COMPARISON OF GENETIC VARIABILITY IN MULTIDRUG RESISTANT *SALMONELLA* NEWPORT ASSOCIATED WITH DAIRY CATTLE AND *S.* KENTUCKY ASSOCIATED WITH POULTRY.

by

JENNIFER ELIZABETH BAUER-TURPIN

(Under the Direction of Mark A. Harrison)

ABSTRACT

Differences in pathogenesis and niches have made *Salmonella* Newport and *S.* Kentucky interesting subjects for the study of genetic variability. *S.* Kentucky is one of the most common serotypes isolated from chickens; however it is not often associated with human infections. *S.* Newport is not only a common serotype isolated from dairy cattle but is also associated with human infections and has developed resistance to multiple antibiotics. In this study a diverse set of *Salmonella* isolates (n=34) were selected from the National Antimicrobial Resistance Monitoring System (NARMS). DNA was extracted from these *Salmonella* isolates and subjected to comparative genomic hybridizations (CGH) to a *Salmonella* whole genome array containing genes from 5 different *Salmonella* serotypes(Typhimurium LT2, Typhi strain CT18, Typhi Ty2, Paratyphi A, and Enteritidis SPT4). Absence versus presence results of the 5,660 genes on the array were analyzed by Bionumerics (V 6.0, Applied Maths Austin, TX, USA). Data from CGH studies and published genomes of *S.* Newport and Kentucky (NCBI database) were compared to the *Salmonella* used as probes for CGH using MAUVE (The University of

Wisconsin-Madison, Madison, WI, USA). CGH of *S.* Newport revealed high genomic similarity within MDR strains of Newport which is consistent with previous findings. *S.* Newport contained most of the phages found in *S.* Typhimurium while these phages were absent in *S.* Kentucky. Also, *S.* Newport contained several *Salmonella* pathogenicity virulence genes. Genetic variability was measured using MAUVE; 100 unique genes were found in *S.* Newport and 162 unique genes were found in *S.* Kentucky. Although these organisms have very similar genomes enough genetic variability exists to allow them to survive in different niches or environments. This may also include the ability of *S.* Newport to invade and colonize the gastrointestinal tracts of humans more efficiently than *S.* Kentucky.

INDEX WORDS: *Salmonella, Salmonella* Newport, *Salmonella* Kentucky, Antibiotic resistance, Comparative genomic analysis, Microarray hybridization, Poultry, Dairy cattle, MAUVE

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DEDICATION

To my Mom, Priscilla Bauer-Corbin, whose own strength inspired me to begin this adventure. You are not forgotten. To my amazing husband, without his encouragement I would have never made it this far.

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CHAPTER 1

INTRODUCTION

As food science and technology advances our food system there is always one staggering step back due to foodborne pathogens. It is these few steps that can cripple a food industry creating fear and distrust in its consumer. It is imperative as the food industry advances its technology that it also knows its enemies. Pathogens have been around billions of years adjusting to even more extreme environments than what the food industry has thrown at them. These organisms learn to adapt to extreme heats, cleaning reagents, and even antibiotics to name just a few. Furthermore, since foods are now shipped from all over, food outbreaks can affect a larger group of people and usually include multiple states throughout the United States. Each year an estimated 76 million people are affected by foodborne diseases of which 325,000 are hospitalized and more than 5,000 die (7). Foodborne diseases can cause symptoms that range from a self-limiting diarrhea to a life threatening blood infection, septicemia (8). Fifteen percent of the estimated 76 million foodborne infections in the U.S. are due to contamination in food or water with *Salmonella (7)*. *Salmonella* are gram-negative, facultative anaerobe, motile rods that are widespread in animals, especially poultry and swine (8). The ability of a *Salmonella* contamination in food to cause infection within a host is dependent on the serovar or serotype (4). Furthermore, the ability of *Salmonella* to cause disease can be host specific such as *S. enterica* serotype Typhi and *S. enterica* serotype Paratyphi or infect a wide range of warm blood species such as *S. enterica* serotype Typhimurium. *S.* Typhimurium, *S. enterica* serotype

Enteritidis, and *S. enterica* serotype Newport were the most common serotypes isolated from humans in the U.S. in 2009 accounting for over 50% of the foodborne infections (1). Infections with *S.* Newport have increased significantly from 1992 to 2006. Furthermore, in 1998 the first multiple drug resistant (MDR) *S.* Newport with a CMY gene conferring resistance to third generation cephalosporins (MDR AmpC) was found domestically (3). Pathogens resistant to multiple antibiotics may cause a more severe infection and may also require hospital treatment especially for the young, elderly, and immunocompromised (6). *S.* Newport resistant to third generation cephalosporins such as ceftriaxone have important clinical repercussions since ceftriaxone is often the drug of choice for bacterial infections. Preventing the spread of the emerging MDR pathogenic *S.* Newport has become a major public health concern.

Another bacterium of interest is *S.* Kentucky due to the fact that it is the predominant serotype found in 50% of samples from broilers but only causes 0.1% of the human infections (5). According to NARMS 2008 report, *S.* Kentucky was the most common serotype isolated from chicken (5). Even though this organism is prevalent in chicken, it was not listed on the CDC NARMS 2007 data for top 20 most common serotypes for *Salmonella* infections in humans (1). Despite the lack of human infections this organism has increased in prevalence in chicken from 25% to 50% in less than 10 years (5). Also, *S.* Kentucky isolated from chicken have developed resistance to tetracycline (47%), streptomycin (35%), and even third generation cephalosporin, ceftiofur (15%) (5). Furthermore, studies have shown that strains of *S.* Kentucky have acquired resistance to multiple antibiotics and have demonstrated increased pathogenicity (2). It is thought that the genomic content of *S.* Kentucky could unlock information concerning virulence in other pathogens, such as *S.* Newport, since this microbe is considered a rare human pathogen.

S. Newport and *S.* Kentucky make for interesting study subjects since they are genetically very similar yet behave very differently. *S.* Newport is predominantly found in dairy cattle, resistant to multiple antibiotics, and known to cause human infections from the consumption of dairy products. On the other hand, *S.* Kentucky colonizes poultry, is increasing in prevalence, and is not associated with human infections. CGH with the microarrays containing the genomes from 5 *Salmonella* can determine the evolutionary relatedness of *S.* Newport or *S.* Kentucky with other *Salmonella* serotypes. It will also help determine which genes are or are not related between pan susceptible and MDR *S.* Newport and other serotypes. Furthermore, genes found to be absent or present as compared to the other *Salmonella* genomes on the chips, will be studied to determine if there are genes that could help increase the survival of these organisms either in poultry or dairy cattle. In addition, these organisms' genomes will be compared to the other *Salmonella* organisms on the chip using Mauve to determine genes that are unique to *S.* Newport or *S.* Kentucky. Unique gene information may determine why these pathogens behave so very differently in foodborne infections and prefer very specific niches.

Project Objectives

S. Newport GOALS 1:

- Compare genomic content of several pan susceptible and MDR *S.* Newport isolates from NARMS on microarray chips that contain genes for *S.* Typhimurium LT2, *S.* Typhi CT18, *S.* Typhi Ty2, *S.* Paratyphi A SARB42, and *S.* Enteritidis PT4 (Sidney Kimmel Cancer Center).

- Determine the differences between strains of MDR *S.* Newport, pan susceptible Newport, and other *Salmonella* as well as evolutionary relationships between strains using dendrograms.
- Compare the genome of *S.* Newport to the other *Salmonella* genomes located on the microarray chips using a genome alignment program called Mauve. Group genes unique to *S.* Newport by location on the genome as well as function and placed into tables.

S. Kentucky GOALS 2:

- Compare genomic content of several pan susceptible and MDR *S.* Kentucky isolates from the National Antimicrobial Resistance Monitoring System (NARMS) on microarray chips that contain genes for *S.* Typhimurium LT2, *S.* Typhi CT18, *S.* Typhi Ty2, *S.* Paratyphi A SARB42, and *S.* Enteritidis PT4 (Sidney Kimmel Cancer Center)
- Determine the differences between strains of MDR S. Kentucky, pan susceptible *S.* Kentucky, and other *Salmonella* as well as evolutionary relationships between strains using dendrograms.
- Compare the genome of *S.* Kentucky to the other *Salmonella* genomes located on the microarray chips using a genome alignment program called Mauve. Group genes unique to *S.* Kentucky by location on the genome as well as function and placed into tables.

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CHAPTER 2

LITERATURE REVIEW

Salmonella

Salmonella are grouped into two species, *Salmonella enterica* and *Salmonella bongori,* each of which contains several serotypes. *Salmonella enterica* can cause enteric fever (septicemia) and gastroenteritis (54). *Salmonella* can be serotyped based on the capsular, flagellar, or envelope antigens by agglutination with antisera. Roughly 2,400 *Salmonella* serotypes capable of causing illness have been identified. The *Salmonella* genus contains pathogens that cause enteric fevers such as *S.* Typhi and *S.* Paratyphi; however, most symptoms of *Salmonella* infections present themselves as food poisoning (54). Unlike *S.* typhi which is a human only pathogen, nontyphodial *Salmonella* strains occur in a wide range of animal hosts which includes human, poultry, cattle, and pigs. Some *Salmonella* are host adapted and are capable of causing human infection. These include *S.* Galinarum (poultry), *S.* Abortus-equi (horse), *S.* Dublin (cattle), *S.* Abortus-ovis (sheep), and *S.* Choleraesuis (swine) (35). Infections from *Salmonella* are usually acquired from contaminated foods such as eggs, poultry, seafood, beef, milk, pork, peanut butter, and vegetables (22, 54). In addition, infections from *Salmonella* can be acquired through contact with an infected animal (22, 45). Interestingly, serotypes of *Salmonella* from food animals contain the same type of *Salmonella* that cause human illnesses (22).

Salmonella infections can occur after the ingestion of 15 to as many as 10^9 bacterial cells but infectious levels can vary depending on the host's age and immune system (58). Infection

dosage is higher, 10^6-10^9, if not accompanied by the ingestion of food because food protects S*almonella* from acidic stomach allowing it to reach the epithelial cells of the intestines (21). Infections from *Salmonella* are usually acquired through the consumption of contaminated foods that have been improperly cooked or handled. Also, *Salmonella* infections can be acquired from other infected individuals via the fecal-oral route (58). Infected individuals can shed up to 10^{11} *Salmonella* cells and in the case of food handlers, can contaminate foods. Also, *Salmonella* in the intestinal tract of animals can contaminate the food product during slaughter. Approximately 24 to 48 hours after the ingestion of contaminated foods, the individual can experience nausea, vomiting, diarrhea, and abdominal pain (54). In some cases the bacteria can cause a more severe infection by invading the blood stream. Further complicating foodborne diseases are the potential sequelae that includes arthritis, Guillian-Barre syndrome, and Rietters syndrome to name a few. Severity of infection varies according to the individual's age and immune system. The young, old, and immunocompromised are susceptible to severe infection and may experience more severe complications due to dehydration (22). In some cases, individuals taking antibiotics or antacids prior to infection are more at risk for *Salmonella* infections (7). Salmonellonosis causes a rapid infection that usually disappears as quickly as it occurred except in about 5% of the population who become carriers. Usually people are not aware they are carriers of *Salmonella* and often infect other individuals via the fecal-oral route (58). *Salmonella* illness usually last 2 to 7 days and is self-limiting thus medical treatment is not typically needed.

However, in cases of foodborne infections that require hospital treatment, antibiotics are used to eliminate the bacteria (22). Third generation cephalosporins and fluoroquinolones are the primary choice for treatment. Children are only treated with third generation cephalosporins since complications arise from the use of fluoroquinolones. Nevertheless, bacteria have

developed resistance to these as well as many other antibiotics which limit the treatment (22). In most cases pathogens resistant to multiple antibiotics are more virulent requiring more people to need hospital treatment as well as more time within the hospital. Since pathogenic bacteria can acquire resistance genes from other pathogenic and non-pathogenic bacteria they may also acquire virulence genes along with resistance genes that produce the more severe infection.

Food Safety

Food safety concerns include many things like pesticides, herbicides, spoilage, chemical additives and microbes. In the food industry, one of the biggest challenges is to control foodborne pathogens. Since many pathogens produce toxins or cause infections, it is imperative that foods are produced and processed to limit the contamination and/or growth of these microbes. As food processing advances to produce safer foods, microbes too evolve. The food processing industry is enormous in the U.S. representing food sales of $1.57 trillion (47). From growing the food to producing the food, profits are usually small due to the competitive nature of food processing. Thus, pathogens can easily cripple this giant industry costing the company millions and even closing the doors of some permanently. It is estimated that yearly for every 100,000 people, 14.6 of them will become infected from *Salmonella* (16). Healthy people 2010 goal for human *Salmonella* infections was much lower than the current numbers thus food companies are constantly trying to find ways to lower contamination of foods with this pathogen. Bacteria can contaminate a food product easily since they are found in soil and water, in the gastrointestinal tracts of animals, on food handlers, on animal hides, and even in the air and dust (35).

