GENETIC APPROACH TO UNDERSTANDING THE BEHAVIOR OF *SALMONELLA* IN MEAT AND POULTRY SYSTEM

by

VIVEK SONI

(Under the Direction of Mark A. Harrison)

ABSTRACT

The genome projects have supplied a rich source of genetic data regarding chromosome structure and gene transfer. In this study, we utilized a genomic approach to assess the epidemiological relatedness of atypical *Salmonella* isolates and the ability of *Salmonella* to acquire antibiotic resistance genes. Genetic relatedness of an atypical serovar (4,5,12:i:-) was investigated to determine whether it was closely related to a common serovar *S.* Typhimurium. We have found that *Salmonella* 4,5,12:i:- strains isolated from poultry do not represent the emergence of a new serovar or clone, rather the result of spontaneous mutations in endemic *S.* Typhimurium strains, affecting phase 2 antigen expression. We also measured different variables involved in transfer of plasmid to *Salmonella* in vitro and assessed what factors affect this process. The data provided information relevant for validating a mathematical model of plasmid transmission to *Salmonella*. Our long term goal is to understand the process underlying the acquisition and spread of multiple drug resistance among major foodborne pathogens.

INDEX WORDS: *Salmonella* Typhimurium, virulence factors, *S. enterica* 4,[5],12:i:-, Antibiotic Resistance, Prebiotic, Biomoss™

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DEDICATION

To my parents and family members for their regular and immense support

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CHAPTER 1

INTRODUCTION & LITERATURE REVIEW

Foodborne diseases are one of the most widespread health problems in the world (WHO, 1984). In the United States, foodborne pathogens have been reported to cause 76 million cases and 5,000 deaths each year. These estimates have been derived by using data from multiple sources including Foodborne Diseases Active Surveillance Network (Foodnet) (Mead *et al*., 1999). *Salmonella enterica*, *Campylobacter* and *Yersinia* species, Shiga toxin-producing *Escherichia coli* (STEC), *Listeria monocytogenes* and *Clostridium perfringens* are the bacterial pathogens constituting the greatest burden of foodborne diseases. While *Campylobacter* is the most common cause of gastroenteritis, *Salmonella* has a higher death rate per year than *Campylobacter* (Mead *et al*., 1999). Modern food production systems are so complex that full control of pathogens at every stage is very difficult (Sanders, 1999).

Salmonella is one of the leading causes of bacterial foodborne diseases in the United States, accounting for an estimated 1.4 million non-typhoidal infections, 16,000 hospitalizations and nearly 600 deaths each year (Mead *et al*., 1999). These infections often result from consuming contaminated foods like poultry, produce, red meats, milk, seafood, eggs and others (Angulo *et al.,* 2004; CDC, 2005a; Dechet *et al*., 2006 Gomez *et al.,* 1997; Mead *et al*., 1999; Zhao *et al*., 2003a). *Salmonella* can also be transmitted via person to person contact, or when a human carrier contaminates food during preparation (Loewenstein *et al*., 1975). Another possible way to transmit *Salmonella* is through direct contact with animals (Dunne *et al*., 2000; Fey *et al*., 2000).

There are over 2,500 different nontyphoidal *Salmonella* serotypes known to date. These serotypes are classified according to antigenic differences in lipopolysaccharide (LPS), capsule (Vi antigen), and flagellin. The O antigen of the LPS is a common starting point for serotyping bacterial species belonging to *Enterobacteriaceae* (Edwards *et al*., 1972). There are 64 *Salmonella* O serogroups (Todar, 2005). Most *Salmonella* serotypes are biphasic producing two antigenically distinct flagellins. However, there are monophasic *Salmonella* serotypes like *S. enterica* Enteritidis that express only the phase 1 antigen (CDC, 2004). Due to antigenic differences in phase 1(H1) and phase 2 (H2) flagellins, *S. enterica* can be differentiated into over 2,000 serotypes based on the O, H1 and H2 antigenic formula that define each serotype. Several *Salmonella* serovars are adapted to its animal host (e.g. *S*. Typhi – humans; *S*. Gallinarum – avian; *S*. Dublin – cattle); others have a broad host range (e.g. *S*. Typhimurium- poultry, cattle, swine, and humans), while some have a limited host spectrum (e.g. *S*. Enteritidis- poultry and humans) (WHO, 2005). *Salmonella* Enteritidis and *S.* Typhimurium are the two most important serotypes responsible for 50% of all cases of salmonellosis worldwide (WHO, 2005).

Salmonella gastroenteritis typically results in an acute, self-limiting illness, not requiring any antimicrobial treatment and characterized by acute onset of fever, diarrhea, abdominal pain and nausea. However, in the case of children, elderly people and persons with weakened immunity, these infections can turn septicemic and become life threatening, requiring effective chemotherapy, and supportive care (Giessing *et al.,* 2003; Lee *et al.,* 2003; Olsen *et al.,* 2000; Olsen *et al.,* 2001). Approximately half of these infections occur in children, (WHO, 2005). *Salmonella* resides in the intestines of mammals, birds, and reptiles and it can survive for long periods in water, soil and on or within foods (Angulo, 1999). The environment can serve as one possible source for *Salmonella* infection in animals (Leibena *et al.,* 2002; Skov *et al.,* 1999a).

***SALMONELLA*, POULTRY AND ON-FARM CONTROL PROGRAMS**

Poultry has been observed as the main vehicle for transmission of *Salmonella* to humans in the United States (Olsen *et al*., 2000; Mead and Impey, 1987; FSIS, 2002). Poultry can easily be colonized by *Salmonella* via drinking water (Stern *et al*., 2002), feed (Davies and Wray, 1997; Whyte *et al.,* 2003; Jones and Richardson, 2004; Chadfield *et al.,* 2001) or contaminated soil or litter (Rehberger, 2002). Although the range of pathogens associated with poultry is large, the most significant organisms in the present context are *Salmonella* and *Campylobacter*. These are responsible throughout the world for 90% of identifiable, bacterial zoonoses that are foodborne (Thorns, 2000). The regular pandemic of human serovar Enteritidis phage type 4 (Europe) and phage type 8 (North America) supports the association of raw or not thoroughly cooked shell eggs and egg containing products with the human salmonellosis (Fantasia and Feletici, 1994, FAO/WHO 2003). The transovarian transmission of *S.* Enteritidis into the interior of egg before shell deposition is the main reason for the persistence of egg related *Salmonella* outbreaks and the current surface sanitizing practices followed by the production facilities are ineffective in eliminating the internalized *S.* Enteritidis from eggs (Thiagarajan *et al.,* 1994; Keller *et al.,* 1995; De Buck *et al.,* 2004). The consumption of large volumes of poultry meat and eggs (Rodenberg, 2004), and the potential for cross-contamination of uncooked products (Sneed *et al.,* 2004; Rooney *et al.,*2004) makes the regulatory control of this pathogen a high priority.

In July 1996, USDA Food Safety Inspection Services (FSIS) mandated to implement HACCP plans in all meat and poultry industries and follow the directions published in "Final Rule on Pathogen Reduction and Hazard Analysis and Critical Control Point Systems" in an attempt to control the pathogen problem. Developed by the USDA FSIS, Hazard Analysis and Critical Control Point (HACCP) is a system that allows the production of safe meat and poultry

products through the analysis of production processes. This includes identification of all hazards that are likely to occur in production, identification of critical control points in the process at which these hazards may be introduced in the product and therefore should be controlled, establishing critical limits for control at these points, verification of these steps, and methods of monitoring how well the plan is working (Hulebak *et al*., 2002). The implementation of the Final rule helped in lowering the contamination rate of *Salmonella* spp. in processed broiler chickens from 24% prior to implementation to 11% in 1999 and the same reductions were observed in other meat animals. Improvements in farm animal hygiene, better in-plant slaughter practices to prevent cross contamination of carcasses, better handling to protect processed foods from contamination, packaging and storage have helped in preventing salmonellosis caused by the contaminated foods (FDA, 2004). Vaccination of breeder and egg-laying flocks, providing salmonellae-free processed feeds and providing training in hygiene practices for all food handling personnel may further help in reducing the number of salmonellosis cases.

Recently, the approval of radiation of poultry to reduce the pathogenic bacteria i.e., *Salmonella* and *Campylobacter* by the U.S. Department of Agriculture has provided a hope in reducing the magnitude of the *Salmonella* problem, but radiation pasteurization has not yet widely accepted in the U.S. (FDA, 1995). Implementation of this technology would greatly help in reducing and tackling this pathogen problem. Further reduction of *Salmonella* contamination on the final product will require pre-harvest, on-farm intervention strategies.

APPLICATION OF MOLECULAR GENETIC METHODS IN DIAGNOSTICS OF FOODBORNE BACTERIAL PATHOGENS

There are several molecular genetic methods available to detect and discriminate foodborne pathogens to serovar and strain level. These molecular genetic techniques have

revolutionized the typing of microbial strains and had overcome the deficiency of phenotypic methods such as biotyping, phage typing and serotyping (Lukinmaa *et al*., 2004). DNA fingerprinting of bacteria as a typing tool was introduced successfully in 1990s, and now infection control in hospitals relies on this approach to identify source of infection (Zaidi *et al*., 2003). Molecular biology has provided us insight into population genetics, epidemiology, evolution and pathogenesis of infectious diseases (Belkum *et al*., 2001).

Molecular typing was initially considered as laborious, expensive and complex method but has since been refined and adapted to accommodate the workflow in high throughput diagnostic labs (Anonymous, 2002). DNA sequencing, PCR-RFLP (Restriction Fragment Length Polymorphism), Pulsed-Field Gel Electrophoresis (PFGE), and ribotyping are the most widely and successfully used molecular tools being used by many diagnostic laboratories to delineate relatedness among microbial isolates. These methods are sometimes referred to as molecular typing and identify small (or large) differences in the genetic makeup of different bacterial strains (Woodruff, 2003). Once full genome sequences for multiple isolates of the single or multiple bacterial species are available; all genetic variables can be identified (Belkum *et al*., 2001) and used to develop new typing tools that discern genetic differences within a bacterial population. This approach has identified repetitive, variable gene sequences that have translated well in development of PCR-based tests that discern strain differences (Rafiee *et al., 2000*; Boxrud *et al.,* 2007).

However, one of the drawbacks associated with several of these molecular typing methods is the lack of internationally standardized (Lukinmaa *et al*., 2004). It is difficult to select the most applicable and appropriate method for the epidemiological investigation amid the large number of available methods. The requirements and logistics behind a small-scale

epidemiological study may be very different than what is needed for investigating large scale epidemics or pandemics. Ideally the molecular typing method should have high degree of reproducibility and significant discriminating power. The method should be easily available, inexpensive, should not be complicated or technically demanding and the results should be easily interpretable (Lukinmaa *et al*., 2004).

PCR is a molecular method that allows one to amplify genetic markers by making multiple copies of the target gene sequence with the help of specific primers for these sequences (Woodruff, 2003). However the target gene sequence should be chosen with care and this gene or sequence should be associated only with the pathogen investigated. PCR can be used to detect and identify non-culturable microorganisms. PCR is very sensitive and can detect a single cell, thus making it sensitive to contamination and false-positives. Single nucleotide differences occurring in the primary annealing regions may affect the performance of PCR, but the PCR conditions can be adjusted to tolerate these mismatches (Fredricks *et al*., 1999). Sometimes presence of inhibitory factors in stool, blood or foods interferes with the amplification of the target sequence (Scheutz *et al*., 2001; Paton *et al*., 2003).

PFGE is a method for separating and comparing large DNA molecules after digestion with a rare restriction enzyme cutter. Because of circular shape of the bacterial chromosome, this digestion yields several linear molecules of DNA (Tynkkynen *et al.,* 1999; Simmons *et al*., 2000; Hager *et al.,* 2001b). PFGE separates large DNA fragments by subjecting them to alternately perpendicularly oriented electrical fields (Al Thawdi *et al*., 2002). PFGE differs from conventional agarose electrophoresis in that the orientation of the electric field across the gel periodically changes in contrast to being unidirectional and constant in standard electrophoresis. The variability in the electric field allows PFGE to resolve the very large fragments (>600 kb).

Before lysing the cells, bacteria are immobilized by mixing the bacterial suspension with melted agarose so as to protect the chromosomal DNA from mechanical breakage (Lukinmaa *et al*., 2004). After multiple washes, the DNA released into the agarose plug is digested with a restriction enzymes and separated by PFGE.

