonly associated with poultry in general (Poppe 1995; Bailey, Stern et al. 2001; Sarwari, Magder et al. 2001), other food animal species (Letellier 1999; Dargatz 2000; Anderson 2001), as well as human illnesses (Olsen, Bishop et al. 2001). Although we observed the same *Salmonella* serotypes, their prevalence varied between farms in this study, as compared to the results from other *Salmonella* regional and national studies (Anonymous 2001).

Although serotyping has been useful to differentiate among *Salmonellae*, it is not discriminatory enough to pin-point the source of a common *Salmonella* serotype like *S.* Typhimurium within a food-production system. While this approach has been useful in our understanding the flow of *Salmonella* within integrated poultry production system (Bailey et al., 2001), it cannot determine whether a *S. enterica* strain is transient or endemic, which is important to know in breaking the flow of *Salmonella* to the finished product. We therefore used PFGE to discern genetic differences among *S. enterica* serotypes, in order to trace-back *Salmonella* within this system and determine where in the poultry production pyramid, a strain might be endemic.

Despite the diversity *of Salmonella* genetic types that have been reported for some of the *S. enterica* serotypes identified in this study (Hoszowski 2001; Sander, Hudson et al. 2001), a single strain was observed for several of the *Salmonella* serotypes isolated on both broiler chicken farms. The *S. enterica* serotypes Typhimurium and Enteritidis isolated from *Salmonella*-contaminated poultry carcasses from Farm One shared the same, indistinguishable PFGE patterns as those isolated from the environment, in which the flocks were reared, as well as isolates from rodents caught in the same flock houses. The poorly controlled rodent population on this farm probably maintains *Salmonella* within the house environment, thereby enabling these serotypes to persist and colonize birds from flock-to-flock (Davies 2003).

Indistinguishable PFGE types of *S.* Typhimurium, *S.* Enteritidis, and *S.* Heidelberg were isolated from processed chicken carcasses, the broiler chicken environment, and chick-box liners, implicating the hatchery as the source for these persistent serotypes on Farm One. Investigation of Company A's breeder farms, producing eggs for chicks to be placed on Farm One, yielded isolates of *S.* Typhimurium and *S.* Enteritidis with the same PFGE patterns as those *Salmonella* isolates resident on Farm One. Thus, vertical transmission from breeders to the broilers appeared to be the ultimate source of the resident *Salmonella* serotypes identified in this integrated production system. With this scenario in mind, it follows then, that prevention of *in ovo* or early infection of broiler chicks with *Salmonella* strains should ultimately reduce carcass contamination with *Salmonella* (Lahellec, Colin et al. 1986). This underscores the need for *Salmonella* monitoring and control at all levels of the integrated production system.

Enteritidis isolates from chicken carcasses and the poultry house environment with the same PFGE and RAPD-PCR typing patterns, confirming the origin of the carcass contamination with *S.* Enteritidis. Molecular typing of *S.* Enteritidis isolates from mice trapped

in the broiler houses demonstrated that the resident strain is being maintained flock-to-flock in the mouse population on this farm. In addition, molecular typing of *S.* Enteritidis isolated from the feces of day-old chicks suggest that the *S.* Enteritidis strains on this farm could have been introduced onto the farm from the breeder hens to the chicks supplying the farm.

We documented transmission and maintenance of *S.* Enteritidis through integrated poultry production by using the combination of PFGE typing, phage typing, and RAPD typing to identify and track individual *Salmonella* strains. Using molecular typing, we were able to observe both horizontal and vertical transmission of *S.* Enteritidis in integrated broiler production in northeast Georgia.

Poultry Salmonella Antiobitic Resistance:

Combining isolates from both farms, 61.9 percent were pan-sensitive to a panel of eighteen antimicrobials used in the National Antimicrobial Resistance Monitoring Service (NARMS) surveillance. Resistance to streptomycin, alone, and in combination with other resistances, was the most common (36.3%) antibiotic resistance phenotype observed. Combinations of resistance against streptomycin, gentamicin, sulfamethoxazole, trimethoprim, and tetracycline, were observed for a variety of *S. enterica* serotypes and PFGE genetic types. Similar to our results, the 2002 NARMS retail meat survey reported that *Salmonella* isolated from chicken were largely pan-susceptible (66.6%), or resistant to the antibiotics: sulfamethoxazole (18.7%), streptomycin (32.3%), gentamicin (3.4%), ampicillin (5.1%), trimethoprim (1.7%), and tetracycline (34.3%) (White DG 2001). In addition, the majority of the 1,526 NARMS 2001 *Salmonella* isolates were sensitive to all antimicrobials tested (51.6%), with resistance to: tetracycline (26.7%), streptomycin (23.7%), sulfamethoxazole (9.1%), gentamicin (6.3%), and ampicillin (15.1%), the most prevalent antibiotic resistances (Anonymous 2001).

The antibiotic resistance phenotypes most frequently observed for 2001 NARMS poultry *Salmonella* isolates are also very similar to those observed in the poultry isolates from this study: GEN, STR, SMX (1.1%), TET, SMX (1.5%), STR, SMX, TET (3.2%), STR, TET (3.8%), and TET alone (5.5%) (Anonymous 2001).

A diversity of antibiotic resistance phenotypes (n = 9) was observed among the few *S.* Typhimurium genetic types identified by PFGE. Combinations of resistance against streptomycin, gentamicin, sulfamethoxazole, and tetracycline accounted for 85.3% of *S.* Typhimurium resistance phenotypes observed (Table 3).

The *S.* Typhimurium PFGE subtype T1.1 from Farm One was identified as phage type (PT) 193, a phage type commonly associated with illnesses in humans (van Leeuwen 1982; Robins-Browne 1983; Thorton 1993; Asensi 1995; Baggesen 1998; Pontello 1998; Ang-Kucuker 2000; Nastasi 2000; Threlfall 2000; Cruchaga 2001; Hannu 2002). The other *S.* Typhimurium PFGE types, T1.2 and T1.3 were identified as phage types DT107 and U302, respectively. Our *S.* Typhimurium phage types DT107 and DT193 appear to be genetically-related as determined by PFGE (Tenover FC 1995). Close genetic-relatedness as determined by PFGE among different *S.* Typhimurium and *S.* Enteritidis phage types has been reported by others (Hudson CR 2000; Olsen, Bishop et al. 2001). *S. enterica* Typhimurium U302 is a phage-type that has been associated with multidrug resistance, having the same complement of antibiotic resistance genes present in DT104 (Cruchaga 2001; Walker 2001), however, we identified only one isolate from this study, having similar multi-resistance pattern: AMP, GEN, STR, SMX but CHL-sensitive, with the remainder of these isolates pan-susceptible. There was a statistically significant association, at $\alpha = 0.05$, between *S.* Typhimurium genetic types and antibiotic resistance for the antibiotic ampicillin (T1.3> T1.1, T1.2). There was no significant difference in resistance

phenotypes between the three *S.* Typhimurium genetic types isolated from Farm One with the exception that PFGE type T1.3 was significantly more likely to be resistant to ampicillin. Fitting the generalized linear model for the three *S.* Typhimurium genetic types, there was a significant difference with regards to the total level of antibiotic resistance, from most-to-least susceptible: T1.3 ($\mu_i = 0.80$); T1.2 ($\mu_i = 0.74$); and T1.1 ($\mu_i = 0.62$).