In the U.S., meat, poultry, and egg products are inspected for safety by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture (USDA) (53). To control

the amount of *Salmonella* on a raw product a Pathogen Reduction Plan and Hazard Analysis and Critical Control Point (PR/HACCP) were enacted in food industries. Under PR/HACCP rules, slaughter facilities must have performance standards set for *Salmonella* incidence. *Salmonella* performance standards are used to determine if a food industry is producing foods in a system that controls the contamination of the raw meat and poultry products with foodborne pathogens. New *Salmonella* performance standards have been enacted to place a limit on the number of samples positive for *Salmonella* within a company (27). Samples are randomly collected by FSIS inspectors and sent to FSIS laboratories for *Salmonella* analysis to verify that standards are met. FSIS is also involved with the Agricultural National Antimicrobial Resistance Monitoring System (NARMS) program along with Animal and Plant Health Inspection Service USDA (APHIS), Center for Veterinary Medicine FDA and the Centers for Disease Control and Prevention (CDC). The animal portion of NARMS is housed in the Bacterial Epidemiology and Antimicrobial Resistance Research unit (BEAR) which studies the changes in resistance/susceptibility of foodborne pathogens and communal microbes from animals, retail meats, and humans to antimicrobials (19). The 2006 NARMS report includes data for *Salmonella*, *Escherichia coli, Enterococcus,* and *Campylobacter* isolated from food animals.

Pathogenesis and Virulence

Salmonellosis can develop from as little as 15 to as many as 10^9 cells of *Salmonella* from contaminated foods (22). As stated previously, this variation is due to host susceptibility, the serotype pathogenicity, and even the fat content of the contaminated food. Ingested *Salmonella* survive the acidic stomach, avoid the host immune system, and reach the small intestine (54). Some bacteria, as is the case with *Salmonella,* have an acid tolerance response that activates the *rpoS* gene (22). The RpoS induces genes needed to survive extreme environments such as

thermotolerance, starvation survival, acid pH and osmotic stress. *Salmonella* that induce an acid tolerance response can survive at pH 3.3. Once the bacteria survive the acidic stomach it has to get past the mucus layer of the intestines to colonize the intestinal walls. Once it colonizes the intestinal walls the pathogen needs to fight off the host defense cells as well as other bacteria in the microflora of the gut. *Salmonella* posses several virulence genes that enable it to colonize the intestines as well as cross the epithelial cell walls (22, 54). These virulence genes include those encoding for fimbriae and pili located on the outer cell membrane that aid in adhesion to the host cell. Some fimbriae genes are located on the virulence plasmid (pSLT) and loss of this plasmid causes the microbe to become avirulent. In addition, the virulence of a *Salmonella* bacterium is determined by the *Salmonella* pathogenicity island (SPI). The SPI contains many genes necessary for invasion into the host cell as well as toxins. The invasiveness of *Salmonella* is dependent on the virulence genes the bacteria contain. Initial colonization of the gut by *Salmonella* is accomplished by the SPI-1 genes. All *Salmonella* contain a type III secretion system (T3SS) within the SPI-1 which is required for endocytosis and epithelial cell invasion. The T3SS is regulated by HilA protein which reacts to various environmental cues to protect the bacterium. The T3SS forms a syringe that can transfer proteins from the bacteria into the host cell. The protein transferred is unique to each pathogen producing different pathogenesis within the host depending on the bacteria. The injected proteins alter the surface of the host cell causing a membrane ruffling necessary for endocytosis. The secreted proteins from the *Salmonella* also induce a secretory response within the intestinal epithelial layer and recruits neutrophils into the intestinal lumen. Several *Salmonella* proteins secreted into the epithelial cells contribute to intestinal inflammation leading to the development of diarrhea (54). The SopB protein is an

important activator of the secretory pathway and proteins SipA, SopA, SopD, and SopE2 also contribute to the development of gastroenteritis.

Another important pathogenicity island, SPI-2, also encodes a T3SS; however, SPI-2 is needed for intracellular survival and systemic infection (22). Regulatory proteins include SsrA and OmpR which can are activated when there is low osmolarity, limited nutrients, and/or decreased pH. The SPI-2 encodes an effector protein, SipC, which protects the microbe from the bactericidal compounds produced by the host cell. Immune cells infected with *Salmonella* can be carried to other areas in the body resulting in septicimia. *Salmonella* is not recognized by the host immune cell due to proteins produced by SPI-2 that suppress the antigen presentation in the host cell. While in the host immune cell, *Salmonella* do not replicate until they come into contact with organs located throughout the body.

Salmonella acquire genes necessary for pathogenesis mainly from horizontal gene transfer (HGT) from other bacteria harboring these genes (37). HGT is the transfer of genetic material between two different microbial cells and thus plays an important role in the evolution of bacteria. Bacteria can acquire genes such as those for virulence and antibiotic resistance by several HGT methods: transduction, conjugation, or transformation. Transduction and transformation involve the transfer of DNA from the environment. Transfer of DNA via transduction or transformation usually occurs within the same bacterial species due to the necessary receptors needed by the recipient bacteria. However, *Salmonella* can acquire virulence genes from different species of bacteria by the transferring DNA directly from the bacterial cell. The process of transferring DNA is called conjugation and can occur as transfer of a plasmid or conjugative transposons (37, 54). Conjugation is believed to play a major role in the spread of antibiotic resistance in bacteria. Mobile elements which include plasmids, bacteriophages,

integrons, genomic islands, transposons and insertion seqeuences (IS) all contribute to the increase in pathogenicity of *Salmonella* as well as the increase in antimicrobial resistance.

Antimicrobials and Resistance

Penicillin was the first antimicrobial discovered by Alexander Fleming (55). His discovery marked the beginning of the change in the treatment of illnesses such as those caused by bacterial infections. This miracle drug saved millions of lives during the World War II. However, even during the initial mass production of penicillin a contamination with *E. coli* containing genes resistant to the antibiotic was soon discovered (55). Antimicrobials are produced by fungi and bacteria as secondary metabolites. It is thought that these secondary metabolites help to inhibit the growth of nearby invaders thus allowing more room for the organism to grow. Microbes that produce these compounds naturally have resistances to the chemical they produce. Resistance to β-lactams occurs through the alteration of cell wall enzymes, penicillin binding proteins (PBPs), and through the production of enzymes (β-lactamases) that breakdown the β-lactam ring (48). Penicillin binding proteins are similar to β-lactamases and it is thought that these enzymes evolved from PBPs. According to the Ambler classification, the β-lactamases make up four groups (A, B, C, and D) that are based on amino acid sequence similarities (32). Most gram negative bacteria contain the genetic information to produce class C β-lactamases, AmpC, on their chromosome or on plasmids (48). The AmpC enzyme confers resistance to most penicillins and cephalosporins including extended spectrum cephalosporins. In addition, AmpC is located on a self-transmissible plasmid that contains other antimicrobial resistance genes. Studies have shown that using antimicrobials such as tetracycline, can select for resistance to extended spectrum cephalosporins because these resistance genes are usually carried together on plasmids (2).

Multidrug resistant strains of *Salmonella* isolated from humans jumped from 16% from 1979-1980 to 24% in 1984-1985 (41). In 1996, MDR *Salmonella* in humans peaked at 31% and then began a gradual decline with only 14.8% of *Salmonella* resistant to 2 or more antibiotics in 2005 (11, 12). According to the 2007 CDC report there was an increase in resistance to extended spectrum cephalosporins from 0.2% in 1998 to 4.5% in 2003 and currently there is a decrease to 3.3% in *Salmonella* isolated from humans (12). Furthermore, NARMS data concerning animal isolates found 39.5% of diagnostic isolates collected from cattle in 2006 to be resistant to extended spectrum cephalosporins while 15% of slaughter isolates collected from cattle were resistant to extended spectrum cephalosporin in 2009 (18). In 2009, *Salmonella* isolated from chicken slaughter samples displayed resistance to several antimicrobials including extended spectrum cephalosporins (12.7%), streptomycin (30.5%) and tetracycline (33.9%) (18). This data provides evidence that antibiotic resistance is found in bacteria associated with food animals which can then cause illness in humans if they eat product contaminated with these microbes. As stated previously, third generation cephalosporins are often the drug of choice to treat severe *Salmonella* infections thus it is important to stop this trend of increasing resistance. Furthermore, not only is the antibiotic rendered ineffective, treatment can possibly further complicate the *Salmonella* infection producing even more severe symptoms.

Salmonella can become resistant to antibiotics through several ways. As stated earlier, acquisition of new genes via horizontal gene transfer is a major contributor to the increase in antibiotic resistance (54). Plasmids containing resistance genes for multiple antibiotics have recently emerged in *S.* Newport. In the past, MDR in *Salmonella* was predominant in servor Typhimurium DT104. Currently, MDR *S.* Newport is resistant to the same 5 antimicrobials as *S.* Typhimurium DT104 along with 5 or more other antimicrobials (12). Thus, *S.* Newport is

notably more worrisome considering the implications of a bacterium resistant to more than 10 antimicrobials. The emerging MDR *S.* Newport is also resistant to extended spectrum cephalosporin which is encoded by a plasmid mediated CMY-2 AmpC ß-lactamase. To further complicate matters, *S.* Newport with the CMY-2 AmpC ß-lactamase gene, co-exists with several other antimicrobial resistance genes on a plasmid. In addition, MDR *S.* Newport contains an IncA/C plasmid backbone conferring resistance to multiple antibiotics was found in the human pathogen *Yersinia pestis* and the fish pathogen *Yersina ruckeri.* The IncA/C backbone consists of plasmid replication and maintenance genes as well as several type IV conjugative transfer genes (61). Thus, this plasmid is capable of transferring itself to other microbes including human pathogens. In fact it was shown that several *Salmonella* serotypes isolated from retail meat contained the IncA/C plasmid which confers resistance to multiple antibiotics. This study provided evidence that not only is this plasmid transferred to other foodborne pathogens but it is also transferred to human pathogens associated with the plague.

In 1996, when MDR was first found domestically in *S.* Newport it was discovered in only 5.9% of the isolates from human sources. In 2007 multidrug resistance with the ceftiofur resistance was found in 7.7% of the *S.* Newport isolates which was a decrease from the 25% in 2001 (12). Resistance to three or more antimicrobials was found in 10.58% of the *S.* Newport isolated from humans in 2007 (12). According to the NARMS 2007 report, the most common multidrug resistance pattern was ACSSuT (resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole/sulfisoxazole, and tetracycline) representing 8.2% of the isolates. Even more daunting is the fact that 77% of *S.* Newport isolated from cattle were resistant to ceftiofur and 3.3% were resistant to ceftriaxone (19). This represents a large number

of possible foodborne infections due to an organism that is resistant to third generation cephalosporins.

Also, in the U.S., *S.* Kentucky isolates from poultry sources have developed resistance to tetracycline, streptomycin, and even extended spectrum cephalosporin, ceftiofur. According to the NARMS 2006 study, *S.* Kentucky was the number one serotype isolated from chicken and 47% were resistant to tetracycline (19). In addition, *S.* Kentucky was the most isolated *Salmonella* serotype from whole broiler carcasses at both the prechill and postchill step (46). More importantly, the amount of *S.* Kentucky recovered from broilers increased from 51.4% to 67.3% from prechill and postchill, respectively. Approximately 73% of the *Salmonella* samples taken from the water used to reduce the temperature of the broiler carcass (postchill) were *S.* Kentucky. Even more alarming is that the majority of these *S.* Kentucky isolates were resistant to tetracycline and β-lactam antibiotics (19). In addition, researchers in France noted that travelers from northeast Africa and Turkey suffered infections with *S.* Kentucky resistant to ciprofloxacin, co-trimoxazole, and exteneded spectrum cephalosporins. Even more troublesome was the fact that antimicrobial treatment for *Salmonella* infection did not work due to the high levels of resistance found in these pathogens (15). Another study found that *S.* Kentucky contained two to three plasmids that were responsible for antimicrobial resistance and could be conjugated into other strains of *Salmonella* (25). Furthermore, 94% of the isolates from chickens in this study contained an avian pathogenic *E.* coli (APEC)-like plasmid that was linked to tetracycline and streptomycin resistance. Acquisition of the APEC plasmid increases the microbes pathogenicity which may explain the increase of colonization of *S.* Kentucky in poultry (25). *S.* Kentucky has increased in prevalence in chicken over the past few years and resistance to tetracycline and streptomycin is the most commonly found resistance phenotype (19). A

group of phylogenetically similar *S.* Kentucky isolates as shown by PFGE, all contained an APEC-like resistance plasmid (25). Suggested reasons for the large percentage of carcasses that contain this APEC-like plasmid include the fact that tetracycline is used in chicken to treat infection which could in turn select for *S.* Kentucky isolates that are resistant to this antibiotic (25). Also, the pathogen could receive a benefit for expressing the plasmid that would allow it to proliferate in certain environments such as poultry. *S.* Kentucky prevalence in chickens has increased over the years while remaining an unsuccessful human pathogen in the U.S. (12, 25). However, in other countries it not only presents with a different resistance pattern it also has a higher association with human infections (15). The fact that *S.* Kentucky could become a successful human pathogen warrants further investigation of this pathogen. More importantly, Welch *et al.* found that *S.* Kentucky isolated from retail meat products contained a plasmid (IncA/C incompatibility group) backbone previously found in multi-drug resistant *S.* Newport and the plague bacterium *Yersina pestis* (61). The IncA/C backbone contains genes that encode for plasmid replication/maintenance and conjugative transfer genes as well as resistance to multiple antibiotics. This plasmid confers resistance to streptomycin, tetracycline, β-lactmases and quaternary ammonium compound resistance (*sugE*). It may be that this innocuous microbe is able to pass resistance genes to other food borne pathogens, thus contributing to a deadly cycle.