PFGE has been considered as the gold standard for subtyping bacteria. The occurrence of point mutation, insertion, deletion, loss or acquisition of plasmids may result in major or minor differences in PFGE profiles between genetically-related strains (Tenover *et al*., 1995). These mutations may result in two to three fragment differences in the PFGE banding patterns. Based on the number of differences between PFGE banding patterns, the isolates are classified as closely related, possibly related or unrelated (Tenover *et al*., 1995). PFGE is a time consuming and labor intensive method. Some strains are not typeable by PFGE as the DNA is sometimes degraded prior to electophoresis (Ridell *et al*., 1998; Liesegang *et al*., 2002). However, PFGE has already been successfully used in short-term epidemiological investigations for many foodborne bacterial pathogens. It is very accurate and reproducible method. Usage of standardized protocols and software by all state public health and worldwide laboratories has made PFGE a gold standard in the epidemiological studies of foodborne outbreaks (Swaminathan *et al*., 2001). In case of *Salmonella* serotype Enteritidis, PFGE exhibits limited discriminatory power (Hudson *et al*., 2001) as two PFGE patterns make up nearly 48% of the *Salmonella* serotype Enteritidis isolates (CDC, 2004).

Ribotyping is another molecular fingerprinting technique for typing microorganisms that identifies polymorphism in regions flanking 2-10 copies of the 16S rRNA (Farber, 1996; Aarnisalo *et al*., 1999; Samadpour, 2002). In this method, the genomic DNA is digested and resolved by electrophoresis before hybridization with the probe specific for the ribosomal genes

(Stull *et al*., 1988). As ribosomal RNA genes are present in the chromosome of all bacteria and they are highly conserved, so a single broad-spectrum probe can be used. Because these ribosomal operons are present in different locations on the chromosome, this technology allows interspecies and intraspecies discrimination (Stull *et al*., 1988; Grimont *et al*., 1991). These ribosomal RNA genes have proven useful for studying the long-term epidemiology and phylogenetic relationships (Ludwig *et al*., 1994; Vandamme *et al*., 1996).

High sensitive molecular techniques are capable of detecting a single pair substitutions and resolving the issue of underlying mechanism of complex variation and epidemiology. For example, in 2002, 2 patients had vancomycin resistant *Staphyloccus aureus* cultures and the presence of *vanA* genetic element in these isolates suggested a transfer of *vanA* vancomycin resistant genes from *Enterococus faecalis* (CDC, 2002). DNA based methods being accurate and rapid are widely used for the determination of the antibiotic resistance and susceptibility. The development of these user-friendly molecular techniques has increased the use of genetic assays and has been of great help in minimizing the spread and better understanding of the antimicrobial resistance bacteria (Forsum *et al*., 2004) and the improved detection and investigation can serve to probe the safety of the food production system at certain levels.

***SALMONELLA* AND ANTIBIOTIC RESISTANCE**

"An antibiotic is a chemical compound that inhibits or abolishes the growth of microorganisms, such as bacteria, fungi or protozoans" (Waksman, 1956). The discovery of antibiotics has improved the well being of humans and animals to a larger extent. Earlier known as miracle drugs, but six decades of use and misuse of antibiotics have become a serious threat to medicine and public health in the effective treatment of infectious diseases. This rise of antimicrobial resistance has made the treatment more difficult and increased the mortality rate of

patients infected with resistant organisms. Infections like HIV and malaria have left us with fewer options due to the increased resistance to the commonly used antimicrobials, and in some cases the infections are almost untreatable (Bloland *et al*., 2000; Bartlett *et al*., 2000). The adaptive nature of bacteria has allowed them to withstand antibiotics and alter and there by inactivate the therapeutic potential of the drug.

The problem of antimicrobial resistance is almost ubiquitous to treatment of various infectious diseases but the various factors associated with it are highly variable (i.e., the rate at which resistance becomes a problem). The development of resistance depends on the type of antimicrobial agent, the pathogen involved and the environment in which transmission occurs. For example, resistance to single anti-infective agents used for treatment of TB and HIV infection was documented almost immediately after these agents became available and only a partial solution was provided by using a combination of drugs (Bartlett *et al*., 2000; Farmer *et al*., 1998).

There are different genetic processes by which bacteria can develop resistance to antibiotics such as preventing the antibiotic from getting to its target, altering the target, inactivating the drug, provide an alternate, drug-resistant pathway, or pump the antimicrobial out of the bacterial cell. Bacteria do this by genetic mutation in the target gene. This can result in changing the permeability of their membranes or by reducing the number of channels available for drugs to diffuse through. Penicillin kills bacteria by interfering with cell wall cross-linking. Resistance is attributed to either amino acid change(s) in the target enzyme (transpeptidase, aka penicillin-binding protein), thus altering binding of the drug to its target, or inactivation of this β-lactam antibiotic. Some mutations derepress expression of enzymes that can pump the drug out of the cell (Piddock, 2006) or inactivate it (Davies, 1994) while other mutations eliminate the

cell target (Douchin *et al*., 2006) or alter its binding properties (Minnick *et al*., 2002). Another possibility is the acquisition of new genes that can deactivate, destroy, or pump the antibiotic out of the cell or modify the drug's target. They do so through genetic exchange involving a process called conjugation which begins with contact between the cell surface of the recipient cell and the tip of a sex pilus of a donor cell, followed by contraction of the pilus, and direct contact between the donor and recipient outermembranes to form a channel through which the replicated, single-stranded DNA molecule passes. Bacterial viruses or phages can serve as another vehicle for passing resistance traits between bacteria (Lipatov *et al*., 2007).

With the exception of quinolone/fluoroquinolone resistance, antibiotic resistance in *Salmonella* is attributed to the acquisition of foreign genes that encode enzymes to destroy, chemically inactivate, or "pump" the noxious drug out of the bacterial cell. The specificity of enzyme ranges from narrow to broad, depending on the drug resistance gene (Keyes *et al*., 2003). Mobile genetic elements like plasmids, transposons and integrons carry the antibiotic resistance genes and transfer the resistance from commensal to the pathogen (Keyes *et al*., 2003). Integrons appear to be responsible for the development and dissemination of multi-drug resistance for many microorganisms, including *Salmonella*. An integron is a mobile DNA element that can capture and carry genes, particularly those responsible for antibiotic resistance. Integrons do this by site specific recombination through recognition of a 59-bp element present in selected genes by its recombinase IntI and then integrating the captured gene into its resident integration site, *attI* (Stokes *et al*., 1989). Integrons occurs widely in nature (Nield *et al*., 2001) and as reported by Naas *et al*, constitute a sizeable reservoir for antibiotic resistance genes.

Class 1 integrons possess various resistance genes and confer resistance to a large number of antibiotics and disinfectants. This includes aminoglycosides, β-lactams,

chloramphenicol, macrolides, quaternary ammonium and trimethoprim (Fluit *et al.,* 1999). Class 1 integrons occur in various *S. enterica* serovars and have been detected in serovars isolated from humans (Nogardy *et al.,* 2005; Randall *et al.,* 2004), animals (Goldstein *et al.,* 2001; Randall *et al.,* 2004) and foods (White *et al.,* 2001).

MDR genomic island, SGI1, which is present in serovar Typhimurium DT104 possess class 1 integron. The *tetG* and *floR* genes are flanked in the chromosome by class 1 integrons and these integrons in DT104 encodes resistance to streptomycin, sulfonamides and ampicilllin (Boyd *et al*., 2001). The SGI1 genomic island, specifically the MDR locus, has also been reported in other serovar Typhimurium phage types (DT 120) and *S. enterica* serovars (serovar Agona, serovar Albany, serovar Paratyphi B) isolated from many animal species (Boyd *et al*., 2001; Doublet *et al*., 2004; Levings *et al*., 2005). This suggested that the MDR gene locus is transferable between serotypes. SGI1 was once thought to be unique to MDR *Salmonella*, but a segment of SGI1 was recently discovered within a 20kb genomic island in *Acinetobacter baumannii* (Fournier *et al*., 2006)

In United States, salmonellosis is an important public health problem and in most cases results in a self-limiting gastrointestinal illness characterized by diarrhea, fever and abdominal cramps. In early 1960s, antibiotic resistance in *Salmonella* was first reported and most of the reported resistance was to a single antibiotic (Bulling *et al*., 1973; Cherubin *et al*., 1981; Van Leeuwen *et al*., 1979). Around mid 1970s, the multi-drug resistant (MDR) *Salmonella* was being reported where isolates were exhibiting resistance to chloramphenicol, streptomycin, sulfonamides and tetracycline (Rowe *et al*., 1979). The factors that influence the emergence and spread of these multi-drug resistant *Salmonella* serovars are presently not known. However, antibiotic usage in veterinary medicine and agriculture may be a major contributor whether by

promoting emergence of novel resistant strains or via enhanced dissemination of selected clones of resistant bacteria (Angulo *et al*., 2000; Hancock *et al*., 2000).

The increasing frequency of multi-drug resistant *Salmonella* strains has become a primary public health concern. Lee and others (1994) compared the proportion of resistant *Salmonella* isolates from human patients in selected U.S. counties and found that the number has increased from 17% in 1979-80 to 26% in 1984 to 1985 and jumped to 31% in 1989-90. The percentage of infections by MDR-strains has increased from 12% in 1979 to 17% in 1984-85 and 25% in 1989-90. *Salmonella* Typhimurium is one of the most common *Salmonella* serotype, comprised 22% of *Salmonella* infections in 2002 (CDC, 2002). A distinct *Salmonella* enterica Typhimurium strain, known as definitive type 104 (DT 104) appeared in late 1980s and raised concern because of its resistance to at least five antimicrobial agents-ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracycline (R-type ACSSuT) (Threlfall *et al*., 1996). This strain has exhibited resistance to other antibiotics including trimethoprim, nalidixic acid and spectinomycin and reduced sensitivity to ciprofloxacin (Threlfall *et al*., 1997, 1998). *Salmonella* DT104 was originally detected in seagulls and then cattle and later identified in other livestock species companion animals and wildlife (Lee *et al*., 1994; Helms *et al*., 2002; Davis *et al*., 1999; Evans and Davies, 1996). In United States, the prevalence of ACSSuT resistant *Salmonella* Typhimurium has increased from 0.6% in 1979 to 34 % in 1996 (Glynn *et al*., 1998; CDC, 2001). Its dual resistance to chloramphenicol and the veterinary analog florofenicol has put the DT104 strain on the forefront of discussion on the usage of antibiotics in the agriculture sector and its impact on public health.

Chloramphenicol/ florofenicol resistance is due to *floR*, a putative drug efflux pump first described in fish pathogen *Photobacterium dansalae* (Kim and Aoki, 1996). *Salmonella*, *E. coli*

and *Klebsiella pneumoniae* also possess this phenicol resistance gene (Cloeckaert *et al*., 2000a, 2000b, 2001; Keyes *et al*., 2000; Sanchez *et al*., 2002). In case of most of *E.coli* isolates, *floR* gene appears to reside on plasmids and on *ampC* plasmids of *Salmonella* Typhimurium and *Salmonella* Newport (Cloeckaert *et al*., 2000a, 2000b, 2001; Keyes *et al*., 2000; Sanchez *et al*., 2002; Doublet *et al*., 2004). Whereas in case of *Salmonella* Typhimurium DT104, this gene occurs next to that of tetracycline efflux (efflux pump *tetG*). Both genes are further flanked in the chromosome by class 1 integrons (Boyd *et al*., 2001). These integrons in DT104 encode for resistance to streptomycin, sulfonamide and ampicillin (Briggs and Fratamico, 1999).

Another drug resistant *Salmonella* serovar, *S.* Newport has emerged while DT104 continues to cause foodborne outbreaks worldwide (Helms *et al*., 2005). This serovar is resistant to β-lactams, the β-lactam inhibitor clavulanic acid and cephalosporins and other classes of antibiotics, and it has been spreading both in animals and humans (Anonymous. 2002; Fey *et al*., 2000). *S.* Newport exhibits resistance to amoxicillin/ clavulanic acid, cephalothin, cefoxitin and ceftiofur in addition to penta-resistance phenotype usually seen in *Salmonella* Typhimurium DT104. This serovar also have decreased susceptibility to ceftriaxone (MIC > 16μg/mL) and some of the *Salmonella* Newport MDR-AmpC strains also shows resistance to kanamycin, trimethoprim and kanamycin (CDC, 2002). The *Salmonella* serovar Newport, like DT104, was also first reported in cattle and subsequently detected in other animal species (Anonymous. 2002; Fey *et al*., 2000; Gupta *et al*., 2003). The prevalence of Newport-MDR-AmpC among *Salmonella* Newport isolates from humans in the United States increased from 0% during 1996 to 1997 to 21% in 2003 (NARMS 2003b).