There was statistically significant difference and ranking among *S. enterica* serotypes from a given poultry farm, with regards to antibiotic susceptibility to gentamicin, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim. Differences in the antibiotic susceptibility profiles observed among certain *Salmonella* serovars or strains may reflect variation in mobile genetic elements that ferry these antibiotic resistance genes within this environment. This integron was present in 83.1% of salmonellae isolated from poultry environment. This prevalence is similar to earlier observations for *Salmonella* isolated from multiple poultry companies (Goldstein et al., 2001). However, there was disparity in the distribution of this genetic element among *Salmonella* serovars, in part explaining higher antibiotic susceptibility observed for *S.* Enteritidis compared to the other *Salmonella* serotypes.

The presence of the class 1 integrase *intI1*, a marker of class 1 integrons, was positively associated with presence of resistance to the following antibiotics: gentamicin ($p = 0.001$), streptomycin ($p = 0.020$), sulfamethoxazole ($p = 0.013$), trimethoprim/sulfamethoxazole ($p = 0.015$), and ampicillin ($p = 0.020$). The association of these particular resistance phenotypes with the presence of the class 1 integron is in agreement with antibiotic resistance gene cassette(s) present within this element (Fluitt and Schmitz, 1999). The most prevalent resistances observed in this study, STR, GEN, and SMX, were statistically positively associated with the presence of *intI1* ($p < 0.05$), and consistent with the most common class 1 integron

antimicrobial resistance gene cassettes found in the *Enterobacteriaceae* (Fluitt 1999; White 2001).

The prevalence of resistance to streptomycin was surprisingly high, considering that streptomycin is not used in poultry production, but can be explained by the high carriage (62.0%) of transposon Tn*21* observed among our streptomycin-resistant isolates.

We have identified the origins of *Salmonella* contamination within the poultry production system, and identified *Salmonella* strains endemic within this system. In this study, we analyzed the physical and temporal patterns of antibiotic resistance in *Salmonella* serotypes isolated from this same system. Analysis of the temporal and physical distribution of the endemic serotypes previously identified with their antibiotic phenotypes, suggests that prevention of antimicrobial resistant *Salmonella* on poultry products is dependent on prevention of vertical transmission of *Salmonella* in the production system.

LITERATURE CITED

Aarestrup, F., A. Seyfarth, et al. (2001). "Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal enterococci from food animals in Denmark." Antimicrob Agents and Chemotherap **45**(2054-2059).

Anderson, R. J., J. K. House, B. P. Smith, H. Kinde, R. L. Walker, B. J. Vende Steeg, and R. E. Breitmeyer (2001). "Epidemiologic and biological characteristics of salmonellosis in three dairy herds." JAVMA **219**: 310-322.

Ang-Kucuker, M., V. Tolun, R. Helmuth, W. Rabsch, O. Buyukbaba-Boral, D. Torumkuney-Akbulut, S. Susever, and O. Ang (2000). "Phage types, antibiotic susceptibilities and plasmid profiles of *Salmonella typhimurium* and *Salmonella enteritidis* strains isolated in Istanbul, Turkey." Clin. Microbiol. Infect. **6**: 593-599.

Anglim, A. M., B. Klym, et al. (1997). "Effect of a vancomycin restriction policy on ordering practices during an outbreak of vancomycin-resistant Enterococcus faecium." Arch Intern Med **157**(10): 1132-6.

Angulo, F. and D. Swerdlow (1998). "*Salmonella enteritidis* infections in the United States." JAVMA **213**(12): 1729-1731.

Anonymous (1984). "The role of food safety in health and development. Report of a Joint FAO/WHO Expert Committee on Food Safety." World Health Organ Tech Rep Ser **705**: 1-79.

Anonymous (1998). Difco Manual, 11th Edition., Difco Laboratories, Division of Becton Dickinson and Co., Sparks, MD.

Anonymous (1999). Food-animal production practices and drug use. The use of drugs in food animals: benefits and risks. T. N. R. Council. Washington D.C., National Academy Press**:** 27-69.

Anonymous (2000). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Villanova PA, National Committee for Clinical Laboratory Standards.

Anonymous (2000). Performance standard for antimicrobial disk susceptibility tests - seventh edition. Villanova PA, National Committee for Clinical Laboratory Standards.

Anonymous (2001). "National antimicrobial susceptibillity monitoring program for veterinary and human isolates, United States Food and Drug Administration."

Asensi, M. D., A. P. Costa, E. Moura, E. M. dos Reis, and E. Hofer (1995). " Lysotypes and plasmidial profile of *Salmonella* serovar typhimurium isolated from children with enteric processes in the cities of Rio de Janeiro, RJ, and Salvador, BA-Brazil." Rev. Inst. Med. Trop. Sao Paulo. **37**: 297-302.

Baggesen, D. L., and F. M. Aarestrup (1998). "Characterization of recently emerged multiple antibiotic-resistant *Salmonella* enterica serovar Typhimurium DT104 and other multiresistant phage types from Danish pig herds." Vet. Rec. **143**: 95-97.

Baggesen, D. L., D. Sandvang, et al. (2000). "Characterization of Salmonella enterica serovar typhimurium DT104 isolated from Denmark and comparison with isolates from Europe and the United States." J Clin Microbiol **38**(4): 1581-6.

Baggesen, D. L. and H. C. Wegener (1994). "Phage types of Salmonella enterica ssp. enterica serovar typhimurium isolated from production animals and humans in Denmark." Acta Vet Scand **35**(4): 349-54.

Baiely J.S., N. A. C., S.E. Craven, D.E. Cosby. (2002.). "Serotype tracking of *Salmonella* through integrated broiler chicken operations." J Food Prot **65**(5): 742-745.

Bailey, J. S. (1988). "Integrated colonization control of *Salmonella* in poultry." Poult Sci **67**(6): 928-32.

Bailey, J. S., N. J. Stern, et al. (2001). "Sources and movement of *Salmonella* through integrated poultry operations: a multistate epidemiological investigation." J Food Prot **64**(11): 1690-7.

Barrett, T. J., H. Lior, J. H. Green, R. Khakhria, J. G. Wells, B. P. Bell, K. D. Greene, J. Lewis, and P. M. Griffin. (1994). "Laboratory investigation of a multistate food-borne outbreak of *Escherichia coli* O157:H7 by using pulsed-field gel electrophoresis and phage typing." J. Clin. Microbiol. **32**: 3013-3017.