Transmission

Humans and animals, including birds, are carries of *Salmonella* and can occasionally become shedders (54). Shedders do not completely rid themselves of the bacterium which reside within the intestines where it can be released into the host feces. Once the animal is slaughtered, the bacteria from the intestine can contaminate the carcass, gaining access into the food chain.

Salmonella is transmitted by the oral-fecal route and maybe found in food and water contaminated with animal feces. In a study of retail ground beef, antibiotic resistant strains of *Salmonella* were found to be a common occurrence (62). Twenty percent of the 200 ground retail beef samples collected had *Salmonella* contamination. Of the positive *Salmonella* isolates, resistance to at least one antibiotic was found in 84% and 53% were resistant to more than three antibiotics.

In poultry houses, *Salmonella* may spread through the air, feed, by contact with infected birds, rodents and by insects (5). The ability of a bird to become colonized depends on the stress of the bird, the health, and the genetics of the bird. Poultry houses contain up to 75,000 birds in close contact, increasing the spread of pathogens (3). Poultry that carry *Salmonella* can shed the bacteria through feces while experiencing no symptoms throughout its lifetime. In general, the chicken and the *Salmonella enterica* bacterium have a symbiotic relationship since the bacterium does not harm the chicken and the chicken does not rid itself of the bacteria. The ceca which is part of the gastrointestinal tract, usually contains the *Salmonella* bacterium. Colonized birds shed *Salmonella* for weeks to months, thus continuously contaminating the environment. Once in the environment, *Salmonella* can survive there for up to 13 months unless the area is properly cleaned (54).

Antimicrobial Use

One issue surrounding antibiotic resistance development in bacteria is the use of antibiotics in animals. Antimicrobials improves animal health producing bigger, healthier animals and thus more product (36). Resistance development in intestinal bacteria found in turkeys fed streptomycin in 1951 was the first noted antimicrobial resistance in food animals (57). Currently, antibiotics are administered to humans and animals treat infections and prevent

infections prophylactically (55). Also, animals are given therapeutic levels and subtherapeutic levels of antibiotics that are very similar in structure to the antibiotics used in humans for growth promotion (55). It is believed that the use of subtherapeutic levels help spread resistance by selecting for pathogens that are resistant to the antibiotic or by allowing a pathogen to acquire resistance genes from non-pathogenic bacteria. By eliminating the use of antibiotics, it is thought that bacteria with resistance genes will decrease due to the metabolic cost of carrying extra genes. This would allow for bacteria not resistant to antibiotics to outgrow the bacteria with resistance genes and potentially reduce the amount of antibiotic resistant pathogens. However, studies have shown that elimination of antibiotics does not reduce the antibiotic resistant population. It is believed that antibiotic resistant bacteria are able to ameliorate the cost of resistance genes and may even have an increased fitness level compared to bacteria sensitive to antibiotics (4).

Antimicrobial use in agriculture began shortly after the discovery of penicillin in the late 1940s (39). Despite issues surrounding the use of antibiotics in animals, there is unequivocal evidence that animals treated with antibiotics grow bigger and faster. There is limited data to explain this phenomenon. Some studies suggest that the antibiotics prevent the growth of bacteria in the gut lining allowing more nutrients to be absorbed into the intestines. Also, energy that would have been spent on fighting potential infections in an animal will be used for growth instead. Researchers feel that more studies are needed to determine the exact mechanisms involved in growth promotion of the animal by antibiotics. This may help to eliminate the use of antibiotics in animals without the loss of growth promotion.

Cattle have been implicated in the spread of antibiotic resistant *Salmonella* to humans via contaminated hamburger meat, milk, cheese and even direct contact with the animals (1). One

reason could be due to the use of antibiotics for growth promotion as well as treatment of illness. The top three *Salmonella* serotypes to cause human infection include *S.* Typhimurium, *S.* Enteritidis, and *S.* Newport while the top three serotypes to cause dairy cattle illness include *S.* Typhimurium, *S.* Newport, and *S.* Agona (1). Ill cattle present with fever, anorexia, reduced milk, depression, and diarrhea (1). Ceftiofur, the only third generation cephalosporin used in dairy cattle, is used to treat respiratory disease, metritis, mastitis, and foot rot (1). Ceftiofur is not used to treat human infections; however, there is a decrease in the human extended spectrum cephalosporin, ceftriaxone, susceptibility in bacteria. This is believed to be due to the use of ceftiofur in food animals. Previous studies found that the use of ceftiofur in dairy cattle produced *E. coli* resistant to extended spectrum cephalosporins (56). The authors felt that the antimicrobial inhibited growth of the susceptible population of bacteria while allowing resistant strains to proliferate. It was suggested that there was not an emergence of resistant bacteria but rather less interference from susceptible strains during isolation of resistant bacteria. Other studies have proposed that by decreasing the susceptible strains, resistant bacteria can out grow the susceptible strains and possibly pass the plasmid containing the *amp*C gene as well as other antimicrobial genes to pathogens previously sensitive to those antimicrobials (36). Singer *et al.* found that after treatment, the sensitive bacterial population returned to the levels found in the cattle not treated with antimicrobials (56). Also Singer *et al.* found the main cause of bacterial resistance was due to a plasmid that contained multiple antibiotic resistant genes including the *bla*CMY-2 gene. It was stated that the multi-drug resistant *E. coli* could be capable of passing the MDR plasmid to other bacteria thus serving as a reservoir for extended spectrum β-lactamase (56).

Infection caused by *Salmonella* in poultry is dependent on the poultry age, the poultry stress level, serovar, and bacterial strain virulence (5). *Salmonella* has many serotypes with a few that can cause disease in poulty (3). *S. pullorum*, pullorum disease, and *S. gallinarum*, fowl typhoid, are both able to cause disease and even death in poultry. Treatment for these diseases involves antibiotics given to poultry supplied in the feed, drinking water, or as an injection. Normally a bird that is sick will not eat but it will continue to drink water, thus antibiotic placed in the water is the best method for treatment of infection. Antimicrobial treatment in water can have varied dosage rates within birds depending on environmental factors that may lead to increased or decreased drinking. Since it is necessary to reach the therapeutic level in order to be effective in the birds, the administration of the antibiotic in water requires extra work to ensure the drug is suspended throughout the drinking water. It is also important to understand where the antimicrobials react in the body of the chicken. For example, the antimicrobial tetracycline is absorbed from the intestines and then distributed throughout the body of the chicken (3). Some drugs pass through the intestines into feces while others or absorbed into the bloodstream. Thus the recommended dose is determined by manufacturers to ensure proper administration of the antibiotic within the chickens. Dosage level is also determined so that the maximum level is reached for which the antibiotic is active within the bloodstream or organ. There is a withdrawal period for antimicrobials to prevent the deposition of drugs in the muscle or eggs of the chicken which can be harmful to humans. FDA proposed a ban in 2008 on the use of extended spectrum cephalosporins in poultry to inhibit the increase of MDR pathogens resistant to cephalosporins (60). Soon after this proposal was withdrawn until more data was available concerning the use of antibiotics in food animals as it relates to human illness from MDR pathogens.

Human use of antibiotics is another major source of the development of antibiotic resistant bacteria (55). The majority patients do not finish the full dose of antibiotics, which allows sensitive bacteria the ability to mutate or acquire resistance to the drug. Also, physicians are prescribing antibiotics for patients who have a viral infection rather than a bacterial infection. Most of the time the patient is the one demanding to have treatment and the physician will give in to appease his/her patient (55). Antibiotics do not affect a viral infection. In fact, by using antibiotics for a viral infection, the bacteria that were sensitive to antibiotics may acquire resistance. If a patient then eats food contaminated with an antibiotic sensitive pathogen, that pathogen can acquire resistance to antibiotics. Now a simple self-limiting diarrheal illness may develop into a life threatening infection. Furthermore, studies have shown that people taking antibiotics are more at risk for acquiring an infection from a MDR microbe such as *S.* Newport (49).

Epidemiology and Incidence

The predominant reservoirs of *Salmonella* are animals which include poultry, pigs, cattle, and reptiles (54). *Salmonella* is the most reported bacterial foodborne pathogen in the U.S. (12). The reported cases could be even higher but confirmation of *Salmonella* infection is difficult because most people are unaware that they have a foodborne infection, a specimen is not obtained by the health care provider, or proper tests are not preformed in the laboratory (44). In 2007, the top three serotypes that caused human infections included *S.* Typhimurium (18.8%), *S.* Enteritidis (18%), and *S.* Newport (10.3%) (12). Of which resistance to at least 1 antimicrobial was found in 42.4% of the *S.* Typhimurium isolates, 9.6% of the *S.* Enteritidis, and 10.5% of *S.* Newport.

Every 20th non-Typhi *Salmonella* isolate from a sick individual is sent from public health laboratories to the NARMS unit within the CDC (12). Minimum inhibitory concentrations (MICs) values for these isolates were determined using a broth microdilution (Sensititre®, Trek Diagnostic Systems, Westlake, Ohio). Isolates were categorized as antibiotic sensitive or resistant based on the Clinical and Laboratory Standards Institute (CLSI, Formerly NCCLS) standards (14). Intermediate susceptibility was designated as sensitive to the antimicrobial. Pan-susceptible isolates were those that were sensitive to all antimicrobials tested. CDC NARMS data found *S.* Newport to be the third most common serotype isolated from cattle (19). *S.* Kentucky is not listed as a common serotype to cause infections because it is not often associated with human infections.

The *Salmonella* surveillance annual summary included human isolates from the Public Health Laboratory Information System (PHLIS) and nonhuman isolates from the U.S. Department of Agriculture, Animal and Plant Health Inspection Services, National Veterinary Services Laboratories (USDA/APHIS/NVSL) (12). PHLIS is an electronic system that tracks foodborne pathogens from human sources while the USDA/APHIS/NVSL data consists of isolates from animals and animal environments. Of the 40,666 *Salmonella* isolates reported to PHLIS, *S.* Newport was in the top three most reported *Salmonella* serotypes. In 2006, there was a reported 121 *Salmonella* outbreaks which caused more than 3,300 illnesses throughout the U.S. Once again *S.* Newport was one of the top three serotypes involved in the *Salmonella* outbreaks. Tomatoes contaminated with *S.* Newport were associated with 119 infections throughout 18 states. Georgia, North Carolina, and South Carolina represent the states with the most *S.* Newport human infections. While *S.* Newport dominated the top serotypes from human sources, *S.* Kentucky was the second most reported serotype from nonclinical, nonhuman sources (52).

Nonclincal results consist of isolates from herd and flock, feed, environmental testing, and from FSIS food testing. Isolation of *S.* Newport has leveled out over the years while *S.* Kentucky has been shown to be increasing in prevalence. Since *S.* Kentucky is also acquiring resistance to antimicrobials as well as increasing in prevalence in poultry, perhaps this pathogen too may be in the top 10 most common serotypes to cause human infections.

As stated above, *S.* Newport has been associated with human infections throughout the U.S. In 1998, the first domestically reported MDR *Salmonella* with CMY-2 AmpC ß-lactamase gene was found in a child and shown to have been acquired from cattle (20). Prior to 1998 extended spectrum cephalosporin resistance in *Salmonella* was not found in the U.S. MDR-AmpC *S.* Newport infections have been found to be associated with dairy cattle. Consuming raw milk and soft cheeses, eating ground beef, and contact with dairy cattle or dairy cattle environment have all been implicated in *S.* Newport infections (31, 59). Furthermore, the use of antimicrobials prior to eating foods contaminated with *S.* Newport increased the chances of becoming infected with a MDR *S.* Newport. Most MDR *S.* Newport foodborne infections were not due to outbreaks but rather sporadic incidents (59). Unlike MDR *S.* Newport infections, foodborne infections from pan-susceptible strains of *S.* Newport are usually associated with consumption of vegetables (29). This is most likely due to the fact that amphibians and reptiles are carriers for pan susceptible *S.* Newport and these animals often come into contact with vegetables located in open fields (59).

In the U.S., states with higher population of dairy cattle also contained higher numbers of MDR *S.* Newport (30). Also, the percentage of MDR-AmpC *S.* Newport isolates was 10-fold lower in the south as compared to the north. Greene *et al.* found that pan-susceptible isolates predominated in southern states and these isolates were seasonal. This pattern of resistance and

susceptibility was found to be consistent with previous theories concerning the use of antimicrobials in animals. The states with the highest density of cows and thus higher usage of antimicrobials had larger amounts of MDR-AmpC *S.* Newport isolates (30). This study also found that pan-susceptible *S.* Newport as compared to MDR *S.* Newport were seasonal and were more likely to occur in the summer and autumn. Given that pan-susceptible strains are associated with vegetables, this could explain the seasonality associated with this microbe since vegetables grow during the summer months.