Fluoroquinolone and ceftriaxone resistant *Salmonella* are of particular concern to public health because of usage of fluoroquinolone, ciprofloxacin and third generation cephalosporins

such as ceftriaxone as commonly used agents for treating invasive *Salmonella* infections in adults and children respectively (Angulo *et al*., 2000; Fey *et al*., 2000). Because the overuse and misuse of antimicrobials can contribute to the dissemination of MDR strains, efforts that promote appropriate use of antimicrobials in both humans and animals are important. We also need to have better understanding of the various resistance mechanisms, the rate at which resistant occurs and effects resistance has on the life cycle of bacteria, in order to slow resistance development. Thus, the need continues for increased surveillance on a global basis of antimicrobial resistant phenotypes among *Salmonella* spp. of animal and human origin, with specific emphasis on susceptibility to drugs used to treat infection.

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CHAPTER 2

MOLECULAR CHARACTERIZATION REVEALS *SALMONELLA* ENTERICA SEROVAR 4,[5],12:i:- FROM POULTRY IS A VARIANT TYPHIMURIUM SEROVAR

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ABSTRACT

While *Salmonella* remains one of the leading causes of foodborne illnesses in the United States, various *S. enterica* serovars and genetic types associated with most infections appears to fluctuate over time. Recently, the Center for Disease Control and Prevention (CDC), has reported an increase in cases of salmonellosis caused by *S. enterica* 4,[5],12: i: -. Similarly, this unusual *Salmonella* serovar has been isolated from cattle and poultry in the state of Georgia. We examined the genetic relatedness of *S. enterica* 4,[5],12: i: -, isolated from several different poultry companies and dairy farms in Georgia, by pulsed-field gel electrophoresis (PFGE). Several *S. enterica* 4,[5],12: i: - isolates had PFGE patterns identical or similar to PFGE patterns of *S.* Typhimurium isolated from numerous animal sources. We identified distinct PFGE patterns for *S. enterica* 4,[5],12:i:- and matching *S.* Typhimurium PFGE patterns, identifying four "distinct" strains. We focused a more specific analysis on the poultry *S. enterica* 4,[5],12:i:- and *S.* Typhimurium isolates and found that of these *S. enterica* 4,[5],12: i: - isolates 32% lacked the entire phase 2 antigen gene, *fljB*; 61% contained partial deletion(s), and 4% had partial deletion(s) in it and an adjacent gene *hin*, 5' to *fljB*. Thirteen percent contained smaller mutations, deletion or point mutations not identified by our DNA probes. The *S. enterica* 4,[5],12:i:- isolates were positive for several genes present in the *S. enterica* serovar Typhimurium including *lpfE* (100%), *sseI* (96%) and *spvC* (93%). Genetic analysis indicates independent, spontaneous mutations in *fljB* in at least 4 distinct *S.* Typhimurium strains of animal origin circulating in nature.

Key Words: *Salmonella* Typhimurium, poultry, virulence, *S. enterica* 4,[5],12:i:-

Abbreviations: CDC = Centers for Disease Control and Prevention; FSIS = Food Safety Inspection Service; PCR = polymerase chain reaction; PFGE = pulsed field gel electrophoresis; USDA = United States Department of Agriculture

INTRODUCTION

Salmonellosis continues to be a major cause of gastroenteritis in the United States, with approximately 40,000 cases reported to the Centers for Disease Control and Prevention (CDC) each year. *Salmonella* outbreaks are often associated with contaminated animal products, such as eggs, milk and meat (CDC, 2005). While there are 2,541 known *Salmonella* serotypes (CDC, 2002), only a few cause significant human illnesses each year. Two of these *Salmonella* serovars, *S.* Typhimurium and *S.* Enteritidis accounts for ~50% of reported cases of salmonellosis every year (CDC, 2005).

A new *Salmonella enterica* serotype has recently emerged in humans with an antigenic formula, 4,[5],12:i:- similar to the formula 4,[5],12:i:1,2, assigned to the serovar *S.* Typhimurium. This new *Salmonella* serovar is often referred to as "monophasic" *S.* Typhimurium due to the absence of a flagellar, phase 2 antigen. *S. enterica* 4,[5],12:i:- has increased in frequency since first being recognized as a separate serotype in 1998 (CDC, 2002). The *S. enterica* 4,[5],12: i: - has jumped from the 18th most frequently reported serotype in human salmonellosis in 2002 to 14th in 2003 to 7th in 2004, to 6th in 2005, probably in part due to more correct classification based on antigenic formula to delineate this serovar (CDC, 2003, 2004, 2005, 2006). *Salmonella* serotype 4,[5],12:i:-also ranks in the top 10 in both broiler and ground chicken samples starting in 2004, the year USDA FSIS began reporting this serovar (FSIS, 2006).

In this paper we focus on the nature of *S. enterica* 4,[5],12:i:- in animal species to determine whether this is a serotype, distinct from *S.* Typhimurium serovar, and a clone disseminating through the food animal production system or if a change is occurring in an already existing *S.* Typhimurium strain population. Pulsed-field Gel Electrophoresis (PFGE) was used to compare chromosomal DNA restriction patterns of both *S.* Typhimurium and *S. enterica* 4,[5],12:i:- isolates to determine the relatedness of the two serotypes. PCR and DNA: DNA hybridizations confirmed that these isolates are truly *S.* Typhimurium variants that possess virulence genes, *spvC*, *sseI*, and *sopC* commonly present in this pathogenic serovar.

MATERIALS AND METHODS

Bacterial strains. A total of 163 *S. enterica* isolates, 131 *S.* Typhimurium and 32 *S.* 4,[5],12:i:- isolates, were examined in this study. Of the 131 *S.* Typhimurium isolates, 57 were from bovine sources, 11 from non-domestic birds (Hudson *et al*., 2000) 36 from poultry, including chickens (n = 31) and quail (n =5; 25), 21 from the SARA (*Salmonella* reference collection A) collection, 4 from the SARB (*Salmonella* reference collection B) collection (Boyd *et al*., 1993), and 10 poultry isolates collected from same farms as the poultry *S. enterica* 4,[5],12;i;-isolates described below. All isolates from bovine source were collected from various dairy and beef cattle farms throughout counties in Georgia. *Salmonella* from commercial quail was from a single company whereas *Salmonella* from commercial chickens were obtained from multiple companies located in the southeastern United States. The SARA and SARB collections consist of a set of *S. enterica* subspecies I, serovar Typhimurium, strains from a wide range of animals isolated from around the world. A total of 32 *S. enterica* 4,[5],12;i;-isolates were included in this study: 28 from poultry, 2 from bovine sources, and 2 from non-domestic birds (Hudson *et al*., 2000). These *S. enterica* 4,[5],12: i:- isolates were collected from several

different poultry companies, and dairy farms in Georgia and identified through Athens Diagnostic Laboratory at the University of Georgia and the Georgia Poultry Laboratory in Gainesville, GA.

Molecular typing by pulsed-field gel electrophoresis (PFGE). CDC PulseNet protocols were used for making agarose plugs and for PFGE conditions (CDC, 2004). Agarose plugs for each *Salmonella* strain was loaded on PFGE comb teeth along with λ ladder, molecular weight standards (Bio-Rad Laboratories; Hercules, CA) in the first, middle, and last lanes and *S.* ser. Braenderup H9812 (standard, QC control) in the second lane. Individual plugs were adhered to the comb teeth by placing a small drop of 1% SeaKem Gold (SKG) Agarose (0.5g agarose; 50ml 0.5X TBE, 55-60°C) behind each plug and allowing it to dry. The comb was positioned and 1% SKG Agarose was poured into the gel form. Thiourea was added (1.65 mM) to the standard 0.5X Tris-Borate EDTA Buffer (TBE), a modification to the CDC protocol (Koort *et al*., 2002). Electrophoresis was done using the CHEF-DRII apparatus (Bio-Rad Laboratories; Hercules, CA) for a total of 20 hours at 14°C at 6 V/cm with pulse times of 2.2 to 54.2 seconds. Following electrophoresis, the gel was stained with ethidium bromide (1µg/ml) for 30 minutes on a platform shaker and pictures were made of the stained gel. The gel was then destained using ultra-pure water for 60 minutes on the platform shaker and pictures were again taken, along with computer images (.tif file).

Cluster analysis of *S. enterica* 4,[5],12;i;- and Typhimurium PFGE patterns. The program Gene Profiler® was used to mark the DNA fragments produced on each PFGE pattern and estimate their size relative to the molecular weight standards. Once a one to one comparison of PFGE profiles was performed and matrix table created, the data was exported to the TreeCon® program which was used to create phylogenetic trees based on PFGE patterns. A

clustered master tree of all 163 isolates was created excluding *S.* Braenderup H9812 and using a match tolerance of 1%. Using the master tree and the agarose gel pictures, the PFGE patterns for each cluster were visually inspected and compared to other clusters using interpretive criteria outlined by Tenover *et al*. In order to better understand the similarities between the *S. enterica* 4,[5],12: i: - and *S.* Typhimurium isolates, representative patterns for the *S. enterica* 4,[5],12:i:- isolates were chosen along with *S.* Typhimurium isolates with similar or the same band patterns. These representative isolates were examined again by PFGE using the restriction enzymes *Xba* I and *Bln* I. DNA pattern recognition software, BioNumerics (Applied Maths, Inc.; Austin, TX) was used to align similar PFGE patterns for comparisons. Cluster analysis involved in this analysis was performed using Dice coefficient.

Colony blots. A master, 96-well plate was made for screening *Salmonella* isolates for several *Salmonella* genes, by DNA: DNA hybridization, as follows. Individual wells containing Lauria-Bertani (LB) with 7% glycerol was inoculated with each isolate. The isolate number was recorded for the position of the well inoculated. The last row for the master plate was reserved for positive and negative controls for each of the seven *Salmonella* genes screened in this study. The plates were incubated overnight at 37°C and subsequently stored at -80°C. This plate served as template for inoculating LB agar plates (150 x 15 mm) using flamed, 96-pin inoculator (Boekel Scientific; Feasterville, PA). Plates were incubated at 37°C for 24 hours. A 32 x 48 mm, nylon membrane was placed onto plates to lift isolates from the agar surface. The nylon membranes were processed for DNA: DNA hybridizations as described by Sambrook *et al*. (24).

DNA probes for the genes of interest were created by PCR with digoxigenin-labeled nucleotides by the procedure of Bass *et al*.. PCR primers are listed in Table 2.1. *S.* Typhimurium SR11 served as positive control and templates for the generation of PCR probes

for DNA-DNA hybridization for *spvC*, *sseI*, *sopE, fliC. fljB*. *Escherichia coli* LE392, *E. coli* HB101, and *S.* Enteritidis χ3968 (*fliC*i, and *fljB* only) were included as a negative control on all colony blots. DNA-DNA hybridizations were performed as described by Sambrook *et al*., with hybridizations and washes done at 68°C for all probes (Sambrook *et al*., 1989). Hybridization was detected with the antidigoxigenin antibody-alkaline phosphatase conjugate provided with the Genius System (Roche; Indianapolis, IN).

PCR. PCR reactions were carried out with the Rapidcycler hot-air thermocycler (Idaho Technology, Salt Lake City, Utah). A 10-µl PCR reaction was prepared by loading 9 µl of PCR master mix and 1 µl of DNA template in round bottom 96-well microtiter plate. The PCR master mix consisted of 2 or 3 mM $MgCl_2$, 50 pmol of each PCR primer, 10mM deoxynucleotide solution (Roche Diagnostic; Indianapolis, IN.), dH_2O and 0.5 U *Taq* DNA polymerase (Roche Diagnostic.). DNA sequence for *S. enterica* Typhimurium LT2 (22; GenBank Accession #: NC 003197) spanning open reading frame (ORF) for *hin* and *fljB* were analyzed, using DNA software analysis program GeneRunner (Hastings software; Hastings, NY), for possible PCR primer pairs that target the terminal 200 bp of *hin* ORF, the gene immediately 5' to *fljB* and its invertible promoter, and the 1st 200 bp of *fljB* ORF (Fig. 2.4). To characterize *fljB*, two PCR primer sets were used which amplified the 1st 200 bp ($fljB_2$), or entire *fljB* ORF ($fljB_1$), labeled with digoxygenin-dNTPs and used as DNA probes in DNA: DNA hybridizations.