Barrow P.A., H. M. B., Lovell M.A. (1994). "Host specificity of Salmonella infection in chickens and mice is expressed in vivo promarily at the level of the reticuloendothelial system." Infect Immun **62**(10): 4602-5610.

Barza, M. (1987). "Potential mechanisms of increased disease in humans from antimicrobial resistance in food animals." Clin Inf Dis **34**(S3): 123-125.

Bass, L., C. Liebert, et al. (1999). "Incidence and characterization of integrons, genetic elements mediating multiple-drug resistance, in avian *Escherichia coli*." Antimicrob Agents and Chemotherap **43**: 2925 - 2929.

Bass, L., C. A. Liebert, et al. (1999). "Incidence and characterization of integrons, genetic elements mediating multiple-drug resistance, in avian Escherichia coli." Antimicrob Agents Chemother **43**(12): 2925-9.

Bayler, A. J. (2003). Compendium of Veterinary Products. Port Huron, MI, North American compendiums, Ltd.

Beal, R., P. Wigley, et al. (2004). "Age at primary infection with Salmonella enterica serovar Typhimurium in the chicken influences persistence of infection and subsequent immunity to re-challenge." Vet Immunol Immunopathol **100**: 151-164.

Beli, E., E. Duraku, et al. (2001). "Salmonella serotypes isolated from chicken meat in Albania." Int J Food Microbiol **71**(2-3): 263-6.

Bhatia, T. R., G. D. McNabb, et al. (1979). "*Salmonella* isolation from litter as an indicator of flock infection and carcass contamination." Avian Dis **23**(4): 838-47.

Blankenship, L., J. Bailey, et al. (1993). "Two-step competitive exclusion floar treatment to diminish *Salmonellae* in commercial broiler chickens." Poult Sci **72**: 1667 - 1672.

Bolton, L., L. Kelley, et al. (1999). "Detection of multi-drug resistant Salmonella enterica serotype typhiumurium DT104 based on a gene which confers cross-resistance to florfenicol and chloramphenicol." J Clin Microbiol **37**(5): 1348-1351.

Bonten, M. J., R. Willems, et al. (2001). "Vancomycin-resistant enterococci: why are they here, and where do they come from?" Lancet Infect Dis **1**(5): 314-25.

Boonmar s., A. B., s. Pornrunangwong, N. Marnrim, K. Kaneko, M. Ogawa. (1998.). "*Salmonella* in broiler chickens in Thailand with special reference to contamination of retail meat with *Salmonella enteritidis*." J Vet Med Sci **60**(11): 1233-1236.

Boyce T.G., D. K., D.L. Swerdlow, T.M. Gomez, B. Serrano, L.N. Nickey,F.W. Hickman-Brenner, G.B. Malcolm, P.M. Griffin. (1996.). "Recurrent outbreaks of *Salmonella enteritidis* infections in a Texas restaurant: Phage type 4 arrives in the United States." Epidemiol Infect **117**(1): 29-34.

Brown, H. J., H. W. Stokes, et al. (1996). "The integrons In0, In2, and In5 are defective transposon derivatives." J Bacteriol **178**(15): 4429-37.

Buchrieser C., R. B., O. Buchrieser, A. Kristl, J.B. Luchansky, C.W. Kaspar. (1997). "Genomic analyses of Salmonella enteritidis phage type 4 strains from Austria and phage type 8 strains from the United States." Zentralbl Bakteriol **285**(3): 379-388.

Bygrave, A. C. and J. Gallagher (1989). "Transmission of *Salmonella enteritidis* in poultry." Vet Rec **124**(21): 571.

Byrd, J. A., J. R. DeLoach, et al. (1999). "Evaluation of *Salmonella* serotype distributions from commercial broiler hatcheries and grower houses." Avian Dis **43**(1): 39-47.

Caldwell, D., BM Hargis, DE Corrier, L Vidal, JR DeLoach. (1995). "Evaluation of persistence and distribution of *Salmonella* serotype isolation from poultry farms using drag-swab sampling." Avian Dis **39**: 617-621.

Carattoli, A. (2001). "Importance of integrons in the diffusion of resistance." Vet Res **32**(3-4): 243-59.

Carlson, S., L. Bolton, et al. (1999). "Detection of multiresistant Salmonella typhimurium DT104 using multiplex and fluorogenic PCR." Mol and Cell Probes **13**: 213-222.

Chanawong, A., F. H. M'Zali, et al. (2001). "SHV-12, SHV-5, SHV-2a and VEB-1 extended-spectrum beta-lactamases in Gram-negative bacteria isolated in a university hospital in Thailand." J Antimicrob Chemother **48**(6): 839-52.

Chiew, Y. F., S. F. Yeo, et al. (1998). "Can susceptibility to an antimicrobial be restored by halting its use? The case of streptomycin versus Enterobacteriaceae." J Antimicrob Chemother **41**(2): 247-51.

Cohen, M., Tauxe RV (1986). "Drug-resistant *Salmonella* in the United States: an epidemiological perspective." Science **234**(964-969).

Collis, C., G. Grammoticopoulos, et al. (1993). "Site-specific insertion of gene cassettes into integrons." Mol Microbiol **9**(1): 41-52.

Collis, C. and R. Hall (1995). "Expression of antibiotic resistance genes in the integrated cassettes of integrons." Antimicrob Agents and Chemotherap **39**(1): 155-162.

Collis, C. and R. Hall (1995). "Expression of antibiotic resistance genes in the integrated cassettes of integrons." Antimicrob Agents and Chemotherap **39**(1): 155-162.

Collis, C. M. and R. M. Hall (1992). "Site-specific deletion and rearrangement of integron insert genes catalyzed by the integron DNA integrase." J Bacteriol **174**(5): 1574-85.

Collis, C. M., M. J. Kim, et al. (1998). "Binding of the purified integron DNA integrase Intl1 to integron- and cassette-associated recombination sites." Mol Microbiol **29**(2): 477-90.

Corrier, D. E., J. A. Byrd, et al. (1999). "Presence of *Salmonella* in the crop and ceca of broiler chickens before and after preslaughter feed withdrawal." Poult Sci **78**(1): 45-9.

Courrier D.E., B. J. A., Hargis B.M., Hume M.E., Bailey R.H., Stanker L.H. (1999). "Presence of *Salmonella* in the crop and ceca of broiler chickens before and after preslaughter feed withdrawl." Poult Sci **78**(45): 153-159.

Cox, N. A., J. S. Bailey, et al. (1991). "Extent of *Salmonellae* contamination in breeder hatcheries." Poult Sci **70**(2): 416-8.

Cruchaga, S., A. Echeita, A. Aladuena, J.Garcia-Pena, N. Frias, and M. A. Usera (2001). "Antimicrobial resistance in salmonellae from humans, food and animals in Spain in 1998." J. Antimicrob. Chemother. **47**: 315-321.