As for *S.* Kentucky, poultry is the main reservoir for this organism throughout the U.S. (25). According to the CDC NARMS 2006 nonhuman data, both *S.* Newport and *S.* Kentucky were the second most recovered *Salmonella* strain from non-human isolates (19). However, *S.* Newport is the third most commonly isolated *Salmonella* from human infections while *S.* Kentucky is predominantly not a human pathogen (19). Travelers in Europe were reported to have acquired a strain of *S.* Kentucky with resistance to ciprofloxacin, extended-spectrum cephalosporins (ESCs) and trimethoprim-sulfamethoxazole. *S.* Kentucky is not usually associated with human infections; however, not only was this pathogen resistant to multiple antibiotics it was also more difficult to treat. Thus these *S.* Kentucky strains acquired multi-drug resistance as well as demonstrated an increase in pathogenicity (15). Another study found strains of *S.* Kentucky with the β-lactamase gene bla_{CMY-2} which confers resistance to extended spectrum cephalosporins (9). As stated previously, *S.* Kentucky has increased in prevalence in poultry and has acquired resistance to multiple antibiotics including extended spectrum cephalosporins. Furthermore, this pathogen has the ability to cause a severe *Salmonella* infection that is difficult to treat. *S.* Kentucky warrants the need for increased scrutiny to prevent it from joining the ranks of the MDR *S.* Typhimurium DT104 and MDR *S.* Newport. Thus it is extremely useful to

compare a pathogenic strain of bacteria, such as *S.* Newport, to a less pathogenic strain, such as *S.* Kentucky, to find differences that could point to mechanisms that would enable the pathogen to cause disease. Comparison of these two foodborne pathogens genomes will provide invaluable information concerning pathogenicity.

Comparative Genomics and Mauve

Pulsed-field gel electrophoresis (PFGE) is the most common method used for epidemiological studies of pathogenic bacteria. PFGE uses restriction enzymes to digest genomic DNA which is then separated by pulses producing a pattern. The patterns from several pathogens can be compared to determine the relationship of the organisms to each other. Those with similar patterns are considered indistinguishable (35). Interpretation of PFGE results can be difficult due to the fact that some bacteria tend to be clonal and thus appear to be epidemiologically related, while some epidemiologically related bacteria appear different due to differences in banding patterns. In the case of *S.* Newport, studies have found that MDR strains were indistinguishable from each other and PFGE clusters correlated based on the antimicrobial susceptibility profiles (33, 64). PFGE is able to link human foodborne infections due to various *Salmonella* serotypes to a contaminated food source or animal source (6, 23, 24). However, due to its clonal nature, PFGE is sometimes unable to differentiate *Salmonella* isolates that are epidemiologically unrelated (38). Thus other techniques are needed to study the genetic variability within foodborne pathogens.

Microarray hybridization is a new technique that can be used to compare the genetic relationship of closely related microorganisms. Microarrays consist of coated glass slides or membranes containing DNA probes which can be in the form of synthetic oligonucleotides or PCR-based product (50, 63). Glass slide microarrays allow for small volumes of DNA to be

deposited thus producing a high density array (8). Oligo-arrays in which the oligonucleotides are synthesized *in situ* onto the glass support produce the highest density array; however, they are expensive (8). PCR-based products are from the open reading frames of a sequenced strain of bacteria. Microarray hybridizations with unknown microbial sequences can be compared to the sequenced strains on the chip and thus determine the absence or presence of genes in the unknown (28). Microarrays have thousands of probes which make it more discriminatory than PFGE. Furthermore, since the probes are physically attached to a chip there is not an issue of variation as in the case of PFGE which is analyzed on a gel.

Comparative genomic hybridizations (CGH) is the process of hybridizing the sequence of an unknown genome to that of a known genome on the same microarray chip (50). The genomic DNA from two bacteria are labeled with different fluorophores, cy3 or cy5 (50). The chips are then scanned with a laser which excites the labeled DNA producing signal intensities. Special software is used to calculate the signal intensity values which can be manipulated in Excel to produce absence or presence values (50). Not all the genomic information concerning the bacteria of interest will be represented on the chip. Genetic information specific to that bacteria will not be spotted on the chip and small deletions will be missed. DNA microarrays can be used to study genome mutations and amplifications, presence of antibiotic resistance genes and pathogenicity as well as identification of mixed microbial samples (10, 26). Several bacterial species have been studied using microarray hybridizations. Studies included pathogenic and non-pathogenic bacteria as well as antibiotic resistant strains. Once the sequence of the *S.* Typhimuium (LT2) genome was completed, the knowledge of the relationship to other *Enterobacteriaceae* as well as the evolution of this bacterium was enhanced tremendously (43). Genomic comparisons with 22 other *Salmonella* strains were made using the PCR product

containing the open reading frames (ORFs) of *S.* Typhimurium LT2 which covered 97% of the genome (51). Using information derived from a phylogenetic tree it was found that based on the relationship between the various *Salmonella,* these organisms acquired new genes via horizontal transfer (51). Horizontal transfer of genes is believed to be the largest contributor to an organism's survival in a new niche (40). Later a microarray chip was constructed that contained 98% of the open reading frame of LT2 genome as well as *S.* Typhi (CT18), *S.* Typhi (STT), *S.* Paratyphi A SARB42 (SPA), and *S.* Enteritidis PT4 (SPT) (50).

Studies have shown that bacteria have a core set of genes that are similar across a group. For example, 54% of the ORFs in *Salmonella* were found to be similar in 24 different serotypes (13). Core genes are usually involved in housekeeping while variable regions are more expendable. The ability of a *Salmonella* serotype to infect mammals and birds is most likely due to the expendable regions of DNA (51). Thus the absences of genes in a particular serotype may limit the organisms to a specific niche. Perhaps this is why MDR *S.* Newport and *S.* Kentucky predominantly reside in dairy cattle and poultry, respectively. As stated in a study, given the variability in genetic content in *Salmonella,* it is most likely that several genes are responsible for the evolution of this pathogen. Genes no longer necessary for a particular niche were most likely lost and/or replaced by other genetic material (42).

As stated previously, genetic information for strains of *Salmonella* not spotted on a microarray chip will not be represented in the hybridization and thus information of the genes unique to the organisms will not be known. *Salmonella* contains approximately 400-600 genes that are specific to each serotype (43). Therefore, there will be about 400-600 genes that are specific to *S.* Newport and *S.* Kentucky that are not represented on the microarray chips. One method to identify unique genomic DNA in these two serotypes involves the comparison of the

S. Newport or *S.* Kentucky genome to the other strains of *Salmonella* located on the microarray chip using an alignment program called Mauve (17). This program allows the identification and alignment of the genome of several bacteria despite rearrangements and horizontal transfer. Locally collinear blocks (LCBs) are identified by Mauve based on regions that are homologous and do not contain any rearrangements. LCBs are represented as colored blocks that represent the alignment of the genome with the other genomes. These colored blocks can be either above or below (reverse orientation) a single black line based on its orientation. Regions located outside of the LCBs do not align with the other genomes and are thus unique to that organism. Inside the block of each LCB, Mauve draws a similarity plot to show the similarity between the genomes. The higher the similarity plot, the more similar the sequence is to the other genomes and the more color within the LCBs. Areas with no similarity plot (no color) are considered unique to that genome. Genetic information from the areas that are colorless and thus unique to that organism will be recorded in Excel (Microsoft Office) and ordered according to location on the genome as well as gene function (17).

Conclusion

The development of resistance to multiple antibiotics in food animals has made a usually self-limiting disease more complicated. In 2004, Helms reported that patients with an infection from a microbe resistant to multiple antibiotics were 4.8 times more likely to die while infections with quinolone-resistant microbes resulted in 10.3 times more death (34). He also found that individuals infected with a MDR *S.* Typhimurium were more at risk for an invasive illness or death within 90 days of the infection as opposed to an infection due to a pan-susceptible bacterium. There are far more complications due to bacteria that acquire resistance to antibiotics than those from non-resistant strains. These risks include the increased likelihood of acquiring a

blood infection, septicima, increased hospitalization, increased chance of complications, sequela, and even death. Food is intended to nourish and strengthen our bodies not to harm us. It is the responsibility of the food industry and science industry to provide safe foods for consumers. It is imperative that this war with MDR pathogen continues to keep pace with the constantly evolving organism. This includes investigating the microbes' genetic makeup to determine not just the genes for resistance but also virulence genes that have allowed this microbe to out compete other sensitive strains as well as cause a more invasive disease in humans. If science can get a step ahead of bacteria by learning more about their genetic abilities, we may be able to predict and prevent future complications due to antibiotic resistance.

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CHAPTER 3

COMPARATIVE GENOMICS OF *SALMONELLA* NEWPORT WITH RESISTANCE TO MULTIPLE ANTIBIOTICS[1]

[1]**Bauer-Turpin, J.E., Frye, J.G., Harrison, M.A., Berrang, M.E. To be submitted to Foodborne Pathogens and Disease.**

Abstract

Resistance to multiple antibiotics is a major concern with foodborne pathogens since they often cause a more invasive human illness, resulting in prolonged hospitalizations, deaths, and sequelae. Multiple antibiotic resistance (MDR) has developed domestically in *S.* Newport and Pulsed-field gel electrophoresis (PFGE) has found an association with strains that cause human gastroenteritis and strains that colonize dairy cattle. In this study, *Salmonella* isolates (n=22) were selected from the National Antimicrobial Resistance Monitoring System (NARMS) to represent the most genetic diversity as determine by PFGE analysis. DNA was extracted from these *Salmonella* isolates and subjected to comparative genomic hybridizations (CGH) on *Salmonella* whole genome arrays containing genes of 5 *Salmonella* (Typhimurium LT2, Typhi strain CT18, Typhi Ty2, Paratyphi A, and Enteritidis SPT4). Absence versus presence results of the 5,660 genes on the array were analyzed by Bionumerics (V 6.0, Applied Maths Austin, TX, USA). *S.* Newport was further investigated with genome alignment program Mauve (The University of Wisconsin-Madison, Madison, WI, USA) to determine genes that were unique to this serotype. Majority of MDR *S.* Newport strains contained the Gifsy-2 prophage which encodes a *sodC* gene which allows for macrophage survival as compared to pan-susceptible strains. On the other hand, several pan-suceptible strains were missing a phosphotransferase system for fructose and mannose metabolism, thus the MDR strains would be capable of using this sugar source. There were 100 unique genes found with the Mauve genome alignment as compared to the *Salmonella* genomes located on the array. They include arsenic resistance, defense mechanisms, cell wall/membrane biogenesis, cell motility, and carbohydrate metabolism. Even though these organisms are genetically similar they behave very differently and are associated with extremely different environments. Some of the variation in genetic

content includes genes involved in virulence and sugar metabolism. Any of these differences could contribute to niche development as well as the ability to cause disease.

Introduction

Microbes associated with foods are adapting to changes in food production or processing. These changes allow microbes to establish new niches on foods not previously associated with that organism; thus, potentially producing a new vehicle for foodborne illness. Modifications within the food production and processing areas include the use of antimicrobials in agriculture, which has allowed bacteria resistant to antibiotics to thrive in environments that were previously established by sensitive bacteria (26). Furthermore, bacteria can acquire multiple antibiotic resistance (MDR) genes via horizontal gene transfer. Antimicrobials used in agricultural settings are involved in the emergence as well as the persistence of multidrug resistant (MDR) strains of bacteria (34). Patients infected with MDR pathogens often require antibiotic treatment, have an increase in the duration of hospital stay, and have a higher likelihood of acquiring septicemia which can result in death. Also, antibiotic treatment of a MDR microbe may eliminate competition by decreasing growth of antibiotic sensitive strains of bacteria while allowing the MDR strain to rapidly multiple in humans.

According to the World Health Organization's survey, *Salmonella enterica* serovar Newport has emerged as the predominant *Salmonella enterica* serotype found in humans worldwide (15). In 2006, approximately 9% of the 1.4 million foodborne infections in the United States are caused by *S.* Newport (22). Dairy cattle are the main vehicle of foodborne infections from *S*. Newport in humans and a large percentage of *S*. Newport in dairy cattle are resistant to multiple antibiotics (14, 18). On the other hand pan-susceptible strains of *S.* Newport are usually associated with vegetable contamination (17). Risks for infection with an MDR *S.* Newport include consumption of ground beef or dairy products, contact with cattle and handling of contaminated pet treats (18, 28). It is unclear why there is a recent increase *S*. Newport

colonization of dairy cattle or why large proportions are resistant to multiple antibiotics. Previous studies have shown that the use of antimicrobials in food animals is linked to the increase in antibiotic resistance in pathogens found in humans (38). Since 1991, ceftiofur, a third generation cephalosporin, has been used in cattle throughout the U.S. for treatment of respiratory disease (42). Furthermore, tetracycline has been added as a milk replacer to treat diseases in cattle (26). It is believed that the use of antibiotics in agricultural settings selects for antibiotic resistant pathogens; therefore, it is possible that the use of ceftiofur and tetracycline in cattle has selected for MDR *S.* Newport. One explanation for this phenomenon is that the use of antibiotics inhibits or kills sensitive bacteria, allowing antibiotic resistant pathogens to overpopulate that area (43). Also, since resistance to third generation cephalosporins is carried on a plasmid along with several other antibiotic resistant genes using one antibiotic can select for resistance to many other antimicrobials (2, 10). Furthermore, non-pathogenic commensal bacteria can transfer antibiotic resistant genes to pathogenic bacteria without the pressure of antimicrobial selection (30, 42). Since bacteria can acquire resistance to antibiotics, the fact that 95% of *Salmonella* illnesses in humans are due to foodborne infections is daunting (27). This could have profound implications as the pathogenic bacteria in food animals acquire resistance to the very drugs humans use for treatment of salmonellosis.