RESULTS AND DISCUSSION

PFGE comparisons of *S. enterica* 4,[5],12;i;- isolates to *S.* Typhimurium reveals genetic similarity between the two serovars. We compared the PFGE of *S. enterica* 4,[5],12:i:- from animals against a PFGE database of *S.* Typhimurium isolated from numerous animal sources including poultry and cattle. We identified 37 distinct PFGE patterns (Tenover *et*

al., 1995) for the *S. enterica* 4,[5],12:i:- and *S. enterica* Typhimurium. *S. enterica* 4,[5],12:i:- strains appeared to cluster with the *S.* Typhimurium strains and fell into four distinct PFGE profile groups with *Xba* I (Fig. 2.5). We identified PFGE profiles for *S. enterica* 4,[5],12;i;- that had 0-3 band differences and corresponded with same or very similar PFGE patterns with *Xba* I for *S.* Typhimurium isolates (Fig. 2.5). We used a second restriction enzyme, *Bln* I to confirm genetic-relatedness of *Salmonella* isolates identified by the first restriction enzyme, *Xba* I (Fig. 2.6). It appears that poultry *S. enterica* 4,[5],12:i:- isolate 192500-A has exactly the same PFGE pattern as poultry *S*. Typhimurium strain 32362-34 (Fig. 2.5: lanes 28, 29). Poultry *S. enterica* 4,[5],12:i:- isolate 190691 B Chick Paper has the same pattern as *S*. Typhimurium isolate 196755FDS (poultry) (Fig. 2.5: lanes 24, 25). Also, *S. enterica* 4,[5],12:i:- isolates 176833 Heart (poultry) and A3-26248 (bovine) have a similar pattern (1-3 band differences) as poultry *S*. Typhimurium strains 47812 and 1483 (Fig. 2.5: lanes 14-17). These apparent matches were confirmed with *Bln* I (Fig. 2.6) except for *S. enterica* 4,[5],12:i:- isolate 190691B Chick Paper which exhibited 4 and 6 band differences with *S*. Typhimurium strains SARA 14 and 196755 FDS, respectively (Fig. 2.6: lanes 8-10). Although only one *S. enterica* 4,[5],12:i:- isolate, 192500-A matched exactly to a corresponding *S*. Typhimurium isolate 32362-34 in both gels (Fig. 2.5: lanes 28, 29 vs. Fig. 2.6: lanes 14, 15), other *S. enterica* 4,[5],12:i:- isolates appeared to have similar PFGE profiles, except for a few band differences. We also observed that *S. enterica* 4,[5],12:i:- isolated from two different sources (poultry vs. bovine) with the exact same PFGE patterns with both restriction enzymes (Fig. 2.5: lanes 1 and 3; Fig. 2.6: lanes 1 and 2). According to Tenovar *et al*. criteria for interpreting PFGE profiles, *S. enterica* isolates are considered closely related if they differ by 2-3 bands and possibly related if they differ by 4-6 bands. We found one example of an *S. enterica* 4,5,12:i:- isolate, 192432, and a *S.* Typhimurium

isolate, 196232YS, isolated on the same date and from the same poultry company, that are closely related (Fig. 2.5: lanes 27, 30; Fig. 2.5: lanes 17, 18). In addition to similarities in PFGE profiles between *S. enterica* 4,[5],12;i;- and *S.* Typhimurium isolates, several *S. enterica* 4,[5],12;i;- possessed 1,000 bp *fliB-A* intergenic region (Table 2.2), characteristic of *S. enterica* Typhimurium.

Work done by Agasan *et al*. also examined *Salmonella* 4,5,12:i:- strains found in humans in New York City using PFGE. The group of *S. enterica* 4,5,12:i:- isolates had 18 PFGE patterns that clustered into two related clusters and one unrelated cluster. These *S. enterica* 4,[5],12;i;- isolates were compared to *S.* Typhimurium isolates, including *S.* Typhimurium DT104, and they found *S. enterica* 4,[5],12:i:- isolates related to some of the *S.* Typhimurium isolates examined. In another study, de la Torre *et al*. examined *S. Enterica* serovar 4,5,12:i:- isolates from swine found in Spain and concluded that *S. Enterica* serovar 4,5,12:i:- strains may belong to a clonal lineage with close similarities to *S. enterica* serovar Typhimurium DT U302. Lastly, a group from Thailand, Amavisit *et al*. compared *S. enterica* serovar Typhimurium DT104, *S.* serovar Typhimurium U302 and *S.* serovar 1,4,[5],12:i:- (from human sources) PFGE profiles. Four of the *S. enterica* 1,4,[5],12:i:- isolates had the same or similar profiles compared to *S.* Typhimurium phage type U302. One *S. enterica* 1,4,[5],12:i:- isolate PFGE pattern was similar to the serovar Typhimurium DT104 profile with only three band differences between these two strains. The authors concluded that *S. enterica* 1,4,[5],12:i:- is most likely a variant of *S.* Typhimurium strains. It therefore appears that these *S. enterica* 1,4,[5],12;i;- isolates are a recent descendant from multiple *S.* Typhimurium strains that have independently acquired mutation(s) responsible for loss of phase 2 antigen expression.

The genetic basis of phase 2 antigen - phenotype of *S. enterica* 4,[5],12:i:-. The majority of *Salmonella* strains are biphasic. The ability of *Salmonella* to alternate phases is a result of a genetic regulatory mechanism that alternates expression of two separate and distinct phase 1 and phase 2 antigens: FliC and FljB (McNab, 1996). In the case of *S. enterica* 4,[5],12:i:- strain ,the *fljB* gene is either not present or contains mutation(s) affecting expression, resulting in the "monophasic" nature of these strains. *Salmonella* isolates were screened by colony blot, as described in materials and methods, for phase 1 and phase 2 antigen genes, *fliC* and *fljB* respectively. We found that 100% and 11% of the *S. enterica* 4,[5],12: i: -isolates were positive for *fliC* and *fljB,* respectively. Approximately 89% of *S. enterica* 4,[5],12:i:- isolates contained complete or partial deletions of the phase 2 flagellin gene, *fljB* (Table 2.3), while 96% of these isolates (Table 2.3) possessed the upstream gene, *hin*, the DNA invertase involved in "flipping" the *fljB* promoter (Fig. 2.4). One *S. enterica* 4,[5],12:i:- isolate was identified that contained deletions in both *hin* and *fljB*, however, retaining enough of *fljB* to be detected using the full length *fljB* DNA probe. A similar mutation was identified in *S. enterica* 9,12:l,v:- that were determined to be derived from *S.* serovar Goettingen (Burnens *et al*., 1996). Deletions in the *fljB* locus, observed in our *S. enterica* 4,[5],12:i:- poultry isolates, varied in the extent of these deletions within the *fljB* locus and may not be as extensive as the mutation reported by Gariazar *et al*., where 16 genes spanning this locus, including *hin*, *fljB*, and *fljA*, were deleted. However, we will need to refine our deletion map to regions immediately upstream and downstream of this locus to know the full extent for some of these deletions. The presence of *fljB* in 11% of the *S. enterica* 4,[5],12: i: - isolates appears to indicate that the mutation event is more subtle, possibly a point mutation or small deletion, affecting either transcription, translation, export, or assembly of *fljB* resulting in the "monophasic" phenotype of 4,[5],12:i:-

strain that went undetected in our screen. Alternatively, the "phase-variable", invertible promoter for the *fljA,*B operon may be in "locked" position, allowing expression of *fliC* while curtailing transcription of *fljB*. We are currently examining the *fljB* locus in greater detail in order to pinpoint mutation(s) to ORF or promoter for these remaining *S. enterica* 4,[5],12:i:- isolates.

Do poultry *S. enterica* 1,4,[5],12:i:- isolates possess the same virulence genes as "true" S. Typhimurium serovar? *S. enterica* 4,[5],12:i:- is generally categorized as a Typhimurium serovar. However, not all strains within the serovar Typhimurium may necessarily contain the full armament of genes necessary for virulence. Due to the increased association of *S. enterica* 4,[5],12:i:- with human illness, we analyzed our animal isolates to determine the distribution of virulence genes generally found in *S.* Typhimurium (Chan *et al.*, 2003; McClelland *et al.*, 2001) are also present in the poultry *S. enterica* 4,[5],12:i:- isolates. We found that all *S. enterica* 4,[5],12:i:- strains and *S. enterica* Typhimurium poultry strains contained the fimbrial gene *lpfE* (Table 2.2). In addition, ~96% of *S. enterica* 4,[5],12:i:- strains harbored the GIFSY-2 prophage associated invasion gene *sseI* compared to ~90% of *S. enterica* Typhimurium isolates. The gene *spvC*, a marker for the virulence plasmid, was present in 93% of the *S. enterica* 4,[5],12: i: - isolates and 90% of *S.* Typhimurium isolates. A detailed genetic analysis, by microarray, of multidrug resistant, *S. enterica* 4,[5],12:i:- strains, isolated from pork meat, sausages and one case of gastroenteritis, revealed that these strains contain many of the same genes as *S.* Typhimurium LT2, with the exception of FELS 1, 2, and GIFSY1 prophages (Garaizar *et al.*, 2002). However, these *Salmonella* strains did possess GIFSY2 prophage and its full complement of virulence associated genes and lambda-like prophage and its resident

virulence gene *sopE*. Therefore, the presence of these virulence genes in *S. enterica* 4,[5],12:i:- indicate their potential for causing significant illnesses in humans.

This is the first study to compare the genetic relatedness of *S. enterica* 4,5,12:i:- to *S.* Typhimurium isolated from commercial poultry in the U.S. We have found that *Salmonella* 4,5,12:i:- strains isolated from poultry do not represent the emergence of a new serovar or clone, rather the result of spontaneous mutations in endemic *S.* Typhimurium strains, affecting phase 2 antigen expression. From the evidence presented, it appears that these *S. enterica* 4,[5],12:i:- isolates are genotypically *S.* Typhimurium strains, possessing the virulence genes commonly associated with this pathogenic serovar. Therefore, this *Salmonella* 4,[5],12:i:- serovar should not be treated any differently than *S.* Typhimurium, especially with regards export or import restrictions concerning poultry with *S.* Typhimurium.

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FIGURES

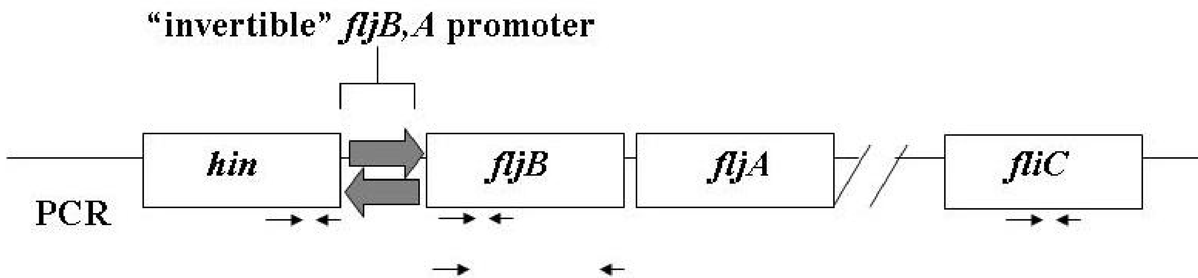


Fig. 2.4. Genetic map of phase 1 (*fliC*) and phase 2 (*fljB*) flagellin loci for *S. enter*ica. Phase 2 flagellin gene, *fljB* is part of an operon consisting of it and the downstream, *fljA* repressor of phase 1 flagellin gene, *fliC*. An "invertible" promoter controls the expression of the *fljB* locus, through *hin* a DNA invertase that causes inversion of the *fljB,A* promoter. Positions of PCR primers used in this study are shown.

Fig. 2.5. Genetic relatedness of *S.* Typhimurium and *S. enterica* 4,[5],12:i:- isolates as determined by pulsed-field gel electrophoresis (PFGE) with restriction enzyme *Xba* I and cluster analysis using the Dice coefficient. Symbol proceeding each isolation number designates source as follows: "bull's-eye"= poultry; star = cattle; square = non-domestic birds; x = other animal source; closed circle = SARA collection; and diamond = SARB collection. *S.* Typhimurium isolates are designated by box around isolate number vs. *S. enterica* 4,[5],12:i:- isolates where the isolate number is left unmarked. Band position tolerance was set a 1.8% for cluster analysis by the Dice coefficient.