Daly, M., J. Buckley, E. Power, C. O'Hare, M. Cormican, B. Cryan, P. G. Wall, and S. Fanning (2000). "Molecular characterization of Irish *Salmonella enterica* serotype Typhimurium: detection of class I integrons and assessment of genetic relationships by DNA amplification fingerprinting." Appl. Environ. Microbiol. **66**: 614-619.

Dargatz, D. A., P. J. Fedorka-Cray, S. R. Ladely, and K. E. Ferris (2000). "Survey of *Salmonella* serotypes shed in feces of beef cows and their antimicrobial susceptibility patterns." J. Food Prot. **63**: 1648-1653.

Davies, R. H., and M. Breslin (2003). "Persistence of *Salmonella enteritidis* phage type 4 in the environment and arthropod vectors on an empty free-range chicken farm." Environ. Microbiol. **5**: 79-84.

Davies, R. H., C. Wray. (1995.). "Mice as carriers of *Salmonella enteritidis* on persistently infected poultry units." Vet Rec **137**: 337-341.

Dickens, J. A., N.A. Cox, J.S. Bailey, J.E. Thomson (1985). "Automated microbiological sampling of broiler carcasses." Poult Sci **64**: 1116 - 1120.

Dunne, E., P. Fey, et al. (2000). "Emergence of domestically acquired ceftriaxone-resistant Salmonella infections associated with AmpC beta-lactamase." JAMA **284**: 3151-3156.

Ebel, E. D., J. Mason, L. A. Thomas, K. E. Ferris, M. G. Beckman, D. R. Cummins, L. Scroeder-Tucker, W. D. Sutherlin, R. L. Glasshoff, and N. M. Smithhisler (1993). "Occurrence of *Salmonella enteritidis* in unpasteurized liquid egg in the United States." Avian Dis. **37**: 135-142.

Ebel, E. D., M. J. David, and J. Mason (1992). "Occurrence of *Salmonella enteritidis* in the U. S. commercial egg industry: report on a national spent hen survey." Avian Dis. **36**: 646-654.

Fey PD, S. T., Rupp ME, Dunne EF, Ribot E, Iwen PC, Bradford PA, Angulo FJ, Hinrichs SH (2000). "Ceftriaxone-resistant *Salmonella* infection acquired by a child from cattle." New England J Med **342**(1242-1249).

Fluitt, A. and F. Schmitz (1999). "Class 1 integrons, gene cassettes, mobility, and epidemiology." Eur J Clin Microbiol Inf Dis **18**: 761-770.

Fluitt, A., Schmitz, FJ (1999). "Class 1 integrons, gene cassettes, mobility, and epidemiology." Eur J Clin Microbiol Inf Dis **18**: 761-770.

Frost, J., A. Kelleher, et al. (1996). "Increasing ciprofloxacin resistance in Salmonellas in England and Wales 1991 - 1994." J Antimicrob Chemother **37**(85-91).

Fuzihara, T. O., S. A. Fernandes, et al. (2000). "Prevalence and dissemination of *Salmonella* serotypes along the slaughtering process in Brazilian small poultry slaughterhouses." J Food Prot **63**(12): 1749-53.

G.C., M. (2000). "Prospects for competitive exclusion treatment to control *Salmonellas* and other foodborne pathogens in poultry." Vet J **159**(2): 107-108.

Gambarotto, K., M. Ploy, et al. (2001). "Occurence of vancomycin-resistant enterococci in pork and poultry products from a cattle-rearing area of France." J Clin Microbiol **39**: 2354-2355.

Gast R.K., H. P. S. (1998). "Persistance of *Salmonella enteritidis* from one day of age until maturity in experimentally infected layer hens." Poult Sci **77**(12): 1759-1762.

Gebreyes, W. A. and C. Altier (2002). "Molecular characterization of multidrug-resistant Salmonella enterica subsp. enterica serovar Typhimurium isolates from swine." J Clin Microbiol **40**(8): 2813-22.

Gebreyes, W. A., and C. Altier. 2002. (2002). "Molecular characterization of multidrug-resistant *Salmonella enterica* subsp. enterica serovar Typhimurium isolates from swine." J. Clin. Microbiol. **40**: 2813-2822.

Girlich, D., L. Poirel, et al. (2001). "Molecular epidemiology of the integron-located VEB-1 extended-spectrum beta-lactamase in nosocomial enterobacterial isolates in Bangkok, Thailand." J Clin Microbiol **39**(1): 175-82.

Glynn MK, B. C., Dewitt W, Dabney P, Mokhta M, Angulo FJ (1998). "Emergence of multi-drug resistant *Salmonella enterica* serotype Typhimurium DT104 infections in the United States." New England J Med **338**(19): 1333 - 1338.

Goldstein, C., M. Lee, et al. (2001). "Incidence of class 1 and class 2 integrases in clinical and commensal bacteria from livestock, companion animals, and exotics." Antimicrob Agents and Chemotherap **45**(3): 723 - 726.

Goldstein, C., M. D. Lee, et al. (2001). "Incidence of class 1 and 2 integrases in clinical and commensal bacteria from livestock, companion animals, and exotics." Antimicrob Agents Chemother **45**(3): 723-6.

Goldstein C, L. M., Sanchez S, Hudson C, Phillips B, Register B, Grady M, Leibert C, Summers O (2001). "Incidence of class 1 and class 2 integrases in clinical and commensal bacteria from livestock, companion animals, and exotics." Antimicrob Agents and Chemotherap **45**(3): 723 - 726.

Gradel K, R. E. (2003). "A questionnaire-based retrospective field study of persistence of *Salmonella entertidis* and *Salmonella typhimurium* in Danish broiler houses." Preventative Vet Med **56**(2003): 267-284.

Gradel, K. O., E. Rattenborg. (2003). "A questionnaire-based, retrospective field study of persistence of *Salmonella enteritidis* and *Salmonella typhimurium* in Danish broiler houses." Prev Vet Med **56**: 267-284.

Gravel, A., B. Fournier, et al. (1998). "DNA complexes obtained with the integron integrase IntI1 at the attI1 site." Nucleic Acids Res **26**(19): 4347-55.

Grinsted, J., F. de la Cruz, et al. (1990). "The Tn21 subgroup of bacterial transposable elements." Plasmid **24**(3): 163-89.

Hakanen, A., H. Jousimies-Soumer, et al. (2003). "Fluorquinolone resistance in Campylobacter jejuni isolates in travelers returning to Finland: association of ciprofloxacin resistance to travel destination." Emerg Infect Dis **9**: 267-270.

Hakanen, A., P. Kotilanen, et al. (2001). "Reduced fluorquinolone susceptibility in Salmonella enterica serotypes in travelers returning from Southeast Asia." Emerg Infect Dis **7**: 996-1003.

Hall, R. and C. Collis (1995). "Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination." Mol Microbiol **15**(4): 593-600.