Another notable issue with MDR *S.* Newport is the fact that these pathogens tend to be clonal making pulsed field gel electrophoresis (PFGE) analysis cumbersome (8). Previous studies found that MDR *S.* Newport from multiple locations contained a high degree of genetic relatedness when analyzed with PFGE (29). Also, genotypic and phenotypic similarities have been demonstrated in *S.* Newport isolated from cattle and humans with PFGEs and antimicrobial susceptibility testing, respectively (18). PFGEs, as well as multilocus sequence typing (MLST),

have found evolutionary differences between older *S.* Newports and the more recent MDR *S.* Newports (19). PFGE results show that genetically related strains of *S.* Newport acquired plasmids with multiple antibiotic resistant genes (19). However, PFGEs provide limited information concerning the natural genetic variability of these bacteria. Additionally, given that these pathogens are clonal they can appear to have epidemiological similar backgrounds and yet come from various sources. This can make identification of sources and transmission routes of outbreak strains extremely difficult. Comparative genomic hybridizations (CGH) can be used to determine the genetic variation of these multidrug resistant pathogens. Furthermore, CGH have previously shown specific genes within a few strains of MDR *S.* Newport related to growth and survival as well as genetic mobile elements (20). Gathering more information concerning the genetic makeup of *S.* Newport will provide a better understanding of the genes that enhances its ability to colonize dairy cattle as well as its development of resistance to multiple antibiotics. Also, this information could provide techniques for rapid identification of outbreak strains of MDR *S.* Newport that could enhance the trace back methods of PFGE, thus providing rapid interventions to reduce the impact of infections on animals and humans.

The objective of the study was to determine the differences between MDR *S.* Newport and pan-susceptible *S.* Newport, isolates from the National Antimicrobial Resistance Monitoring System (NARMS). Comparative genomic analysis was used to compare the genomic content of *S.* Newport to the sequenced genomes of *Salmonella* to determine differences within the strains as well as the evolutionary relationships between these strains. This allowed for the determination of the presence or absence of genes of *S.* Newport as compared to 5 other *Salmonella,* but differences between MDR strains and pan-susceptible strains were also determined. In addition, the genome of *S.* Newport was aligned with Mauve to the *Salmonella*

used as probes on the microarray chip to determine genes that are unique to *S.* Newport. Information concerning the genetic makeup of S. Newport will provide a better understanding of the genes that enhance its ability to colonize dairy cattle and its development of resistance to multiple antibiotics.

Material and Methods

***Salmonella* Newport stains.** Isolates (n=24) for this study were obtained from the National Antimicrobial Resistance Monitoring System to represent the most genetic diversity as determined by PFGE analysis. Antimicrobial resistance profile was determined by the National Antimicrobial Resistance Monitoring System (NARMS) using the Sensititer system (Trek Diagnositc Systems, Inc., Westlake, OH) which included amikacin, amozicillin-clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim-sulfamethoxazole as previously described (39). Isolates were defined as being susceptible, intermediate, or resistant following the Clinical and Laboratory Standards Institute breakpoints (7). PFGE analysis was performed following the CDC PulseNet protocol using the restriction enzyme XbaI (16). Bionumerics 5.10 software (Appled Maths, Sint-Martens-Latem, Belgium) was used to analyze images of gels and dendrograms of the PFGE patterns were generated with Dice correlation coefficients with a tolerance of 1.5%. Dendrograms of mulitdrug resistant patterns as well as pan-suceptible patterns were generated from a large group of *S.* Newport from the VetNet database to determine similarity clusters (6). Isolates were selected from the larger group based on distance on the dendrogram, date strain was obtained, source, and location. PFGE patterns selected from the larger group of *S.* Newport were analyzed again to determine the similarity cluster of the selected group.

Microarray design. The *Salmonella* microarray contained 5,660 PCR products which covered 95% of all genes in genomes of *S.* Typhimurium strain LT2, *S.* Typhimurium strain SL1344, *S.* Typhi strain CT18, *S.* Typhi strain Ty2, *S.* Paratyphi A strain SARB42, and *S.* Enteritidis strain PT4. Each multiserotype microarray contained the genomes in triplicate thus producing 3 identical experiments on one chip (32).

Genomic isolation, labeling and hybridization. Genomic DNA was isolated following manufacturer's instructions using the GenElute bacterial DNA kit (Sigma, St Louis MO, USA). Genomic DNA (1.5 µg) from the control, *S.* Typhimurium LT2, and *S.* Newport were labeled overnight as previously described with cy5 and cy3, respectively (32). Pre hybridization was done as described (Corning, Inc.) *S.* Newport along with *S.* Typhimurium LT2, control, were hybridized as previously described onto the *Salmonella* genomic chips overnight at 42°C in a hybridization chamber (Corning, Inc.) (32). Post hybridization washes were done as previously described. Genomic chips were scanned with the GenePix Personal 4100A (Molecular Devices, Sunnyvale, CA) using the GenePix Pro software to acquire an image.

Data acquisition. QuantArrray 3.0 software (Packard BioChip Technologies, Billerica, MA) was used to analyze the spot intensities. Data was normalized by subtracting the background noise from the intensity values resulting in the signal of the spot in each channel. The *Salmonella* genomic chip was printed in triplicate resulting in 3 data points for each spot. The median of the 3 spots was used to determine the absence or presence of the *S.* Newport genomes as compared to the other *S. enterica* genomes. Data was analyzed to determine the absence (0) or presence (1) values as previously described (31).

Bionumerics analysis. Microarray absence presence data was imported into the Bionmuerics program (V 6.0, Applied Maths Austin, TX, USA) using the import fields and character function.

Imported data was manipulated using cluster analysis with unweighted-pair group method using arithmetic averages (UPGMA) to determine the phylogentic relationship based on the genetic content of the *S.* Newport used in this study. Statistic tools in Bionumerics were used to determine the percentage of genes absent or present in *S.* Newport as compared to the *Salmonella* genomes on the microarray chip.

Sequence alignment. All *Salmonella* genomes located on the microarray chips were aligned with the genome of a MDR *S.* Newport GenBank number CP00113 using the Mauve genome alignment program (The University of Wisconsin-Madison, Madison, WI, USA). Alignments were used to determine the genes that were unique to *S.* Newport as compared to the other *Salmonella* genomes located on the microarray chips. Locally collinear blocks (LCBs) were identified by MAUVE based on regions that were homologous and did not contain any rearrangements. LCBs were presented as colored blocks that represent the alignment of the genome with the other genomes. These colored blocks were either above or below (reverse orientation) a single black line based on its orientation. Regions located outside of the LCBs did not align with the other genomes and are thus unique to that organism. Inside the block of each LCB, Mauve creates a similarity plot to show the similarity between the genomes. The higher the similarity plot, the more similar the sequence is to the other genomes and the more color within the LCBs. Areas with no similarity plot (no color) are considered unique to that genome. Genetic information from the areas that are colorless and thus unique to that organism were recorded in Excel and placed in order according to location on the genome as well as gene function (9).

Results

Strain information. Upon further PFGE analysis, 3 of the isolates were found not to be *S.* Newport and were left out of the study (less than 60% similarity). Eight isolates were pan-susceptible, 1 was resistant to tetracycline only, and 13 were resistant to multiple antibiotics of which 7 were resistant to third generation cephalosporin. Most isolates had antimicrobial resistance to at least amoxicillin/clavulanate, ampicillin, cefoxitin, ceftiofur, cephalothin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim-sulfamethoxazole. Eight isolates were from cattle, 4 from equine, 2 from swine, 2 from reptiles, 1 from turkey, 4 from humans, and with 1 from an unknown source (Table 3.1). Isolation of *S.* Newport from the various organisms ranged in dates from 1999-2006.

PFGE. To determine the relationship of the MDR *S.* Newport to each other as well as to pan-susceptible strains of *S.* Newport, isolates were analyzed with PFGE and dendrograms were built using Bionumerics software using Dice correlation coefficients with a tolerance of 1.5% (Figure 3.1). Similarity clusters from PFGE patterns revealed 4 clusters which all consisted of a mixture of pan-susceptible and MDR *S.* Newports. Cluster 1 consisted of 3 pan-susceptible and 1 tetracycline resistant *S.* Newport and they were approximately 65% identical. Cluster 2 on the other hand was more than 80% identical and included 3 MDR *S.* Newports that were indistinguishable. Cluster 2 also included 2 more MDR isolates and 2 pan-susceptible isolates. Cluster 3 consisted of 6 MDR isolates including all the *S.* Newport isolated from humans and were approximately 80% identical. Cluster 4 which was less than 65% identical, consisted of 2 MDR and 2 pan-susceptible *S.* Newport.

Microarray analysis. The genomic content of 24 *S.* Newport was compared with *S.* Typhimurium LT2, *S.* Typhi CT18, *S.* Typhi Ty2, *S.* Paratyphi A SARB42, and *S.* Enteritidis

PT4 genomes. The microarray results were compiled into one Excel spreadsheet to compare the genetic variations within the *S.* Newport isolates. Initially, analysis was performed by finding large sections of adjacent absent/present probe hybridization indicating contiguous sections of the chromosome. Further analysis included looking for the absence/presence of specific genes with functions that would correlate with survival in an environmental niche or virulence development. Table 3.2 demonstrates the genes of interest that were absent or present within the various *S.* Newports as they relate to location on the *S.* Typhimurium LT2 (STM) genome. Several pan-susceptible isolates were missing genes from the phosphotransferase system (STM0571 to STM0577) for fructose and mannose metabolism. Three pan-susceptible isolates were missing metabolic pathway genes such as those involved in valine, leucine, and isoleucine biosynthesis (STM0328 to STM0335). Two pan-susceptible isolates from cattle were missing the region STM0761 to STM0769, genes involved in the citrate cycle (TCA cycle) and glyoxylate and dicarboxylate metabolism. The *rfb* cluster which is involved in O-antigen biosynthesis was missing in 15 *S.* Newport isolates. Eleven MDR *S.* Newport and 3 pan-susceptible *S.* Newport isolates were missing a putative gene cluster that included the *agaR* operon, transcriptional regulator of sugar metabolism, as well as genes for galactose, fructose, and mannose metabolism. Interestingly, 5 MDR *S.* Newport isolates have the region STM2741 to STM2768 which is absent in the other isolates. This region encodes genes for fructose and mannose metabolism as well an ATP-binding protein involved in virulence.

Virulence genes include flagella, which allows the bacteria to come into close contact with the host cell, and pili, which functions by adhering to the host cell. A large group of pan-susceptible and MDR *S.* Newport were missing the *stc* cluster encoding for pili assembly (STM2149-STM2152). Four pan-suceptible isolates were missing the *lpf* cluster which encodes

fimbrial genes. Eleven MDR *S.* Newport which includes all isolates from human sources, were missing the *yih* cluster that encode for capsule assembly.

All of the *S.* Newport isolates were missing the plasmid PLST genes and the Fels-1 prophage genes found in LT2. A few of the isolates were missing some of the Gifsy-1 and 2 as well as the Fels-2 prophage genes. Of the 4 functional prophage genomes found in LT2, most of the isolates contained the Gifsy-1 and Girsy-2 prophage and a few contained genes from the Fels-2 prophage. Also most isolates contained the prophage region from STM4196 to 4219. Twelve MDR *S.* Newport contained the *sodC* gene from the Gifsy-2 prophage which provides the bacterium defense against macrophage killing during the infection process. All human *S.* Newport isolates contained this important virulence factor. A few isolates had one or 2 of the *spv* genes found in the PSLT virulence plasmid (Table 3.2). All isolates contained genes for the *Salmonella* pathogenicity island (SPI-1) Type III Secretion Systems (TTSS) and SPI-2 TTSS. Proteins from SPI-1 included *sopE*2 responsible for actin cytoskeleton rearrangements and internalization, *sopA* , *sopB*, and *sopD*. The protein from SPI-2 included the gene cluster *ssaB* to *ssaV*.

Bionumeric analysis. After compilation of each isolates microarray results, there were 5305 genes which were analyzed in Bionumerics. Using statistics tools in Bionumerics it was found that *S.* Newport had 77% to 86% of the genes present as compared to the *Salmonella* genomes on the microarray chip (Table 3.3). Cluster analysis with UPGMA was based on the absence or presence of 5305 genes on the microarray chip (Figure 3.2). Mulitdrug resistant *S.* Newport clustered into 2 distinct groups. One cluster of MDR *Salmonella* included isolates from one human, 2 horses, 1 turkey, 1 cattle, and 1 unknown with approximately 97% similarity. The other cluster with more than 96% similarity contained isolates from 3 human, 1 snake, 1 cattle,

and even 1 pan-susceptible isolate from swine. Another cluster included pan-susceptible and antimicrobial resistant *Salmonella* strains with approximately 92% similarity. This group included 4 cattle sources, 1 ground beef source, 1 swine source, and 1 horse source. The antimicrobial resistant strains in this cluster included 1 isolate resistant to tetracycline and 1 isolate resistant to ampicillin, streptomycin, sulfisoxazole and tetracycline.