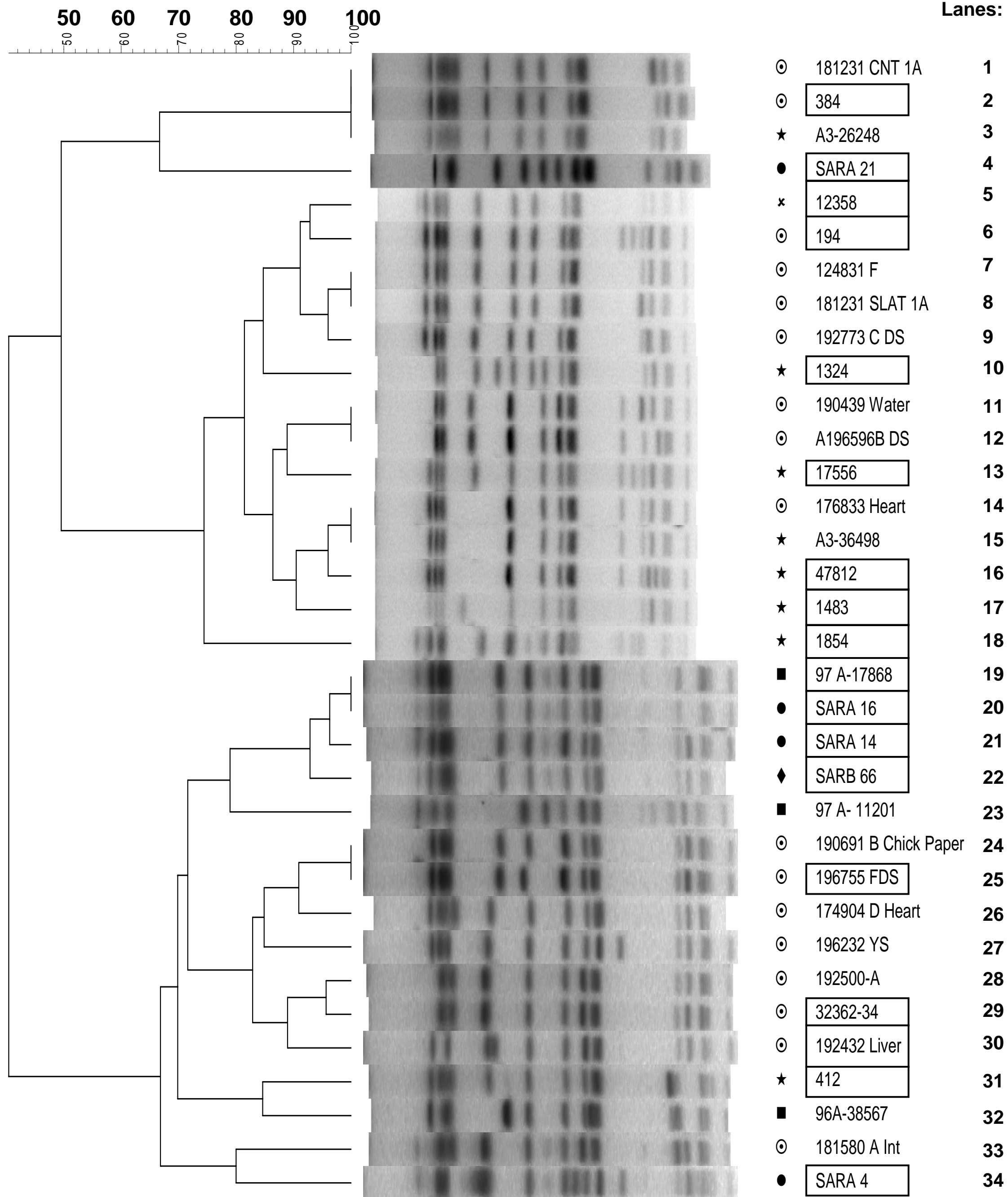
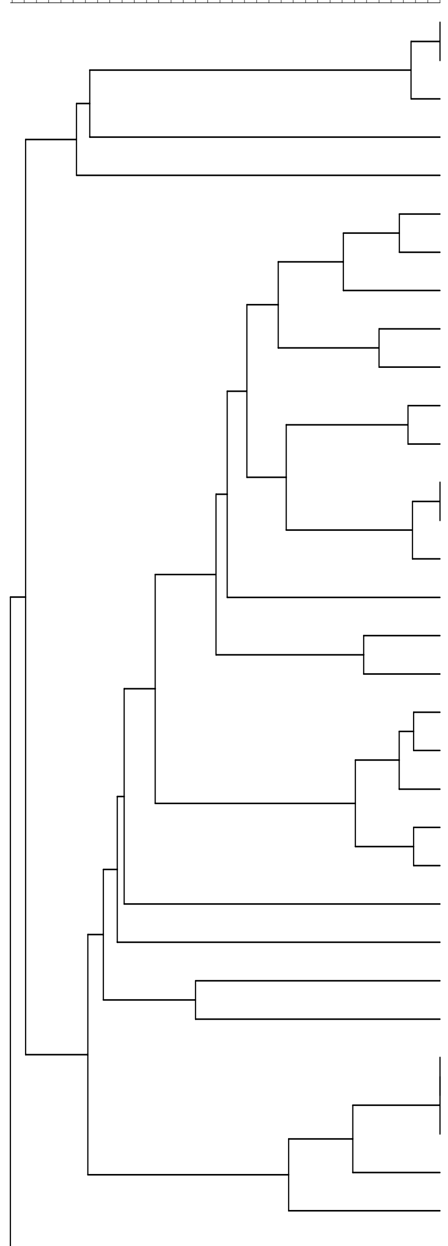
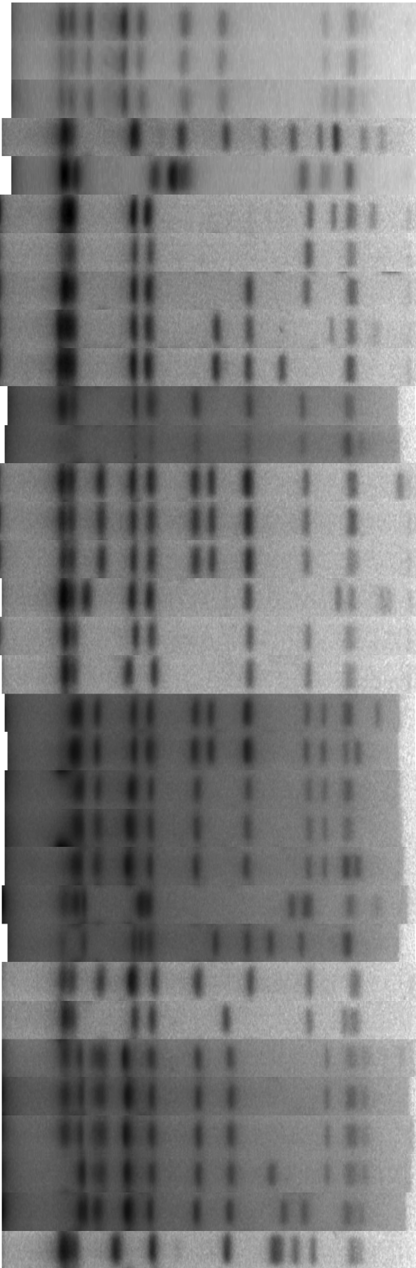

50
60
70
80
90
100
Lanes:
181231 CNT 1A 1
384 2
A3-26248 3
SARA 21 4
12358 5
194 6
124831 F 7
181231 SLAT 1A 8
192773 C DS 9
1324 10
190439 Water 11
A196596B DS 12
17556 13
176833 Heart 14
A3-36498 15
47812 16
1483 17
1854 18
97 A-17868 19
SARA 16 20
SARA 14 21
SARB 66 22
97 A- 11201 23
190691 B Chick Paper 24
196755 FDS 25
174904 D Heart 26
196232 YS 27
192500-A 28
32362-34 29
192432 Liver 30
412 31
96A-38567 32
181580 A Int 33
SARA 4 34

Fig.2.6. Genetic relatedness of *S.* Typhimurium and *S. enterica* 4,[5],12:i:- isolates as determined by pulsed-field gel electrophoresis (PFGE) with restriction enzyme *Bln* I and cluster analysis using the Dice coefficient. Symbol proceeding each isolation number designates source as follows: "bull's-eye"= poultry; star = cattle; square = non-domestic birds; x = other animal source; closed circle = SARA collection; and diamond = SARB collection. *S.* Typhimurium isolates are designated by box around isolate number vs. *S. enterica* 4,[5],12:i:- isolates where the isolate number is left unmarked. Band position tolerance was set a 0.8% for cluster analysis by the Dice coefficient. PFGE with *Bln* I was not done for non-domestic bird, *S. enterica* 4,[5],12:i:- 97 A-11201.

30 40 50 60 70 80 90 100





Lanes:

⊙	181231 CNT 1A	**1**
★	A3- 26248	**2**
⊙	384	**3**
★	412	**4**
●	SARA 21	**5**
■	97 A-17868	**6**
●	SARA 16	**7**
●	SARA 14	**8**
⊙	190691 B Chick Paper	**9**
⊙	196755 F DS	**10**
★	1324	**11**
★	1483	**12**
⊙	181580A- Int	**13**
⊙	32362-34	**14**
⊙	192500 A DS	**15**
■	96 A- 38567	**16**
⊙	192432 Liver	**17**
⊙	196232 YS	**18**
⊙	190439 Water	**19**
⊙	A 196596 B DS	**20**
★	A3- 36498	**21**
⊙	176833 Heart	**22**
★	47812	**23**
★	1854	**24**
★	17556	**25**
⊙	174904 D Heart	**26**
♦	SARB 66	**27**
×	12358	**28**
⊙	124831 F	**29**
⊙	181231 SLAT 1A	**30**
⊙	194	**31**
⊙	192773 C DS	**32**
●	SARA 4	**33**

TABLES

Table 2.1. PCR primers.

Gene	Sequence	PCR Conditions (°C; mM) [A]	Expected Size (bp)	Reference
Phage-Associated Virulence Genes				
sopE	F: GCAACACACTTTCACCGAG	50 °C, 2mM	400	This Study
	R: ATGCCTGCTGATGTTGATTG			
sseI	F: ATTTATCGTATTGCCTGGTC	45°C, 2mM	200	This Study
	R: TCCTCCCATCCGTCATAC			
Virulence Plasmid				
spvC	F: CGGAAATACCATCTACAAATA	42°C, 2mM	669	26
	R: CCCAAACCCATACTTACTCTG			
Adhesin Gene				
lpfE	F:TTTGATGCCAGCGTGTTTACTG	50°C, 2mM	611	4
	R:TAGACCACCAGCAGAGGGAAAG			

Flagellar Genes				
fliC	F:AACGAAATCAACAACAACCTGC	55°C, 2mM	508	This study
	R:TAGCCATCTTTACCAGTTCCC			
$fljB_1^B$	F:CAAGTAATCAACACTAACAGTC	55°C, 2mM	1500	15
	R:TTAACGTAACAGAGACAGCAC			
$fljB_2^B$	F:CTACGCGCTTCAGACAGATTG	45°C, 2mM	200	This study
	R:GCCGCTGGTCATGATTTC			
hin^B	F:GCGCTACTGGTATCAATAC	45°C, 2mM	200	This study
	R:GACCGTATCAGTGGCAAG			
fljB-fliA	F:CTGGCGACGATCTGTCGATG	55°C, 3mM	1000, 250	17
	R:GCGGTATACAGTGAATTCAC			

[A] Annealing temperature and $MgCl_2$ concentration. [B] PCR amplifies 1st 200 bp ($fljB_2$),or entire *fljB* open reading frame ($fljB_1$). We targeted PCR to the terminal 200 bp of *hin* open reading frame, the gene immediately 5' to *fljB* and its invertible promoter.

Table 2.2. Distribution of flagellar and virulence genes in poultry *S. enterica* 4,[5],12:i:- and *S.* Typhimurium isolates

[A] Isolates positive by DNA: DNA hybridization using PCR generated probes. PCR was used to amplify and label the 1st 200 bp (*$fljB_2$*), or entire *fljB* open reading frame (*$fljB_1$*). We also targeted the terminal 200 bp of *hin* open reading frame, the gene immediately 5' to *fljB* and its invertible promoter, to generate *hin*-specific DNA probe.