Hall, R. M., D. E. Brookes, et al. (1991). "Site-specific insertion of genes into integrons: role of the 59-base element and determination of the recombination cross-over point." Mol Microbiol **5**(8): 1941-59.

Hall, R. M. and C. M. Collis (1995). "Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination." Mol Microbiol **15**(4): 593-600.

Hannu, T., L. Mattila, A. Siitonen, and M. Lerisalo-Repo (2002). "Reactive arthritis following an outbreak of *Salmonella typhimurium* phage type 193 infection." Ann. Rheum. Dis. **61**: 264-266.

Heidelberg, J. F., J. A. Eisen, et al. (2000). "DNA sequence of both chromosomes of the cholera pathogen Vibrio cholerae." Nature **406**(6795): 477-83.

Heilig, S., P. Lee, et al. (2002). "Curtailing the use in Agriculture: it is time for action: This use contributes to bacterial resistance in humans." Western J Medicine **176**: 9-11.

Helms M, V. P., Gerner-Smidt P, Molbak K,. (2002). "Excess mortality associated with antimicrobial drug-resistannt *Salmonella typhimurium* DT104." Emerg Infect Dis **8**: 490-495.

Henzler, D. J., H.M. Opitz. (1992.). "The role of mice in the episootiology of *Salmonella enteritidis* infection on chicken layer farms." Avian Dis **36**: 625-631.

Hinton A., B. R. J., Ingram K. (1998). Feed withdrawl and carcass microbiological counts. Georgia Poult. Conf., Athens, GA.

Hinton, M., E. A. Ali, and A. H. Linton (1983). "The excretion of *Salmonella typhimurium* in the faeces of calves fed milk substitute." J. Hyg. **91**: 33-45.

Hofacre, C. (2002). "The health and management of poultry production." Int J Infect Dis **6**: 353-357.

Hofacre, C. L., D. G. White, J. J. Maurer, C. Morales, C. Lobsinger, C. R. Hudson and S. G. Thayer (2001). "Antibiotic resistant bacteria in rendered animal products." Avian Dis **45**: 953-961.

Hollinger K, W. C., Evans D, Pascoe S, Chappell S, Jones Y (1998). "*Salmonella typhimurium* DT104 in cattle in Great Britain." JAVMA **213**(12): 1732 - 1733.

Hong, Y., T. Liu, C. Hofacre, M. Maier, S. Ayers, D. G. White, L. Wang, and J. J. Maurer (2003). "A restriction fragment length polymorphism based polymerase chain reaction as

an alternative to serotyping for identifying *Salmonella* serotypes." Avian Dis **47**(2): 387-395.

Hoszowski, A., and D. Wasyl (2001). "Typing of *Salmonella enterica* subsp. *Enterica* serovar Mbandaka isolates." Vet Microbiol **80**: 139-148.

Hudson, C., C. Quist, et al. (2000). "The genetic-relatedness of *Salmonella* from non-domestic birds in the Southeastern United States." J Clin Microbiol **38**: 1860 - 1865.

Hudson CR, Q. C., Lee MD, Keyes K, Dodson SV, Morales C, Sanchez S, White DG, Maurer JJ (2000). "The genetic-relatedness of *Salmonella* from non-domestic birds in the Southeastern United States." J Clin Microbiol **38**: 1860 - 1865.

Hudson, C. R., C. Quist, et al. (2000). "Genetic relatedness of Salmonella isolates from nondomestic birds in Southeastern United States." J Clin Microbiol **38**(5): 1860-5.

Iba A.M., B. J. A., Barrow P.A. (1992). "Interference between *Salmonella* serotypes in intestinal colonisation of chickens: correlation with in vitro behavior." FEMS Microbiol Lett **131**(2): 153-159.

Iovine, N. and M. Blaser (2004). "Antibiotics in animal feed and spread of resistant Campylobacter from poultry to humans." Emerg Infect Dis **10**(6): 1158-1159.

Jolley KA, F. E., Chan MS, Maiden MC (2001). "Sequence type analysis and recombinational tests (START)." Bioinformatics **17**(12): 1230-1231.

Keyes, K., C. Hudson, et al. (2000). "Detection of florfenicol resistance genes in Escherichia coli isolated from sick chickens." Antimicrob Agents and Chemotherap **44**(2): 421-444.

Keyes, K., C. Hudson, et al. (2000). "Detection of florfenicol resistance genes in Escherichia coli isolated from sick chickens." Antimicrob Agents Chemother **44**(2): 421-4.

Koort, J., S. Lukinmaa, et al. (2002). "Technical improvement to prevent DNA degradation of enteric pathogens in pulsed-field gel electrophoresis." J Clin Microbiol **40**(9): 3497-3498.

Koort JM, L. S., Rantala M, Unikila E, Siitonen A (2002). "Technical improvement to prevent DNA degradation of enteric pathogens in pulsed-field gel electrophoresis." J Clin Microbiol **40**(9): 3497-3498.

Kusunoki J., A. K., Y. Yanagawa, C. Monma, M. Shingaki, H. Obata, T. Itoh, K. Ohta, Y. Kudoh, A. Nakamura. (1999.). "Biochemical and molecular characterization of *Salmonella* ser. *enteritidis* phage type 1 isolated from food poisoning outbreaks in Tokyo." Kansenshogaku Zasshi **73**(5): 437-444.

Laconcha I., N. L.-M., A. Rementeria, A. Audicana, I. Perales, J. Garaizar. (1998.). "Phage typing combined with pulsed-field gel electrophoresis and random amplified polymorphic DNA increases discrimination in the epidemiological analysis of *Salmonella enteritidis* strains." Int J Food Microbiol **40**(1-2): 27-34.

Lahellec, C., P. Colin, et al. (1986). "Influence of resident *Salmonella* on contamination of broiler flocks." Poult Sci **65**(11): 2034-9.

LaHellec C., P. c., G. Bennejean. (1986.). "Influence of resident *Salmonella* on contamination of broiler flocks." Poult Sci **65**: 2034-2039.

Lee, M. and S. Sanchez (2002). "Class 1 integron-associated tobramycin-gentmycin resistance in Campylobacter jejuni isolated from the broiler chicken house environment." Antimicrob Agents and Chemotherap **46**(11): 3660-3664.

Lee Y.J., K. M. S., Woo Y.K., Mo I.P., Tak R.B. (2001). "Competitive exclusion against *Salmonella gallinarum* of *Salmonella enteritidis* infected chickens." J Vet Sci **2001**(2): 1.

Letellier, A., S. Messier, J. Pare, J. Menard, and S. Quessy (1999). "Distribution of *Salmonella* in swine herds in Quebec." Vet. Microbiol. **67**: 299-306.

Leverstein-van Hall, M. A., M. B. HE, et al. (2003). "Multidrug resistance among Enterobacteriaceae is strongly associated with the presence of integrons and is independent of species or isolate origin." J Infect Dis **187**(2): 251-9.