Analysis of S. Newport genome sequences. To determine genes that are unique to *S.* Newport and potentially provides niche adaption and virulence development, a sequenced strain of MDR *S.* Newport was analyzed using the genome alignment program Mauve. The whole genome sequence for *S.* Newport SL254 including the MDR plasmid SN254 were used for genomic comparison to all *Salmonella* genomes located on the microarray chip. *Salmonella* SL254 sequence contains 4912 genes and the plasmid SN254 contains 198 genes (J. Craig Venter Institute, Rockville, MD, USA). The *S.* Newport genome contained 290 more genes than *S.* Typhimurium genome. Also, *S.* Newport contained 144 pseudogenes which are single-base mutations that cause a frameshift or stop codon causing the gene to no longer function (24). Analysis of SNSL254 using the Mauve software revealed 100 genes that were unique as compared to the other *Salmonella* genomes used as probes on the microarrays. Also, there were 79 hypothetical proteins unique to the SNSL254 genome. Table 3.4 illustrates the unique genes with known functions found in SNSL254. This pathogen contained unique genes involved in transcription (4 genes), replication, recombination and repair (2 genes), defense mechanisms (3 genes), signal transduction mechanisms (3 genes), cell wall/membrane biogenesis (5 genes), cell motility (2 genes), carbohydrate transport and metabolism (5 genes), general function prediction only (18 genes), and several genes not found in the Clusters of Orthologous Groups (COGs) database. Transcription genes included regulators for arsenic resistance, phage regulators, and

mannitol transcription. Two genes involved in replication, recombination, and repair included a replicative DNA helicase, and a site-specific recombinase phage protein. Defense mechanisms included 3 unique putative type I restriction-modification system, a S subunit, M subunit, and EcoEI R protein. Signal transduction includes mannitol-specific phosphtransferase enzyme, arsenate reductase, and adenine methylase genes. Unique genes associated with the cell wall included rhamnosyl, mannosyl and glycosyl transferases. Other notable unique genes involved in carbohydrate transport and metabolism included phosphotransferasese for mannitol and fructose-1-6 bisphophatase. In addition there were 2 arsenical proteins involved in inorganic ion transport. Also, *S.* Newport contained unique genes for cell motility that included periplasmic chaperones and flagellin. Several genes predicted with general function and several not found in the COG database included phage proteins.

Discussion

Foodborne pathogens resistant to multiple antibiotics which have been implicated in human foodborne infections have emerged in dairy cattle causing foodborne outbreaks from beef and milk products. Tracing outbreak sources can be difficult due to the clonal nature of MDR *S.* Newport and thus inhibit finding the outbreak source. In this study, CGH analysis was used to determine the phylogentic relationship of the 22 *S.* Newport based on the microarray absence/presence data which was used to construct dendrograms. The length of the arms of the phylogentic trees demonstrates the level of similarity of the pathogens. Originally, isolates were selected from PFGE dendrogram based on distance from each other. The purpose was to select isolates that were dissimilar based on PFGE distance, year, and source. This would then allow us to determine the genetic variability within this clonal organism. The majority of the isolates that clustered together with the PFGE dendrogram also clustered together with the dendrogram

from the microarray data. However, several isolates were found to cluster more strongly with different groups using the microarray data as compared to PFGE. More importantly, the percentage of similarity used in the cluster analysis to differentiate the *S.* Newport microarray data found the isolates to be 92% or more similar, while PFGE results was only 80% similar. These results suggest that microarray analysis is less discriminatory than PFGE. Previous genome sequencing of *Salmonella* serotypes found these microbes to range up to 97% identical within the different serotypes (25). Researchers found that CGH of various *Salmonella* serotypes were sometimes found to be genotypically close due to similar genetic content (31). Our research has shown *S.* Newport whether MDR or pan-susceptible to be genotypically closely related as opposed to results from PFGE analysis. Since these organisms are closely related genotypically, it may be easy to find genes responsible for developing specific niches such as MDR *S.* Newport association with dairy cattle and pan-susceptible *S.* Newport association with fruits and vegetables. Also, virulence genes found in MDR *S.* Newport could lead to understanding the increased pathogenicity of this microbe.

Salmonella have horizontally acquired genes found in prophage and pathogenicity islands which confer genes for virulence (11). One of the genomes used as a probe on the microarray chip, *S.* Typhimurium, contains 4 functional prophages, Fels-1, Fels-2, Gifsy-1 and Gifsy-2. Certain prophages are rarely found outside of *S.* Typhimurium and several prophages from *S.* Typhimurium encode toxins that can be inserted into the host cell (4). In this study, all of the *S.* Newport isolates were missing the Fels-1 prophage. However, most of the isolates contained genes for Gifsy-1 and Gifsy-2 prophages. Three *S.* Newport contained genes that encoded for approximately 72% of the Fels-2 prophage. Nine MDR *S.* Newport and 4 pan-susceptible *S.* Newport contained the entire Gifsy-1 cluster. Of the nine MDR *S.* Newport that contain the

entire Gifsy-1 cluster, 8 of those also contained the Gifsy-2 cluster. Previous studies have shown that Gifsy-1 and Gifsy-2 contain virulence factors, like *sodC,* that make *S.* Typhimurium a more pathogenic organism (37). Finding Gifsy-1 in organisms other than *S.* Typhimurium is usually rare. However, not only did the isolates in this study show the presence of the Gifsy-1 prophage but the genome of the sequenced SNSL254 *S.* Newport also contained this prophage. Gifsy-1 has shown a less pronounced effect on virulence as compared to Gifsy-2 prophage. The Gifsy-2 prophage enables *S.* Typhimurium to cause systemic infections in mice (12). Virulent strains of *Salmonella* contain the *sodC* gene located in the Gifsy-2 prophage which is needed for intracellular survival in the host (1). Since MDR *S.* Newport is considered more virulent due to its higher rate of infection in humans as compared to pan-suceptible strains, then perhaps the *sodC* protein has contributed to this increase in virulence.

Genetic variation is often found in genes coding for fimbriae, flagella, and LPS in *Salmonella*. Variation in cell surface affects the virulence of *Salmonella* allowing it to colonize and infect the host as well as evade the host immune system (11). Eight MDR *S.* Newport and 4 pan-susceptbile *S.* Newport were missing the *stc* cluster which is important in adhesion to a host surface as well as other bacterium. Without this cluster for pili the bacterium would need an alternative gene for adhesion necessary to transfer plasmids to other bacterium and toxins to a host cell. Perhaps this alternative adhesion provides MDR *S.* Newport the ability to acquire a new niche in dairy cattle. Alternatively, losing adhesion genes may limit this pathogen to certain environments thus forcing *S.* Newport to form a new niche in cattle. Furthermore, 4 pan-susceptible *S.* Newport were missing the *lpf* cluster which encodes fimbrial genes. As stated earlier, outer membrane protein genes including fimbrial genes differ throughout various bacteria (35). These differences can allow pathogens to survive in new environments.

Plasmids that confer virulence have been found in host-adapted serotypes such as *S.* Dublin which is found in cattle (3). Thus, it may be possible to discover virulence genes in MDR *S.* Newport which have enabled this pathogen to adapt to a new niche such as dairy cattle or cause infections in humans. Studies found a MDR plasmid that shares an IncA/C backbone with *Yersina pestis* and *Yersina ruckeri (41).* Interestingly, this plasmid encodes genes for replication/maintenance and type IV conjugative transfer thus this plasmid is easily transferred to other microbes. Other notable virulence plasmids are found in *S.* Typhimurium which contains 5 SPIs which are considered recent additions into the *Salmonella* chromosome (33). Most of the MDR *S.* Newport contained an important virulence gene for defense against the host macrophage. SPI-1 allows *Salmonella* to invade non-phagocytic cells while SPI-2 is needed for intracellular survival in phagocytes (35). The TTSS located in *S.*Typhimurium is used to inject toxins into a host cell. All isolates in this study contained the genes for both SPI-1 and SPI-2 and thus are likely capable of invasion of non-phagocystic cells as well as survival and proliferation in phagocytes. Furthermore, TTSS is usually found in gram-negative microbes that are associated with an animal or plant (4). Almost all of the PLST virulence plasmid was absent in all *S.* Newport isolates. Although a few of the isolates contained various *Salmonella* plasmid virulence (*spv)* genes. The *spv* operon is largely involved in the pathogencity of *Salmonella* and more specifically involved in systemic virulence (40). Therefore, *Salmonella* that lack the *spv* genes are more likely to cause gastroenteritis while those with this operon are more likely to invade the bloodstream. The *spv* operon consists of 5 genes *spvA, spvB, spvC, spvD,* and *spvR.* A mutation in the *spvR* will affect the expression of the *spv* operon thus this gene is often used to determine the Spv phenotype in *Salmonella* (11). Six *S.* Newport from this study contained the *spvR* gene and 4 of these isolates were also MDR. One of these MDR *S.* Newport isolates with

the *spvR* gene was isolated from cattle and also contained the *spvD* gene. Another MDR *S.* Newport from an equine source hybridized with the *spvC* and *SpvD* gene. The *spvC* gene is a toxin released by TTSS and has been shown to down regulate cytokine release from infected cells (23). *In vivo* studies have shown that *spvC* prevents a host immune response and thus helping the pathogen to proliferate (23). Studies have found that *S.* Newport is not usually associated with blood infections therefore, it may be necessary to have the entire *spv* operon to be able to cause septicemia.

In addition, sugar metabolism plays an important role in the establishment of a niche in a food animal. Livestock feed is formulated such that the carbohydrates not digested by the animal can be easily digested by the intestinal microflora (36). Cattle feed is formulated with mannanoligosaccharids so that microbes can metabolize the mannose as a source of energy while coliforms cannot. Another function of this diet is so to prevent colonization from enteric pathogens with the addition of a mannose residue to the cell wall lectin. Results from this study found that all strains of MDR *S.* Newport contained a phosphotransferase system capable of fructose and mannose metabolism while only a few pan-susceptible strains carried these genes. Since MDR *S.* Newport have the genes necessary for mannose metabolism it is possible this pathogen has an advantage in the gut of cattle.

CGH can only analyze genes that are located in the genome of the other bacterium located on the chip. *Salmonella* contains as many as 400-600 genes specific or unique to each particular serotype. Therefore, there will be about 400-600 genes that are specific to *S.* Newport that will not be represented on the microarray chips used in this study. Recent genome sequence of *S.* Newport makes it possible to analyze this organism with genome alignment. The sequence strain of *S.* Newport contained a large number of pseudogenes (144) as compared to *S.*

Typhimurium. McClelland *et al* found several pathogens that also had large numbers of pseudogenes to have evolved into human specific pathogens (24). It was suggested that the large numbers of pseudogenes was due to the bacterium eradicating genes from itself because these genes were no longer needed in the new niche (24). To study the genes that are unique in *S.* Newport the software Mauve was used to align the genomes of the 5 *Salmonella* along with the genome of *S.* Newport. Table 3.4 demonstrates the genes that were unique based on COG. Since *S.* Newport was compared to 5 other *Salmonella* genomes, there were only 100 genes that were specific to this organism as compared to the other bacteria. Some genes of interest found only in *S.* Newport include genes for arsenic resistance which is an efficient way to inhibit many antimicrobial biosynthetic pathways (13). Operons for arsenic resistance are distributed throughout bacteria, however, this operon was specific to *S.* Newport as compared to the other *Salmonella* genomes. These sequences were analyzed with the NCBI Basic Local Alignment Search Tool (BLAST) which reveal similarities with *S.* Kentucky, *S.* Tennessee, *S.* Agona, and *S.* Saintpaul. Studies have found that dairy cattle eat large quantities of arsenic as well as other heavy metals as these metals are found in the feed and as well as the soil (21, 36). Perhaps *S.* Newport acquired resistance to arsenic which has allowed this pathogen to survive in an arsenic rich environment and outcompete sensitive strains of bacteria. Another gene responsible for cell defense, SNSL254_A4881, is a restriction-modification system that helps protect the pathogen against invasion of foreign DNA by preventing cleavage of DNA. Bacteria produce restriction enzymes to destroy the DNA of other bacteria and viruses which then allows the microbe to proliferate (35).