Serovars	Flagella Loci (%)							Virulence Genes (%)			
		fljB locus			*fliB-fliA* intergenic region						
	fliC	*hin*[A]	*$fljB_1$*[A]	*$fljB_2$*[A]	1,000 bp	250 bp	No Amplicon	*lpfE*	*spvC*	*sseI*	*sopE*
4,[5],12; i; -	28 (100)	27 (96)	19 (68)	2 (7)	17 (61)	1 (4)	10 (35)	28 (100)	26 (93)	27 (96)	0(0)
Typhimurium	10 (100)	10 (100)	10 (100)	10(100)	4 (40)	0 (0)	6 (60)	10 (100)	9 (90)	9 (90)	0 (0)

Table 2.3. Mutation(s) affecting phase 2 flagellin expression in *S. enterica* 4,[5],12:i:-.

Genotype[A]	*S. enterica* 4,[5],12; i; - isolates (%)
No deletion [B]	3 (11)
Δ*fljB*$_1$ [C,F]	9 (32)
Δ*fljB*$_2$ [D,F]	17 (61)
Δ*hin-fljB* [E]	1 (4)

[A]*fljB* is the structural gene for the phase 2 antigen in biphasic *S. enterica*. *hin* is the DNA invertase gene, 5' to *fljB* and responsible for inversion of the *fljB* promoter to "on" or "off" position.

[B] Isolates were positive by DNA: DNA hybridization for both *hin* and *fljB*. Nature of mutation affecting phase 2 antigen expression in these isolates is currently unknown. [C] Deletion of the entire *fljB*. [D] Partial deletion in the amino-terminal region of the *fljB* ORF. [E] Deletion of terminal 200 bp of *hin* and at least the 1st 200 bp of *fljB*. [F] The *hin*, DNA invertase, 5' of *fljB* is still present in isolates.

Chapter-3

IN VITRO MEASUREMENT OF DIFFERENT VARIABLES INVOLVED IN TRANSFER OF MULTIDRUG RESISTANT NEWPORT PLASMID IN *SALMONELLA*

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ABSTRACT

The emergence of multiple-drug resistant *Salmonella* has made antibiotic resistance an important, health issue in medicine and food safety. Surveillance data over the past 2 decades indicates that salmonellosis has been caused by multiple waves of multi-drug resistant serovars that emerge initially in animal populations, often calves. The factors that influence the emergence and spread of these multi-drug resistant serovars are unknown; however antibiotic usage in animals is hypothesized to be a major contributor. Antibiotic resistance is encoded by transposons and plasmids that are frequently transferable by conjugation. In order to study the factors that affect gene transfer, we designed a series of experiments to quantitate transfer of the *Salmonella* Newport multi-drug resistance (MDR) plasmid. In contrast to published studies that reported the plasmid as non-conjugative, we have successfully transferred a Newport plasmid to *E. coli* and the transconjugants are able to transfer the plasmid to other *Salmonella* serovars. Results from these studies will enable us to model plasmid transfer dynamics and determine factors that affect the rate of transfer and plasmid stability in the farm environment.

INDEX WORDS: *Salmonella* Typhimurium, MDR Newport plasmid, Prebiotic, Biomoss™

INTRODUCTION

Salmonella is one of the leading causes of bacterial foodborne diseases in the United States, accounting for an estimated 1.4 million non-typhoidal infections, 16,000 hospitalizations and nearly 600 deaths each year (Mead *et al*., 1999). These infections often result from consuming contaminated foods like poultry, produce, red meats, milk, seafood, eggs and others (Angulo *et al.,* 2004; CDC, 2005a; Dechet *et al*., 2006 Gomez *et al.,* 1997; Mead *et al*., 1999; Zhao *et al*., 2003a). *Salmonella* can also be transmitted from person to person contact, or when a carrier contaminates food during preparation. Another possible way to transmit *Salmonella* is through direct contact with animals (Dunne *et al*., 2000; Fey *et al*., 2000).

The emergence of multiple-drug resistant *Salmonella* has made antibiotic resistance an important, health issue in medicine and food safety (Glynn *et al*., 1998). Surveillance data over the past 2 decades indicates an increase in cases of salmonellosis caused by multiple waves of multi-drug resistant (MDR) *Salmonella* serovars. The MDR serovars emerged initially in animal populations, often seagulls and then calves (Lee *et al*., 1994, Helms *et al*., 2002, Davis *et al*., 1999; Evans and Davies, 1996). These MDR *Salmonella* strains have reduced susceptibility to $\geq$ 5 antimicrobials. Two of these multiple MDR *Salmonella* serovars; *S.* Newport and *S.* Typhimurium, are widely recognized for their high morbidity in humans and cattle (Threlfall *et al*., 1996; Cobbold *et al*., 2006). *Salmonella enterica* serotype Typhimurium, known as definitive type 104 (DT 104) appeared in late 1980s and raised concern because of its resistance to at least five antimicrobial classes - ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracycline (R-type ACSSuT). It has been of particular concern to public health because of increased rates of hospitalization and greater morbidity and mortality (Threlfall *et al*., 2000; Glynn *et al*., 1998).

Another MDR *Salmonella* serovar Newport has recently emerged and increased in its reported incidence in the U.S. (Dunne *et al*., 200, Winokur *et al*., 2000, Fey *et al*., 2000) while DT104 continues to cause significant foodborne outbreaks worldwide (Helms et al., 2005). The decreased susceptibility to cephamycins and third generation cephalosporins is due to the plasmid-mediated AmpC-like β-lactamase gene bla_{CMY-2} possessed by these MDR isolates (Zhao *et al*., 2003). It is the possession of these MDR phenotypes, specifically resistance to extended spectrum cephalosporins, by some strains that has greatly complicated the treatment and control of disease both in humans and cattle (Akkina *et al*., 1999; Cobbold *et al*., 2006; Dunne *et al*., 2000; Fey *et al*., 2000; Rankin *et al*., 2002; Zansky *et al*., 2002).

The factors that influence the emergence and spread of these multi-drug resistant *Salmonella* serovars are presently not known. However, antibiotic usage in veterinary medicine and agriculture has been hypothesized to be a major contributor whether by promoting emergence of novel resistant strains or via enhanced dissemination of selected clones of resistant bacteria (Angulo *et al*., 2000; Hancock *et al*., 2000; WHO, 2000). Once antibiotic pressure is introduced in an environment, antimicrobial resistance quickly develops, spreads and persists even without continuing selective pressure from antibiotics. Antibiotic resistance genes can be borne on transposons and plasmids that are frequently transferable by conjugation. Conjugation is considered to be an important mechanism of gene transfer and establishing new genetic traits (Ashelford *et al*., 1998; Bale *et al*., 1987; Hoffmann *et al*., 1998; Hausner *et al*., 1999; Kroer *et al*., 1998; Kuehn *et al*., 1998). In order to better understand how rapidly the drug resistance develops requires a complete knowledge of significant variables that are involved in genetic exchange.

European Union has put the emphasis on lowering the use or ban antibiotics in livestock feeds, and there is a strong possibility that other countries may follow the same track in order to reduce the prevalence of resistance to antibiotics in humans (White *et al*. 2002). These constraints have raised the need of an alternative to antibiotics, which can improve animal growth and intestinal health, improve integrity of the intestinal mucosa and suppress enteric pathogens. Probiotics and prebiotics are two available alternatives to antibiotics with a potential to reduce enteric diseases in poultry and the contamination of poultry with *Salmonella* or *Campylobacter*, which are primary concerns to the poultry industry (Patterson *et al.* 2003).

Probiotic has been defined as “a live microbial feed supplement, which beneficially affects the host animal by improving the intestinal balance” (Fuller, 1989). Probiotics include live microorganisms, including species of *Bifidobacterium*, *Bacillus, Enterococcus, Escherichia coli, Lactobacillus, Lacococcus, Streptococcus*, and a variety of yeast species (Patterson *et al.* 2003). *Bacillus, Enterococcus,* and *Saccharomyces* yeast have been the most common microbes used in livestock whereas *Lactobacillus* and *Bifidobacterium* species have been the most extensively used in humans (Simon *et al*. 2001).

Prebiotics are defined as “non digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon” (Gibson and Roberfroid, 1995). Thus, to be effective, prebiotics must escape digestion in the upper gastrointestinal tract and be used by a limited number of the microorganisms comprising the colonic microflora. Prebiotics are principally oligosaccharides and the dominant prebiotics are fructooligosaccharide products (FOS, oligofructose, inulin) (Patterson *et al.*, 2003). They mainly stimulate the growth of healthy intestinal bacteria (such as bifidobacteria and lactobacilli) at the expense of the less friendly putrefactive bacteria (such as bacteroides,

clostridia, and other coliforms) (Kaplan *et al.*, 2000). These bacteria produces lactic acid and increases the concentration of short-chain fatty acids in the gut, resulting in lowering of the pH in the large intestine which may inhibit the growth of pathogenic bacteria and stimulate intestinal peristalsis (Juven *et al*., 1991). Mannan oligosaccharides have been used in the same manner as the above listed prebiotics, but they do not selectively enrich for beneficial bacterial populations. Their mechanism of action is thought to act by binding and removing pathogens from the intestinal tract and stimulation of the immune system (Spring *et al*. 2000).

"BIOMOSS" is a commercial synthetic mannan-oligosaccharide derived from the cell wall of the yeast *Saccharomyces cerevisiae*. It is made from the lysed cell culture which has been centrifuged and washed before being spray dried (Spring *et al*., 2000). Oligosaccharides occur naturally as an ingredient in many feeds, and raffinose series oligosaccharides are the most common ones. These oligosaccharides have been researched for their impact on the reduction of intestinal pathogens and on immune modulation. *Biomoss* has shown some promise in suppressing enteric pathogens, improving the integrity of the intestinal mucosa and modulating the immune response when studied in chickens and turkeys (Olsen, 1996). Though the results seem somewhat inconsistent, some research suggests that mannan-oligosaccharide may improve growth performance and help in suppressing enteric pathogens like *Salmonella* and *Campylobacter* (Spring *et al*. 2000; Fernandez *et al.,* 2000). Some studies have also shown that these mannan oligosaccharides reduce the risk of disease by reducing the proliferation of pathogenic species. It also has the added benefit of improving the digestibility of various dietary fractions.

The objective of this study was to develop, apply and validate a mathematical model on plasmid transmission dynamics of *Salmonella*, which will eventually help us to better understand

the processes underlying the acquisition and spread of multiple drug resistance among major foodborne pathogens. In order to study the factors that affect gene transfer, we designed a series of experiments to quantify transfer of the *Salmonella* Newport multi-drug resistance plasmid and measure the plasmid's host range (broad vs. narrow), plasmid transfer rate, plasmid segregation rate, associated fitness costs, growth rates by measuring the changes in donor, recipient and transconjugants (recipients with plasmids) population over time, for both the prebiotic *"BIOMOSS"* treatment group and the control. Results from these studies will enable us to model plasmid transfer dynamics and determine factors that affect the rate of transfer and plasmid stability in the farm environment.

MATERIALS AND METHODS

Bacterial Strains:

Salmonella Typhimurium strain 934R and *E.coli* strain 14407 x 1932 were used as recipient and donor, respectively, in this study. The MDR plasmid from *S.* Newport strain 14407 was first introduced into *E. coli* K12 strain 1932 through filter matings. The resulting transconjugant *E. coli* (14407 x 1932) served as the donor strain in this study. The donor possessed a diverse antibiotic resistance genotype with *intI1, floR, blaCMY2, strA, tet(A)* genes borne on the conjugative plasmid. The *floR* gene, which encodes florofenicol resistance, was used as a marker in screening for the *S.* Newport plasmid in presumptive *S.* Typhimurium transconjunts. *Salmonella* Typhimurium strain 934R was made rifampin (64µg/ml) resistant and used as recipient in this study.