Leverstein-Van Hall, M. A., A. Paauw, et al. (2002). "Presence of integron-associated resistance in the community is widespread and contributes to multidrug resistance in the hospital." J Clin Microbiol **40**(8): 3038-40.

Levesque, C., L. Piche, et al. (1995). "PCR mapping of integrons reveals several novel combinations of resistance genes." Antimicrob Agents and Chemotherap **39**(1): 185-191.

Liebana, E., L. Garcia-Migura, et al. (2002). "Investigation of the genetic diversity among isolates of Salmonella enterica serovar Dublin from animals and humans from England, Wales, and Ireland." J Appl Microbiol **93**(5): 732-744.

Liebana, E., L. Garcia-Migura, et al. (2002). "Investigation of the genetic diversity among isolates of Salmonella enterica serovar Dublin from animals and humans from England, Wales and Ireland." J Appl Microbiol **93**(5): 732-44.

Liebert, C., R. Hall, et al. (1999). "Transposon Tn21, flagship of the floating genome." Micro. and Molec. Biol. Rev. **63**(9): 507-522.

Limawongpranee, S., H. Hayashidani, et al. (1999). "Prevalence and persistence of *Salmonella* in broiler chicken flocks." J Vet Med Sci **61**(3): 255-9.

Lin A., M. A. U., T.J. Barrett, R.A. Goldsby. (1996). "Application of random amplified polymorphic DNA analysis to differentiate strains of Salmonella enteritidis." J Clin Microbiol **34**(4): 870-876.

Lipstitch, C., R. Singer, et al. (2002). "Antibiotics in agriculture: when is it time to close the barn door?" Proc. Natl. Acad. Sci. USA **99**: 5752-5754.

Liu, T., K. Liljebjelke, et al. (2002). "Application of nested polymerase chain reaction to detection of *Salmonella* in poultry environment." J Food Prot **65**(8): 1227 - 1232.

Lopes VC, V. B., Halvorson DA, Lauer DC, Gast R, Nagaraja KV (2004). "Comparison of methods for differentiation of *Salmonella enterica* serovar Enteritidis phage type 4 isolates." Am J Vet Res **65**(5): 538-543.

Lukefahr, S. (1999). "Teachig international animal agriculture." *J Anim Sci.* **77**(11): 3106-3113.

Maguire, A. J., D. F. Brown, et al. (2001). "Rapid screening technique for class 1 integrons in Enterobacteriaceae and nonfermenting gram-negative bacteria and its use in molecular epidemiology." Antimicrob Agents Chemother **45**(4): 1022-9.

Malorny, B., A. Schroeter, et al. (1999). "Incidence of quinolone resistance over the period 1986 to 1998 in veterinary Salmonella isolates from Germany." Antimicrob Agents and Chemotherap **43**: 2278-2282.

Marshall, B., D. Petrowski, and S. B. Levy (1990). "Inter-and intraspecies spread of *Echerichia coli* in a farm envoronment in the absence of antibiotic usage." Proc. Natl. Acad. Sci. USA **87**(6609-6613).

Martin J.L., M. F., K. Dore, J.A. Buxton, F. Pollari, B. Henry, D. Middleton, R. Ahmed, F. Jamieson, B. Ciebin, S.A. McEwen, J.B. Wilson. (2004). "Increased burden of illness associated with antimicrobial resistant *Salmonella enterica* serotype Typhimurium infections." J Infect Dis **189**: 377-384.

Martinez, E. and F. de la Cruz (1988). "Transposon Tn21 encodes a RecA-independent site-specific integration system." Mol Gen Genet **211**(2): 320-5.

Martinez-Freijo, P., A. C. Fluit, et al. (1998). "Class I integrons in Gram-negative isolates from different European hospitals and association with decreased susceptibility to multiple antibiotic compounds." J Antimicrob Chemother **42**(6): 689-96.

Martinez-Freijo, P., A. C. Fluit, et al. (1999). "Many class I integrons comprise distinct stable structures occurring in different species of Enterobacteriaceae isolated from widespread geographic regions in Europe." Antimicrob Agents Chemother **43**(3): 686-9.

Mauldin, J. M. (2002). The breeder and hatchery industries. Commercial Chicken Meat and Egg Production. W. W. Bell DD. Norwell Park, Kluwer Academic Publishers**:** 623-775.

McManus, P., V. Stockwell, et al. (2002). "Antibiotic use in plant agriculture." Ann Rev Phytopathology **40**: 443-465.

Mead, G. (2000). "Prospects for competitive exclusion treatment to control *Salmonellas* and other foodborne pathogens in poultry." Vet J **159**(2): 107-109.

Mead, P. S., L. Slutsker, V. Deitz, L.F. McCaig, J.S. Breese, C. Shapiro, P.M. Griffin, R.V. Tauxe. (1999.). "Food-related illness and death in the United States." Emerg Infect Dis **5**: 607-625.

Mead, P. S., L. Slutsker, et al. (1999). "Food-related illness and death in the United States." Emerg Infect Dis **5**(5): 607-25.

Murase, T., K. Senjyu, et al. (2001). "Monitoring of chicken houses and an attached egg-processing facility in a laying farm for *Salmonella* contamination between 1994 and 1998." J Food Prot **64**(12): 1912-6.

Nandi, S., C. Hofacre, et al. (2004). "Gram positive bacteria are a major reservoir of class 1 antibiotc resistance integrons in poultry litter." PNAS **101**(8): 7118-7122.

Nandi, S., J. Maurer, et al. (2004). "Gram-positive bacteria are a major reservoir of class 1 antibiotic resistance integrons in poultry litter." PNAS **101**(18): 7118-7122.

Nandi S., M. J. J., Hofacre C., Summers A.O. (2004). "Gram-positive bacteria are a major reservoir of class 1 antibiotic resistance integrons in poultry litter." PNAS **101**(18): 7118-7122.

Nastasi, A., and C. Mammina (2000). "Surveillance of multidrug-resistant strains of *Salmonella enterica* serotype Typhimurium in southern Italy in the years 1992-1997." Eur. J. Epidemiol. **16**: 135-139.

Nield, B., A. Holmes, et al. (2001). "Recovery of new integron classes from environmental DNA." FEMS Microbiol Lett **195**(59-65).

Nield, B. S., A. J. Holmes, M. R. Gillings, G. D. Recchia, B. C. Mabbutt, K. M. Nevalainen, and H. W. Stokes.195:59-65. (2001.). "Recovery of new integron classes from environmental DNA." FEMS Microbiol. Lett. **195**(59-65).

Nield, B. S., A. J. Holmes, et al. (2001). "Recovery of new integron classes from environmental DNA." FEMS Microbiol. Lett. **195**: 59-65.

Notermans, S. and A. Hoogenboom-Verdegaal (1992). "Existing and emerging foodborne diseases." Int J Food Microbiol **15**(3-4): 197-205.