Metabolic differences in pathogens, such as sugar metabolism, can allow bacteria to survive in new niches based on its ability to utilize carbohydrates in that environment. One

metabolic function found in the genome of *S.* Newport was a sugar phosphotransferase system (PTS) which is involved in the regulation of several metabolic and transcriptional processes. The PTS allows for the uptake of sugars across the cytoplasmic membrane, thus this system allows *S.* Newport to use sugar sources not available to the other *Salmonella* used in this comparison. Another PTS found in *S.* Newport was mannitol specific. Mannitol is a sugar alcohol found throughout the environment particularly in tree saps and fresh mushrooms. Furthermore, studies comparing the pathogen *Salmonella* to the commensal organism *E. coli* have shown that carbon utilization differed between these organisms. *Salmonella* utilized a wide range of carbons that included amino acids and sugar alcohols which may have helped them adapt to new niches as compared to *E. coli.*

There were several phage proteins found in *S.* Newport SNSL_254 that were not found in the other *Salmonella* genomes. This could indicate that *S.* Newport has acquired extra genes such as those involved in virulence or niche development. Bacteriophages have co-evolved with *Salmonella* and in some cases this pathogen can contain multiple prophages throughout their genome (5). It is these differences in prophages that contribute to the diversity of *Salmonella.* Studies have shown that the prophage genes encode virulence genes such as those for surface proteins which alter the antigenicity of the pathogen, intracellular survival genes, and even TTSS (25). The *S.* Newport used in this study contained genes for phage tail assembly, phage enzymes for degradation of peptidoglycan found in bacterial cell walls, and even phage replication proteins.

Comparative genomic studies have shown that even after *Salmonella* diverged from *E. coli* by acquiring the virulence genes via horizontal gene transfer 100 million years ago, these organisms have acquired another 1000 plus genes that have further divided *Salmonella* into

subspecies and serovars (33). As research has shown, *S.* Newport has developed into an important pathogen capable of acquiring resistance and spreading throughout dairy cattle. This study reports that MDR *S.* Newport has acquired several important virulence genes and phage genes. Other studies have shown that the loss of genetic functions can also contribute to the development of niches (24). Since the strains of *S.* Newport in this study lost several functions related to sugar metabolism, perhaps this organism is limited to an area such as the dairy cattle gut which provides a source of nutrients not seen elsewhere. Also, several of the MDR *S.* Newport contained genes for fructose and mannose metabolism as well an ATP-binding protein involved in virulence allowing these organisms to survive on these carbohydrates. Understanding the biology of antibiotic resistant strains of *S.* Newport can help to inhibit the colonization of food sources and thus human infections. More importantly, understanding foodborne bacteria such as *S.* Newport may help to prevent emerging pathogens from establishing niches as well as causing human illnesses.

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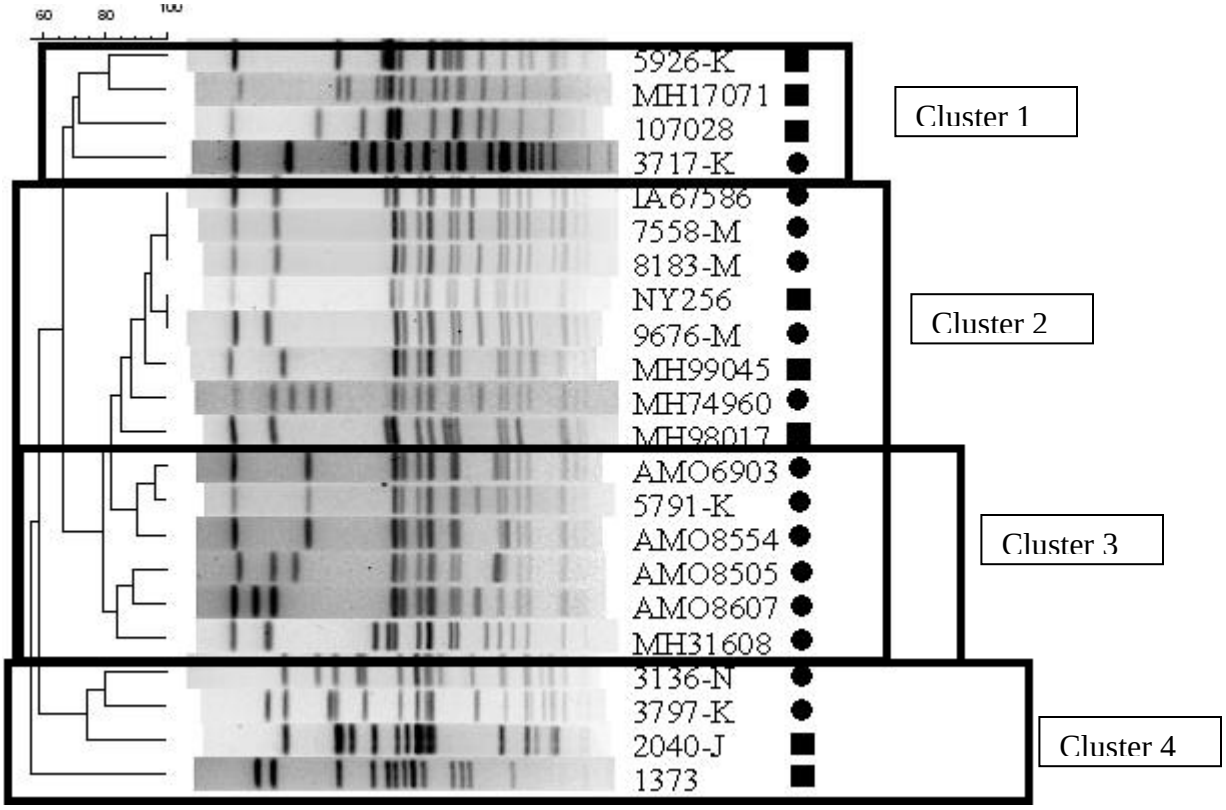

Figure 3.1 Cluster anaylsis from PFGE patterns from *S.* Newport isolates from various animal and clinical sources using Dice correlation coefficients with a tolerance of 1.5%. Circles denote antimicrobial resistance while squares represent isolates that are pansuscpetible.

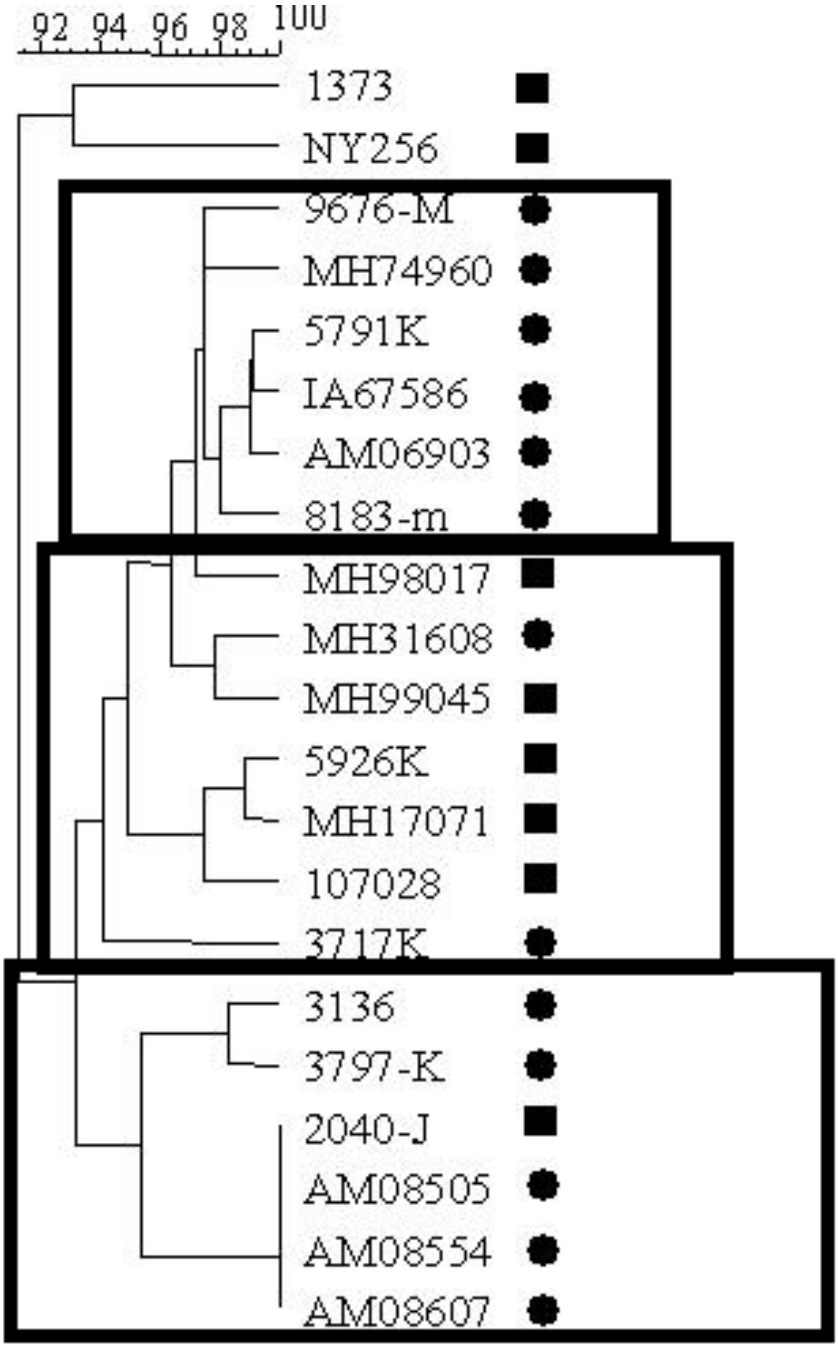

Figure 3.2 Cluster analysis of multidrug resistant *S.* Newport and pan-susceptible *S.* Newport from comparative genomic hybridization data using categorical clustering Unweighted Pair Group Method with Arithmetic Mean. Circles denote antimicrobial resistance while squares represent isolates that are pansuscpetible.

Table 3.1 *Salmonella* Newport isolates with source, year, and susceptibility patterns.

Isolate number	Year	Source	Susceptibility pattern
9676-M	2001	Equine	AmoAmpFoxTioCepChlStrSulTetTri
1373	2004	Cattle	Pan-susceptible
8183-M	2001	Equine	AmoAmpFoxTioCepChlKanStrSulTet
3797-K	2001	Cattle	AmoAmpFoxTioCepChlGenKanNalStrSulTet
2040J	2001	Swine	Pan-susceptible
AMO 8554	--[a]	Human	AugAmpFoxTioChlStrSulTet
AMO 8505	--[a]	Human	AugAmpFoxTioChlKanStrSulTet
AMO 8607	--[a]	Human	AugAmpFoxTioChlStrSulTet
AMO 6903	--[a]	Human	AugAmpFoxTioChlStrSulTet
MH99045	1999	Cattle	Pan-susceptible
MH74960	2004	Ground Turkey	AmoAmpFoxTioChlGenKanStrSulTetTri
3136-N	2001	Snake	AmoAmpFoxTioCepChlKanStrSulTetTri
MH98017	2002	Cattle	Pan-susceptible
5791-K	2001	Cattle	AmoAmpFoxTioCepChlKanStrSulTetTri
NY256	2002	Dairy Cattle	Pan-susceptible
107028	2003	Equine	Pan-susceptible
5926-K	2001	Cattle	Pan-susceptible
3717-K	2001	Cattle	Tet
IA67586	2001	Unknown	AmoAmpFoxTioCepChlGenKanStrSulTet
MH31608	2006	Ground beef	Ampicillin, Streptomycin, Sulfisoxazole and Tetracycline
MH17071	1999	Swine	Pan-susceptible

[a] Date of isolation unavailable.

Table 3.2 *S.* Newport genes of interest that did or did not hybridize with genes as they relate to location on *S.* Typhimurium LT2 (STM) genome. Gene name when applicable is in parenthesis.
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STM no.	0291-0293 (Saf)	0327-0335	0571-0577	0654-0659 (Ybe)	0716-0727	0761-0769	1350-1362 (Ydi)
9676-M	+	+	+	+	+	+	+
1373	+	-	-	-	+	-	-
8183-m	+	+	+	+	+	+	+
3797-K	+	+	+	+	+	+	+
2040-J	+	-	+	+	+	+	+
AM08554	-	+	+	+	+	+	+
AM08505	-	+	+	+	+	+	+
AM08607	-	+	+	+	+	+	+
AM06903	+	+	+	+	+	+	+
MH99045	+	+	+	+	+	+	+
MH74960	+	+	+	+	+	+	+
3136	+	+	+	+	+	+	+
MH98017	+	+	+	+	+	+	+
5791K	+	+	+	+	+	+	+
NY256	+	-	-	-	-	-	+
107028	+	+	-	+	+	+	+
5926K	+	+	-	+	+	+	+
3717K	+	+	+	+	+	+	+
IA67586	+	+	+	+	+	+	+
MH31608	+	+	+	+	+	+	+
MH17071	+	+	-	+	+	+	+

STM no.	2082-2097 (Rfb)	2937-2944 (Ygc)	3080-3085	3251-3256 (AgaR, GatY)	3277-3278	3516-3522 (Rtc)	3636-3640 (Lpf)	3651-3653
9676-M	-	-	+	-	-	-	+	-
1373	-	-	-	+	-	-	-	+
8183-m	-	-	+	-	-	-	+	-
3797-K	+	+	+	+	+	+	+	+
2040-J	+	+	+	-	-	+	+	+
AM08554	+	+	+	-	-	+	+	+
AM08505	+	+	+	-	-	+	+	+
AM08607	+	+	+	-	-	+	+	+
AM06903	-	-	+	+	-	-	+	-
MH99045	-	-	+	-	-	-	+	-
MH74960	-	-	+	-	-	-	+	-
3136	+	+	+	+	+	+	+	+
MH98017	-	-	+	-	-	-	+	-
5791K	-	-	+	-	-	-	+	-
NY256	-	+	-	+	-	-	-	+
107028	-	+	+	+	-	-	+	+
5926K	-	+	+	+	-	-	+	+
3717K	-	+	+	-	-	-	-	+
IA67586	-	-	+	-	-	-	+	-
MH31608	-	-	+	-	-	-	+	-
MH17071	-	+	+	+	-	-	+	+