Plasmid Transfer Frequency:

The selective screening method was used to differentiate recipients (*S. Typhimurium*), donor (*E. coli* 14407 x 1932) and transconjugants (*Salmonella* recipient with plasmid). On the

first day, 5 ml of Lauria-Bertani (LB) broth was inoculated with a single colony of either recipient or donor and incubated overnight at 37°C. Five different groups of dilutions were made, four with different concentrations (i.e., 1%, 0.5%, 0.25%, 0.125%) of *Biomoss*™ and one without *Biomoss*™ so as to compare the effect of this mannan oligosaccharide on the plasmid transfer frequency from donor to the recipient. For each treatment group, recipient only, donor only and 1:1 recipient + donor mix, with cell density of 2.5 x 10^8 cfu/ml, were added to their respective tubes. The same protocol was followed for all the different *Biomoss* concentrations and one without *Biomoss*, incubating the cultures at 37°C. After 2 and 24 hr of incubation, the 10^{-6} to 10^{-8} dilutions for recipient and donor alone and 10^{-0} to 10^{-3} dilutions of the 1:1 donor-recipient mix were plated on LB + rifampin (64µg/ml), LB + florofenicol (32µg/ml), and LB + rifampin (64µg/ml) + florofenicol (32µg/ml) plates, and cell densities were recorded after 18-20 hr of incubation at 37°C. The antibiotics florfenicol, rifampin and the combination florfenicol/rifampin selects for donor, recipient, and transconjugants (recipient with plasmid), respectively. The plasmid transfer frequency was calculated by taking the ratio of total number of transconjugants divided by the total number of recipients.

Determining Fitness Cost of *S.* Newport Plasmid on Recipient:

To measure the fitness cost of the *S.* Newport plasmid on *Salmonella* Typhimurium strain 934R, *Salmonella* transconjugants and recipient strains were grown in the presence of antibiotics and plated on LB with rifampin (64µg/ml) + florofenicol (32µg/ml) and LB + rifampin (64µg/ml) alone. Loop full of an overnight, static culture was used to inoculate 5ml of LB + florofenicol (32µg/ml) broth and LB broth only for transconjugant and recipient respectively and tubes were incubated overnight. The next day, 1ml of the overnight broth culture was transferred to microfuge tubes and centrifuged at 4,500 x g for 10 minutes to pellet the cells. The

supernatant was decanted and the cells were resuspended in 1ml of water. 50µl of the each resuspended, cell culture was used to inoculate 10 ml of LB with no antibiotics, with 10-fold serial dilutions (10^{-4} to 10^{-7}) made and plated onto LB & LB + florofenicol (32µg/ml) plates at 0 hr Recipient and transconjugants were grown alone in separate tubes also, to serve as the controls necessary for determining fitness cost. After 24 hr, we plated 10^{-5} to 10^{-9} dilutions onto appropriate LB plates, with/without antibiotics, and recorded cell densities following 20-24 hr incubation at 37°C.

Plasmid Segregation Rate:

Plasmid segregation rate was performed for five consecutive days by passaging 100 µl of overnight grown culture into fresh 100 ml LB broth sans antibiotics. On the first day, 5 ml of LB broth was inoculated with a single colony of *Salmonella* transconjugant strain and grown overnight under the selective pressure of florofenicol (32µg/ml). On the following day, 100 µl was transferred to fresh 100ml of LB broth. 100 µl inoculum was passaged to fresh sterile LB broth and the inoculum was plated on LB and LB + florofenicol (32µg/ml) plates for five consecutive days and the cell densities (CFU/ml) were recorded daily from both types of plates to calculate the plasmid segregation rate. Plasmid segregation rate was calculated as described by Modi and Adams, 1991.

DNA Template:

Isolated colonies from LB with florfenicol and rifampin were streaked onto the LB agar with rifampin (64µg/ml) plates and incubated overnight in 37°C. The next day, cells were scraped from plate with sterile cotton swabs and resuspended in 1ml of double distilled (dd) H_2O to a turbid cell suspension of 1.0 OD units (λ 600 nm). The cell suspension was boiled for 10

min, followed by centrifugation at 4,500 x g for 10 minutes. The supernatant was diluted 1:20 in dd H_2O and used as template for PCR.

PCR:

PCR reactions were carried out with the Rapidcycler hot-air thermocycler (Idaho Technology, Salt Lake City, Utah). A 10-µl PCR reaction was prepared by loading 9 µl of PCR master mix and 1 µl of DNA template in round bottom 96-well microtiter plate. The PCR master mix consisted of 2 or 3 mM $MgCl_2$, 50 pmol of each PCR primer, 10mM deoxynucleotide solution (Roche Diagnostic; Indianapolis, IN.), dH_2O and 0.5 U *Taq* DNA polymerase (Roche Diagnostic.). Primers were designed to confirm presumptive transconjugants as *Salmonella* and verify the presence of the MDR S. Newport plasmid using *floR* gene as its marker. The *invA* PCR primer set designed for *Salmonella* confirmation yields a product of 437 bp and the program parameters were 30 cycles of 93°C for 1 s, 42°C for 10 s, and 72°C for 15 s (Liu *et al*., 2002). The *floR* primers used to detect *floR* genes yields a product of 399 bp and the program parameters for the hot-air thermocycler were 30 cycles of 94°C for 1 s, 40°C for 1 s, and 72°C for 15 s (Keyes *et al*., 2000).

Agglutination test with *Salmonella* O Poly A-I and Vi:

Serological confirmation involves the procedure in which the microorganism (antigen) reacts with its corresponding antibody. This *in vitro* reaction produces macroscopic clumping of the cell suspension.

For in vitro confirmation of *Salmonella*, a drop of *Salmonella* O Poly A-I and Vi antisera (BD Difco) were spotted onto a clean glass slide. With a sterile toothpick, a loopful of cells from a tryptic soy agar (TSA) was mixed in the drop of antisera. The slide was rocked for 10-15 s before recording results. If the agglutination occurs then it confirms the strain as *Salmonella*.

Agglutination of the somatic antigen in the slide test appears as granular clumping of the cell suspension (Difco Manual).

RESULTS AND DISCUSSION

Conjugation is an important mechanism for gene transfer and establishing new genetic traits (Ashelford *et al*., 1998; Bale *et al*., 1987; Hoffmann *et al*., 1998; Hausner *et al*., 1999; Kroer *et al*., 1998; Kuehn *et al*., 1998). Conjugation is a multi-step process in the transfer of plasmid or transposon from one cell (donor) to another (recipient). In the case of gram-negative bacteria, conjugation begins with contact between the cell surface of the recipient cell and the tip of a sex pilus of a donor cell, followed by contraction of the pilus, and direct contact between the donor and recipient outermembranes to form a channel through which the replicated, single-stranded DNA molecule passes. The donor plays host to conjugative or mobilizable (plasmid requiring a second, "helper" plasmid for conjugation) genetic element transferred from one cell to the next.

In order to study the plasmid transmission dynamics of *Salmonella*, the MDR *S.* Newport 14407 plasmid was first introduced into *E.coli* K12 strain 1932 through matings. The resulting transconjugant *E. coli* (14407 x 1932) served as the donor strain in this study. The *S.* Newport plasmid possessed a diverse antibiotic resistance genotype including class 1 integron, the DT104 florfenicol/chloramphenicol resistance gene *floR* (Bolton et al., 1999), bla_{CMY2} (Rankin *et al*., 2002), *strA,B,* and *tet(A)*. To find out whether this plasmid is conjugative, non-conjugative or mobilizable, several broth and filter mating experiments were performed with *Salmonella* Typhimurium strain 934R as the recipient. We found that MDR *S.* Newport 14407 plasmid was in itself conjugative, in contrast to one published study where investigators found that the plasmid as transferable with the assistance of a helper plasmid (Rankin *et al*.2002, Winokur *et*

al., 2000). The plasmid's host range was not limited to *S*. Newport but could be transferred to other *S. enterica* serovars. Plasmid transfer frequency was highest for *S*. Typhimurium (8.0×10^{-6}) in comparison to *S*. Heidelberg and other *Salmonella* serovars where transfer frequencies ranged from 1.8×10^{-7} to 3.8×10^{-7} (see Table 3.1). Transfer of the plasmid to *Salmonella* Typhimurium strain 934R was confirmed by *floR* PCR (see Figure 3.4 & 3.5).

Plasmid segregation is another factor that affects the maintenance and persistence of antibiotic resistance in the new as well as residential bacterial population (Lee and Maurer, 2006). The ability to propagate genetic information faithfully is a prerequisite for the evolutionary success of bacterial plasmid and many bacterial plasmid encode their own machinery to ensure their proper segregation and subcellular positioning (Gerdes *et al*., 2000). High copy plasmids, which are commonly used in molecular cloning are randomly distributed to the two daughter cells upon cell division. However, low copy plasmids have active mechanisms to ensure their precise segregation such that daughter cells inherit an equal number of plasmids (Gerdes *et al*., 2000, Gitai, 2006).

In this study, MDR *S*. Newport 14407 plasmid appeared to be relatively stable. Even after 50 generations in the absence of antibiotic selection pressure, florofenicol, the plasmid was maintained. The cell densities recorded for five consecutive days on LB and LB + Florofenicol (32µg/ml) plates were similar (see Table 3.3). We calculated the plasmid segregation rate (v) after 50 generations as described by Modi *et al*., and we found that the segregation rate was low ($v = 8.6 \times 10^{-2}$) as compared to their results. Results suggest that MDR *S*. Newport 14407 plasmid is relatively stable.

With the development of antibiotic resistance in bacteria either through the acquisition of resistance elements or mutation, usually there is a fitness cost associated with resistance

(Andersson *et al.,* 1999). This fitness cost is observed as reduced transmission between hosts, reduced persistence and growth within and outside hosts, with resistant bacteria out competed by susceptible strains in the absence of drug and the decline of the frequency of resistance over time (Mariam *et al*., 2003). However, in some cases, the fitness cost associated with some resistance mutations is less than 1%. For example, *katG* mutations (isoniazid resistance) in *M. tuberculosis* (Pym *et al.,* 2002), *rpsL* mutations (streptomycin resistance) in *M. tuberculosis* (Bottger *et al.,* 2000), *Escherichia coli (*Schrag *et al.,* 1996; Schrag *et al.,* 1997) and *Salmonella enterica* serovar Typhimurium (Bjorkman *et al.,* 1998; Hughes *et al.,* 1998), and *gyrA* and *parC* mutations (fluoroquinolone resistance) in *Streptococcus pneumoniae* (Gillespie *et al.,* 2002) confer no measurable fitness cost.

We measured in-vitro fitness cost of our *Salmonella* transconjugant (recipient with plasmid) in comparison to the *Salmonella* recipient strain. Our results indicate that there was some fitness cost associated with the transconjugants. Cell density of the transconjugant and recipient were higher when grown independently, as compared to when they were grown together. The cell density of transconjugant was 9.6 x 10^8 cfu/ml when grown alone in comparison to 2.4 x 10^8 cfu/ml when grown in competition with recipient *Salmonella* Typhimurium strain 934R. We found that the plasmid adversely impaired the growth of the *Salmonella* transconjugant strain compared to the parental, recipient strain (Figure 3.6). Our results were in agreement with Meriam *et al* showing that there is an associated fitness cost with the acquisition of drug-resistance elements (Mariam *et al*., 2003).

In this study, a prebiotic "*BIOMOSS"* was included to see it could block plasmid transfer to *Salmonella*. It has been proposed that *Biomoss*™ reduces colonization of the intestine by enteric pathogens through the binding of the oligomannan to the type 1 fimbriae, an adesin

involved in bacterial attachment to epithelial surfaces (Howard *et al*., 1995, Spring *et al*., 1999). We did not find any significant difference in the plasmid transfer frequency of the *S.* Newport plasmid between the prebiotic *Biomoss* treatment groups and the control group (see Table 3.4). Data was analyzed by analysis of variance (ANOVA) and P-value was not significant ($P = 0.64$).

While this prebiotic does not prevent plasmid transmission in vitro, it may have a profound effect in vivo. This might be possible that the incorporation of Biomoss™ in the diet may prevent or reduce the colonization of enteric pathogens like *Salmonella*, possibly by lowering the pH of the large intestine as a result of lactic acid and short chain fatty acids produced by bifidobacteria. (Juven *et al*., 1991).

We have measured different variables involved in plasmid transfer in vitro and assessed whether a prebiotic like Biomoss™ might interfere with this process. These experiments have provided us the information necessary for assessing and validating a mathematical model of plasmid transmission dynamics of *Salmonella*. We have assessed the affect of the prebiotic Biomoss™ on plasmid transfer and did not find any significant difference on the plasmid transfer frequency.

Our long term goal is to understand the process underlying the acquisition and spread of multiple drug resistance among major food borne pathogens. The development of this model will allow us to predict the emergence and prevalence of resistance in *Salmonella* within the gastrointestinal tract of food animals, especially in response to veterinary use of antibiotics in poultry and other food animals. Knowing how resistance actually disseminates in these ecosystems will help identify the best points for novel interventions.