Olsen S.J., R. B., F.W. Brenner, T.H. Roels, N. Bean, R.V. Tauxe, L. Slutsker. (2001.). "The changing epidemiology of Salmonella: Trends in serotypes isolated from humans in the United States, 1987-1997." J Infect Dis **183**: 753-761.

Olsen, S. J. (1999). "Antibiotic resistance: genetic mechanisms and mobility." Acta Vet Scand Suppl **92**: 15-22.

Olsen, S. J., R. Bishop, et al. (2001). "The changing epidemiology of *Salmonella*: trends in serotypes isolated from humans in the United States, 1987-1997." J Infect Dis **183**(5): 753-61.

Olsen, S. J., L. C. MacKinnon, et al. (2000). "Surveillance for foodborne-disease outbreaks--United States, 1993-1997." MMWR CDC Surveill Summ **49**(1): 1-62.

Phillips, I. (1999). "Assessing the evidence that antibiotic growth promoters influence human infections." J Hosp Infect **43**: 173-178.

Pontello, M., L. Sodano, A. Nastasi, C. Mammina, M. Astuti, M. Domenichini, G. Belluzzi, E. Soccini, M. G. Silvestri, M. Gatti, E. Gerosa, and A. Montagna (1998). "A community-based outbreak of *Salmonella enterica* serotype Typhimurium associated with salami consumption in Northern Italy." Epidemiol. Infect. **120**: 209-214.

Pope, M. J. and T. E. Cherry (2000). "An evaluation of the presence of pathogens on broilers raised on poultry litter treatment-treated litter." Poult Sci **79**(9): 1351-5.

Poppe, C., R. J. Irwin, et al. (1991). "The prevalence of Salmonella enteritidis and other Salmonella spp. among Canadian registered commercial layer flocks." Epidemiol Infect **106**(2): 259-70.

Poppe, C., J. J. Kolar, W. H. B. Demczuk, and J. E. Harris (1995). "Drug resistance and biochemical characteristics of *Salmonella* from Turkeys." Can. J. Vet. Res. **59**: 241-248.

R., T. (1991.). "Salmonella: A postmodern pathogen." J Food Prot **54**(7): 563-568.

Rajashekara, G., E. Haverly, D. A. Halvorson, K. E. Ferris, D. C. Lauer, and K. V. Nagaraja (2000). "Multidrug-resistant *Salmonella typhimurium* DT104 in poultry." J. Food Prot. **63**: 155-161.

Rampling, A., J. R. Anderson, et al. (1989). "*Salmonella enteritidis* phage type 4 infection of broiler chickens: a hazard to public health." Lancet **2**(8660): 436-8.

Rampling A., J. R. A., R. Upson, E. Peters, L.R. Ward, B. Rowe. (1989.). "Salmonella enteritidis phage type 4 infection of broiler chickens: A hazard to public health." The Lancet **August**: 436-438.

Rankin, S., H. Aceto, et al. (200). "Molecular characterization of cephalosporin-resistant *Salmonella enterica* serotype Newport isolates from animals in Pennsylvalia." J Clin Microbiol **40**(4670-4684).

Rankin SC, A. H., Cassidy J, Holt J, Young S, Love B, Tewari D, Munro DS, Benson CE (200). "Molecular characterization of cephalosporin-resistant *Salmonella enterica* serotype Newport isolates from animals in Pennsylvalia." J Clin Microbiol **40**(4670-4684).

Recchia, G. D., H. W. Stokes, et al. (1994). "Characterisation of specific and secondary recombination sites recognised by the integron DNA integrase." Nucleic Acids Res **22**(11): 2071-8.

Rigby, C., J. Petit, et al. (1982). "The relationships of *Salmonellae* from infected broiler flocks, transport crates or processing plants to contamination of eviscerated carcasses." Can J Comp Med **46**: 272 - 278.

Robins-Browne, R. M., B. Rowe, R. Ramsaroop, A. D. Naran, E. J. Threlfall, L. R. Ward, D. A. Lloyd, and R. E. Mickel (1983). "A hospital outbreak of multiresistant *Salmonella typhimurium* belonging to phage type 193." J. Infect. Dis. **147**: 210-216.

Rowe-Magnus DA, G. A., Ploncard P, Dychinco B, Davies J, Mazel D (2001). "The evolutionary history of chromosomal super-interons provides an ancestry for multiresistant integrons." Proc. Natl. Acad. Sci. USA **98**(2): 652-657.

Rowe-Magnus, D. A., A. M. Guerout, et al. (1999). "Super-integrons." Res Microbiol **150**(9-10): 641-51.

Rowe-Magnus, D. A. and D. Mazel (2002). "The role of integrons in antibiotic resistance gene capture." Int J Med Microbiol **292**(2): 115-25.

Russell, S. M. (2003). "The effect of airsacculitis on bird weights, uniformity, fecal contamination, processing errors and populations of *Campylobacter* spp. and *Escherichia coli*." Poult Sci **82**(8): 1326-1331.

Russell, S. M. (2003). "The effect of airsacculitis on bird weights, uniformity, fecal contamination, processing errors and populations of *Campylobacter* spp. and *Escherichia coli*." Poultry Science **82**(8): 1326-1331.

Salyers, A. and C. Amabile-Cuevas (1997). "Why are antibiotc resistance genes so resistant to elimination?" Antimicrob Agents and Chemotherap **41**: 2321 - 2325.

Sanchez, S., C. L. Hofacre, M. D. Lee, J. J. Maurer, and M. P. Doyle. 2002.J. Am. Vet. Assoc. 221:492-497. (2002). " Animals sources of human salmonellosis." J. Am. Vet. Assoc. **221**(492-497).

Sander, J., C. R. Hudson, et al. (2001). "Dynamics of *Salmonella* contamination in a commercial quail operation." Avian Dis **45**(4): 1044-9.

Sandvang, D. and F. M. Aarestrup (2000). "Characterization of aminoglycoside resistance genes and class 1 integrons in porcine and bovine gentamicin-resistant Escherichia coli." Microb Drug Resist **6**(1): 19-27.

Sandvang, D., F. M. Aarestrup, et al. (1998). "Characterisation of integrons and antibiotic resistance genes in Danish multiresistant Salmonella enterica Typhimurium DT104." FEMS Microbiol Lett **160**(1): 37-41.

Sarwari, A. R., L. S. Magder, et al. (2001). "Serotype distribution of *Salmonella* isolates from food animals after slaughter differs from that of isolates found in humans." J Infect Dis **183**(8): 1295-9.

Schlosser, W., Hogue A., Ebel E., Rose B., Umholtz R., Ferris K., James W. (2000). "Analysis of *Salmonella* serotypes from selected carcasses and raw ground products sampled prior to implementation of the Pathogen Reduction; Hazard Analysis and Critical Control Point Final Rule in the US." Int J Food Microbiol **58**: 107-111.