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TABLES

Table 3.1 Host range and transfer frequency of *S.* Newport 14407 MDR plasmid in different *Salmonella* serovars.

Different *Salmonella* Serovars	**Plasmid Transfer frequency (# transconjugants / # recipients)**
***S.* Typhimurium**	8.0×10^{-6}
***S.* Heidelberg**	1.8×10^{-7}
***S.* Infantis**	3.8×10^{-7}
***S.* Kentucky**	3.3×10^{-7}
***S.* Enteritidis**	1.4×10^{-7}
***S.* Montevideo**	1.7×10^{-7}

Table 3.2 Effect of Biomoss™ on plasmid transfer frequency of *S.* Newport 14407 plasmid between donor *E. coli* 1932 x 14407 and recipient *Salmonella* Typhimurium strain 934R via conjugation.

Biomoss %age	**Transfer frequency (# transconjugants /# recipients)**		
	First Trial	**Second Trial**	**Third Trial**
0.125%	0.33×10^{-6}	0.82×10^{-6}	0.42×10^{-6}
0.25%	0.58×10^{-6}	0.96×10^{-6}	0.43×10^{-6}
0.5%	0.58×10^{-6}	0.82×10^{-6}	0.36×10^{-6}
1.0%	0.30×10^{-6}	0.74×10^{-6}	0.40×10^{-6}
Without Biomoss	0.21×10^{-6}	0.64×10^{-6}	0.20×10^{-6}

Table 3.3. Stable maintenance of the MDR *S.* Newport 14407 plasmid in the absence of antibiotic selection pressure.

Time	**No Antibiotics (CFU/ml)**	**Florofenicol (CFU/ml)**	**Ratio of Resistant Cell to Total Population**
Day 1	1.1×10^9	8.9×10^8	0.81
Day 2	1.0×10^9	7.7×10^8	0.77
Day 3	2.0×10^9	1.2×10^9	0.60
Day 4	1.6×10^9	1.5×10^9	0.94
Day 5	2.6×10^9	2.7×10^9	1.04

FIGURES

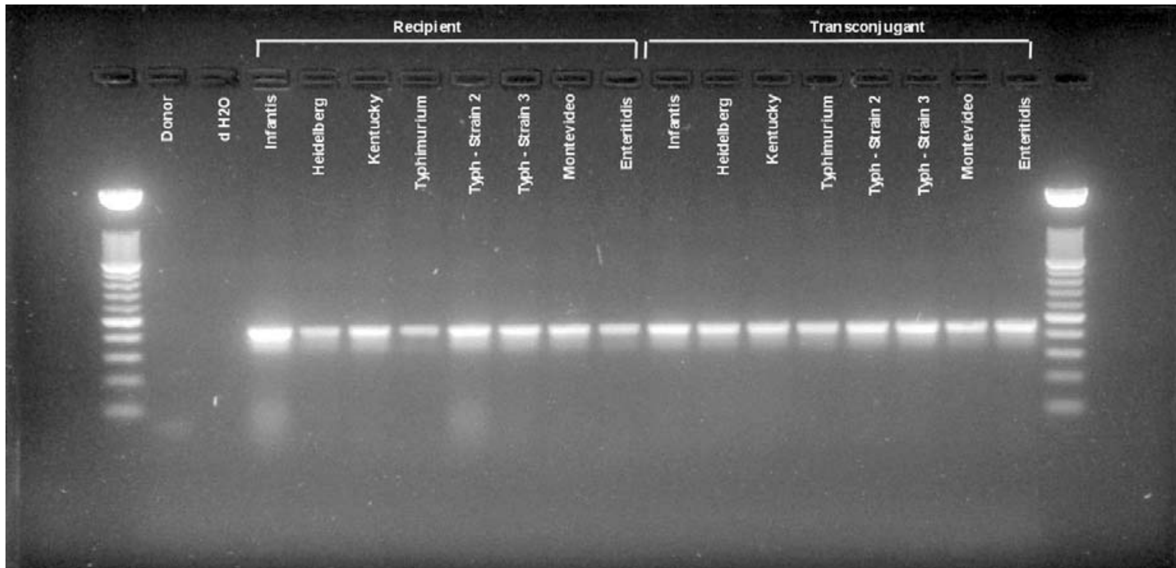

Figure 3.4 PCR confirmation presumptive transconjugants as *Salmonella* as determined by *invA* PCR (expected product size- 437bp)

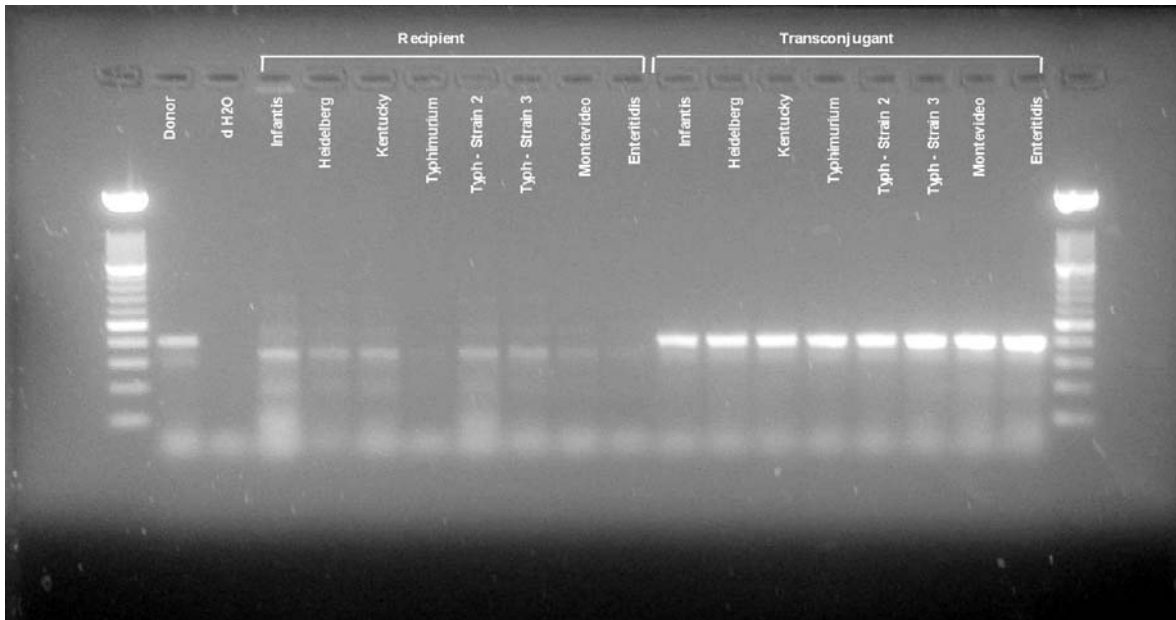

Figure 3.5 PCR confirmation of the *S.* Newport plasmid to other *S. enterica* Serovars as determined by *floR* PCR. (expected product size -399bp)

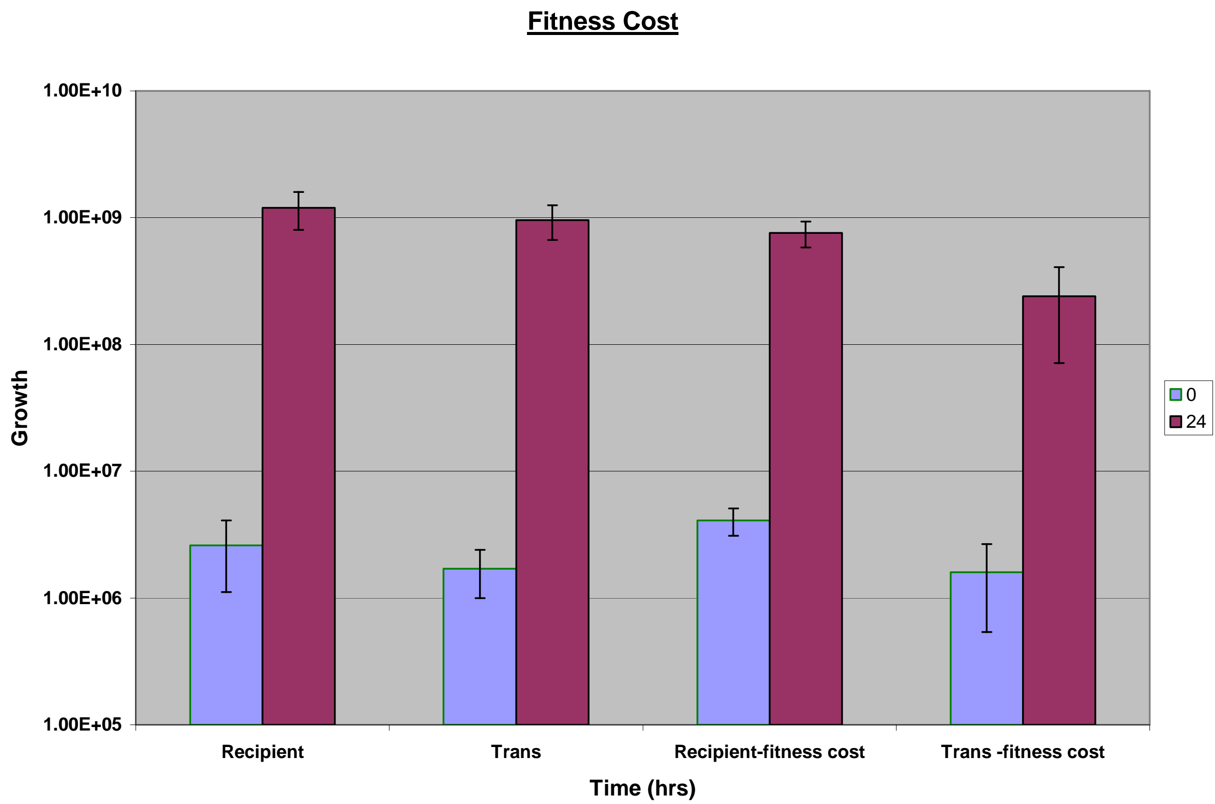

Figure 3.6 Growth of recipient *Salmonella* Typhimurium strain 934R and transconjugant (recipient with plasmid) grown together or independently.`

CHAPTER 4

CONCLUSION

In this study we utilized a genomic approach to assess the epidemiological relatedness of atypical *Salmonella* isolates and the ability of *Salmonella* to acquire antibiotic resistance genes. We have found that *Salmonella* 4,5,12:i:- strains isolated from poultry do not represent the emergence of a new serovar or clone, rather the result of spontaneous mutations in endemic *S.* Typhimurium strains, affecting phase 2 antigen expression. From the evidence presented, it appears that these *S. enterica* 4,[5],12:i:- isolates are genotypically *S.* Typhimurium strains, possessing the virulence genes commonly associated with this pathogenic serovar. Therefore, this *Salmonella* 4,[5],12:i:- serovar should not be treated any differently than *S.* Typhimurium, especially with regards export or import restrictions concerning poultry with *S.* Typhimurium.

We have measured different variables involved in plasmid transfer in vitro and assessed whether a prebiotic like Biomoss™ might interfere with this process. While this prebiotic does not prevent plasmid transmission in vitro, it may have a profound effect in vivo. It might be possible that the incorporation of Biomoss™ in the diet may prevent or reduce the colonization of enteric pathogens like *Salmonella*, possibly by lowering the pH of the large intestine as a result of lactic acid and short chain fatty acids produced by bifidobacteria. (Juven *et al*., 1991) or may affect bacterial concentrations in the gastrointestinal tract by adsorbing bacteria and keeping them away from adhering to the gut wall (Spring et al., 2000).

Our long term goal is to understand the process underlying the acquisition and spread of multiple drug resistance among major foodborne pathogens. These experiments have provided us the information necessary for assessing and validating a mathematical model of plasmid

transmission dynamics of *Salmonella*. The development of this model will allow us to predict the emergence and prevalence of resistance in *Salmonella* within the gastrointestinal tract of food animals, especially in response to veterinary use of antibiotics in poultry and other food animals.

Overall, the high frequency of resistant *Salmonella* serovars to the various antimicrobials could be an indication of misuse or overuse of antimicrobials both in veterinary and public health. Efforts that promote appropriate use of antimicrobials in both humans and animals are required. We also need to have better understanding of the various resistance mechanisms, the rate at which resistant occurs and effects resistance has on the life cycle of bacteria, in order to slow resistance development. Knowing how resistance actually disseminates in these ecosystems will help identify the best points for novel interventions.