Schlosser W., A. H., E. Ebel, B. Rose, R. Umholtz, K. Ferris, W. James. (2000). "Analysis of Salmonella serotypes from selected carcasses and raw ground products sampled prior to implementation of the pathogen reduction; Hazard analysis and critical control point final rule in the US." Int J Food Microbiol **58**: 107-111.

Silver, S. and L. T. Phung (1996). "Bacterial heavy metal resistance: new surprises." Annu Rev Microbiol **50**: 753-89.

Smith KE, B. J., Hedberg CW, Leano FT, Bender JB, Wicklund JH, Johnson BP, Moore KA, Osterholm MT (1999). "Quinolone-resistnat *Campylobacter jejuni* infections in Minnesota, 1992 - 1998." New England J Med **340**(1525-1531).

Soto, S. M., B. Guerra, et al. (2001). "Outbreaks and sporadic cases of Salmonella serovar panama studied by DNA fingerprinting and antimicrobial resistance." Int J Food Microbiol **71**(1): 35-43.

Stokes, H. W. and R. M. Hall (1989). "A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons." Mol Microbiol **3**(12): 1669-83.

Stokstad, E., Jukes TH (1949). "The multiple nature of the animal protein factor." J Biol Chem **180**: 647-654.

Sunde, M. and H. Sorum (1999). "Characterization of integrons in Escherichia coli of the normal intestinal flora of swine." Microb Drug Resist **5**(4): 279-87.

Sunde, M. and H. Sorum (2001). "Characterization of integrons in Escherichia coli of the normal intestinal flora of swineq." Microb. Drug Resist. **5**(4): 279-287.

Sunde, M. and H. Sorum (2001). "Self-transmissible multidrug resistance plasmids in Escherichia coli of the normal intestinal flora of healthy swine." Microb Drug Resist **7**(2): 191-6.

Sundstrom, L. and O. Skold (1990). "The dhfrI trimethoprim resistance gene of Tn7 can be found at specific sites in other genetic surroundings." Antimicrob Agents Chemother **34**(4): 642-50.

Tauxe, R. (1991). "*Salmonella*: A Postmodern Pathogen." Journal of Food Protection **54**(7): 563-568.

Tenover, F., R. Arbeit, et al. (1995). "Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: Criteria for bacterial strain typing." J Clin Microbiol **33**(9): 2233 - 2239.

Tenover FC, A. R., Goering RV, Mickelson PA, Murray BE, Persing DH, Swaminathan B (1995). "Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: Criteria for bacterial strain typing." J Clin Microbiol **33**(9): 2233 - 2239.

Thorton, L., S. Gray, P. Gingham, R. L. Salmon, D. N. Hutchinson, B. Rowe, D. Newton, and Q. U. Syed (1993). "The problems of tracing a geographically widespread outbreak of

salmonellosis from a commonly eaten food: *Salmonella typhimurium* DT193 in northwest England and north Wales in 1991." Epidemiol. Infect. **111**: 465-471.

Threlfall, E. J., J. A. Frost, et al. (1994). "Epidemic in cattle and humans of Salmonella typhimurium DT 104 with chromosomally integrated multiple drug resistance." Vet Rec **134**(22): 577.

Threlfall, E. J., L. R. Ward, J. A. Skinner, and A. Graham (2000). "Antimicrobial drug resistance in non-typhoidal *Salmonellas* from humans in England and Wales in 1999: decrease in multiple resistance in *Salmonella enterica* serotypes Typhimurium, Virchow, and Hadar." Microb. Drug Resist. **6**: 319-325.

Todd, E. C. D. (1989). "Preliminary Estimates of Costs of Foodborne Disease in the United States." Journal of Food Protection **52**(8): 586-594.

Uyttendaele, M. R., J. M. Debevere, et al. (1998). "Prevalence of Salmonella in poultry carcasses and their products in Belgium." Int J Food Microbiol **40**(1-2): 1-8.

van Leeuwen, W. J., C. E. Voogd, P. A. Guinee, and A. Manten (1982). "Incidence of resistance to ampicillin, chloramphenicol, kanamycin, tetracycline and trimethoprim of *Salmonella* strains isolated in the Netherlands during 1975-1980." Antoine Van Leeuwenhoek **48**: 85-96.

Walker, R. A., E. Lindsay, M. J. Woodward, L. R. Ward, and E. J. Threlfall (2001). "Variation in clonality and antibiotic-resistance genes among multiresistant Salmonella enterica serotype typhimurium phage-type U302 (MR U302) from humans, animals, and foods." Microb. Drug Resist. **7**: 13-21.

Waltman, W., A. Horne, et al. (1991). "Use of delayed secondary enrichment for the isolation of *Salmonella* in poultry and poultry environments." Avian Dis **35**: 88 - 92.

Waltman, W. D., A.M. Horne, and C. Pirkle. 1993. Influence of enrichment incubation time on the isolation of Salmonella. Avian Dis. 37:884-887. (1993). "Influence of enrichment incubation time on the isolation of *Salmonella*." Avian Dis **37**: 884-887.

Waltman WD, C. P., T. Dickson. (1991.). "Use of delayed secondary enrichment for the isolation of Salmonella in poultry and poultry environments." Avian Dis **35**: 88-92.

White, D., S. Zhao, et al. (2001). "The isolation of antibiotic-resistant *Salmonella* from retail ground meats." The New England Journal of Medicine **345**(16): 1147-1154.

White, D. G., S. Zhao, et al. (2003). "Characterization of integron mediated antimicrobial resistance in Salmonella isolated from diseased swine." Can J Vet Res **67**(1): 39-47.

White, D. G., S. Zhao, et al. (2001). "The isolation of antibiotic-resistant salmonella from retail ground meats." N Engl J Med **345**(16): 1147-54.

White DG, Z. S., Sudleer R, Ayers S, Friedman S, Chen S, McDermott PF, McDermott S, Wagner Dd, Meng J (2001). "The isolation of antibiotic-resistant *Salmonella* from retail ground meats." The New England Journal of Medicine **345**(16): 1147-1154.

White, P., McIver, CJ, Rawlinson, WD (2001). "Integrons and gene cassettes in the Enterobacteriaceae." Antimicrob Agents and Chemotherap **45**(9): 2658-2661.

Zansky, S., B. Wallace, et al. (2002). "Outbreak of multi-drug resistant Salmonella Newport - United States." JAMA **288**: 951-953.

Zhao, C., J. DeVillena, et al. (2001). "Prevalence of *Campylobacter* spp., *Escherichia coli*, and *Salmonella* serovars in retail chicken, turkey, pork, and beef from the greater Washington, D. C., area." Appl Environ Microbiol **67**(12): 5431-5436.

Zhao, T., M. P. Doyle, et al. (2002). "Occurrence of Salmonella enterica serotype typhimurium DT104A in retail ground beef." J Food Prot **65**(2): 403-7.