Table 3.2 Continued

STM no.	3696-3698	3752-3755 (SugR, RhuM	3779-3785	3845-3846	3940-3942	4010-4020 (Ysh)	4110-4116 (PtsA, Frw, Pfl)	4195-4199
9676-M	+	-	+	-	-	-	+	-
1373	-	-	+	-	+	-	-	-
8183-m	+	-	+	-	+	-	+	+
3797-K	+	+	+	+	+	+	+	+
2040-J	+	+	+	-	-	-	-	+
AM08554	+	+	+	-	-	-	-	+
AM08505	+	+	+	-	-	-	-	+
AM08607	+	+	+	-	-	-	-	+
AM06903	+	-	+	-	-	-	+	-
MH99045	+	-	+	-	-	-	+	-
MH74960	+	-	+	-	+	-	+	-
3136	+	+	+	+	+	+	+	+
MH98017	+	-	+	-	-	-	+	-
5791K	+	-	+	-	-	-	+	-
NY256	-	-	+	+	+	+	-	-
107028	+	-	-	-	-	+	+	-
5926K	+	-	-	-	-	+	+	-
3717K	+	-	+	-	+	-	-	-
IA67586	+	-	+	-	-	-	+	-
MH31608	+	-	+	-	+	-	+	+
MH17071	+	-	-	-	-	+	+	-

Table 3.2 Continued

STM no.	4200-4217	4312-4313	4317-4318	4417-4436 (SrfJ)	4488-4498	4522-4529 (YjiW, Hsd, FliC	1959
9676-M	+	+	+	-	-	-	-
1373	-	+	+	-	-	-	+
8183-m	+	+	+	-	-	-	-
3797-K	+	+	+	+	+	+	+
2040-J	+	+	-	-	-	+	+
AM08554	+	+	-	-	-	+	+
AM08505	+	+	-	-	-	+	+
AM08607	+	+	-	-	-	+	+
AM06903	+	-	+	-	-	-	-
MH99045	+	-	+	-	-	-	-
MH74960	+	+	+	-	-	-	-
3136	+	+	+	+	+	+	+
MH98017	+	+	+	-	-	-	-
5791K	+	+	+	-	-	-	-
NY256	-	+	+	-	-	-	-
107028	+	+	-	-	-	-	-
5926K	+	+	-	-	-	-	-
3717K	+	+	+	-	-	+	-
IA67586	+	-	+	-	-	-	-
MH31608	+	-	+	-	-	-	-
MH17071	+	+	+	-	-	-	-

Table 3.2 Continued

Table 3.3 Percentage of genes present within *S.* Newport genes as compare to *Salmonella* whole genome microarray.

Newport Isolates	Present	Percentage present
9676-m	4485	85
1373	4341	82
8183-m	4487	85
3797-K	4574	86
2040-J	4452	84
AM08554	4452	84
AM08505	4452	84
AM08607	4452	84
AM06903	4454	84
MH99045	4459	84
MH74960	4500	85
3136	4570	86
MH98017	4473	84
5791K	4476	84
NY256	4215	79
107028	4309	81
5926K	4404	83
3717K	4404	83
IA67586	4456	84
MH31608	4416	83
MH17071	4416	83

Table 3.4 Unique genes in *S.* Newport SNSL254 identified with Mauve genome alignment

Locus tag	**Product**	**Function**
SNSL254_A3317	mannitol operon repressor	Transcription genes
SNSL254_A4842	phage transcriptional regulator, AlpA	
SNSL254_A2190	arsenic resistance transcriptional regulator	
SNSL254_A1143	adenine methylase	
SNSL254_A1140	replicative DNA helicase	Replication, recombination and repair genes
SNSL254_A4348	site-specific recombinase, phage integrase family	
SNSL254_A4358	Fic protein family	Cell cycle control, mitosis and meiosis genes
SNSL254_A4880	putative type I restriction-modification system, S subunit	Defense mechanisms genes
SNSL254_A4881	type I restriction-modification system, M subunit	
SNSL254_A4882	type I restriction enzyme EcoEI R protein	
SNSL254_A3321	mannitol-specific cryptic phosphotransferase enzyme iia component	Signal transduction mechanisms genes
SNSL254_A2189	arsenate reductase	
SNSL254_A1143	adenine methylase	
SNSL254_A2267	rhamnosyl transferase	Cell wall/membrane biogenesis genes
SNSL254_A2265	second mannosyl transferase	
SNSL254_A2264	glycosyl transferase, group 1	
SNSL254_A2271	CDP-abequose synthase	
SNSL254_A2269	glycosyl transferase, group 2 family protein	
SNSL254_A4669	periplasmic chaperone	Cell motility genes
SNSL254_A2120	flagellin	
SNSL254_A4669	periplasmic chaperone	Intracellular trafficking and secretion genes

Locus tag	Product	Function
SNSL254_A2006	putative O-actetyl transferase related protein	Carbohydrate transport and metabolism genes
SNSL254_A3321	mannitol-specific cryptic phosphotransferase enzyme iia component	
SNSL254_A2271	CDP-abequose synthase	
SNSL254_A3318	fructose-1,6-bisphosphatase, class II	
SNSL254_A3320	pts system mannitol-specific eiicba component	
SNSL254_A3319	L-sorbose 1-phosphate reductase	Amino acid transport and metabolism genes
SNSL254_A2187	arsenical pump-driving ATPase	Inorganic ion transport and metabolism genes
SNSL254_A2186	arsenical-resistance protein	
SNSL254_A4372	glycoside hydrolase, family 24	General function prediction only genes
SNSL254_A4837	putative dead/deah box helicase domain protein	
SNSL254_A4388	gp25	
SNSL254_A4389	late control gene D protein	
SNSL254_A4378	baseplate assembly protein W	
SNSL254_A4847	bacteriophage P4 DNA primase	
SNSL254_A4380	phage tail protein I	
SNSL254_A4383	phage tail sheath protein	
SNSL254_A4384	phage major tail tube protein	
SNSL254_A4377	baseplate assembly protein V	
SNSL254_A4370	phage Tail Protein X	
SNSL254_A4363	phage portal protein, pbsx family	
SNSL254_A4379	baseplate assembly protein J	
SNSL254_A3319	L-sorbose 1-phosphate reductase	

Table 3.4 continued

Locus tag	Product	Function
SNSL254_A2964	bacteriophage P4 DNA primase	General function prediction only genes
SNSL254_A2918	gp19	
SNSL254_A4381	gp19	
SNSL254_A4382	phage tail assembly protein	
SNSL254_A4364	putative conserved hypothetical protein	Function unknown genes
SNSL254_A4679	SrgB	
SNSL254_A4387	gp24	
SNSL254_A4367	gpM	Not in COGs genes
SNSL254_A4374	P2 phage tail completion protein R	
SNSL254_A1145	hypothetical phage-related protein	
SNSL254_A4665	integrase, catalytic region	
SNSL254_A4375	phage virion morphogenesis protein	
SNSL254_A0314	putative cytoplasmic protein	
SNSL254_A1154	gp33 TerL	
SNSL254_A2188	arsenical resistance operon trans-acting repressor ArsD	
SNSL254_A4536	tail assembly chaperone gp38	
SNSL254_A1666	transcriptional regulator, LysR family	
SNSL254_A4365	phage capsid scaffolding protein GpO	
SNSL254_A4369	phage head completion protein	
SNSL254_A1181	phage tail assembly protein	
SNSL254_A2834	replication protein O	
SNSL254_A4675	thiol:disulfide interchange protein DsbA	

Table 3.4 continued

Locus tag	Product	Function
SNSL254_A2835	gifsy-1 prophage cI	Not in COGs genes
SNSL254_A1149	phage-holin analog protein	
SNSL254_A4351	putative replication gene B protein	
SNSL254_A2266	O-antigen polymerase	
SNSL254_A2943	gp91	
SNSL254_A4879	endoribonuclease SymE	
SNSL254_A1152	bacteriophage lysis protein	
SNSL254_A4672	putative invasin protein	
SNSL254_A1146	gifsy-2 prophage protein	
SNSL254_A1129	site-specific recombinase, phage integrase family	
SNSL254_A1139	replication protein	
SNSL254_A4373	protein LysB	
SNSL254_A2185	integrase	
SNSL254_A4844	phage immunity repressor protein	
SNSL254_A2917	gp20	
SNSL254_A4668	repressor protein	
SNSL254_A2833	replication P family protein	
SNSL254_A2832	Eaa1	
SNSL254_A4841	putative capsid morphogenesis protein encoded in CP-933I	
SNSL254_A4371	putative prophage Hp1 family holin	
SNSL254_A4357	replication gene A protein	
SNSL254_A2958	glycoprotein 3	

Table 3.4 continued

Locus tag	Product	Function
SNSL254_A4349	Cox protein	Not in COGs genes
SNSL254_A4296	putative domain of unknown function	
SNSL254_A2961	phage immunity repressor protein	
SNSL254_a4840	putative polarity suppression protein encoded in CP-933I	
SNSL254_A1151	putative bacteriophage protein	
SNSL254_A4356	gp83	
SNSL254_A2956	phage transcriptional activator, Ogr/delta	
SNSL254_A0313	probable secreted protein	
SNSL254_A2830	putative bacteriophage protein	
SNSL254_A4366	phage major capsid protein, P2 family	
SNSL254_A4839	putative Ogr family transcription activator	

Table 3.4 continued

CHAPTER 4

COMPARITIVE GENOMICS OF *SALMONELLA* KENTUCKY ASSOCIATED WITH POULTRY COLONIZATION[1]

[1]**Bauer-Turpin, J.E., Frye, J.G., Harrison, M.A., Berrang, M.E. To be submitted to Foodborne Pathogens and Disease.**

Abstract

The most common *Salmonella enterica* isolated from poultry are serotype Kentucky yet this microbe is rarely associated with human disease. Studies have shown that *S.* Kentucky contains several genes necessary for pathogensis; however, little is known concerning the genetic content as compared to serotypes that are human pathogen. Genetically diverse isolates (n=12) for this study were acquired from the National Antimicrobial Resistance Monitoring System (NARMS). DNA was extracted from these *Salmonella* isolates and subjected to comparative genomic hybridizations (CGH) on *Salmonella* whole genome arrays containing genes of 5 *Salmonella* (Typhimurium LT2, Typhimurium SL1344, Typhi strain CT18, Typhi Ty2, Paratyphi A, and Enteritidis sPT4). Absence versus presence results for the 5,660 genes on the array were analyzed by Bionumerics (V 6.0, Applied Maths Austin, TX, USA). *S.* Kentucky was further investigated with genome alignment program Mauve (The University of Wisconsin-Madison, Madison, WI, USA) to determine genes that were unique to this serotype. The majority of *S.* Kentucky isolates were missing 2 regions involved in sugar transportation and the *rfb* cluster which encodes genes for fructose and mannose metabolism. In addition, all isolates were missing the phosphotransferase system for fructose and mannitol metabolism. Region STM4417 to STM4436 which encodes a major facilitator superfamily (MFS) for transport of various substrates was absent in 10 isolates. All isolates in this study contained a variety of genes from *Salmonella* pathogenicity island (SPI) 1-5. There were 168 unique genes found with the Mauve genome alignment as compared to the *Salmonella* genomes located on the array. Some unique genes found in *S.* Kentucky include those encoding for 10 fimbrial proteins, 8 type VI secretion proteins, 4 phage proteins, 4 arsenic resistance proteins, 4 β-lacatmase proteins, 6 outer membrane lipoproteins, 2 quaternary ammonium compound-resistance proteins, *sugE,* and

11 transposase metabolic pathways. *S.* Kentucky contains virulence genes similar to other pathogenic strains of *Salmonella* associated with human illness, yet this organism is rarely associated with human disease. Any of the genetic variations within this organism could have contributed to the colonization of poultry and the decreased pathogenicity in humans. Perhaps the genes that have made this microbe able to colonize poultry have prevented it from causing human disease.

Introduction

The poultry industry is in a constant battle to keep product safe for human consumption. This can be a daunting task since poultry are the ecological niche for numerous food borne pathogens. *Salmonella* serotypes differ in virulence and host specificity thus not all of these organisms are equal in pathogenicity. According to the Centers for Disease Control and Prevention (CDC), the top 3 *Salmonella* to cause human infections included *S.* Typhimurium, *S.* Enteritidis, and *S.* Newport (11). However, the most commonly isolated *Salmonella* serotype from poultry is Kentucky, which is not usually associated with human illness. In fact according to CDC 2007 data, *S.* Kentucky was not even in the top 30 serotypes from human sources (11). In addition, the prevalence of *S.* Kentucky in poultry has increased in recent years and almost 50% of *Salmonella* isolated from poultry are this serotype (11). Recently strains of *S.* Kentucky have also acquired resistance to multiple antibiotics (11). According to the National Antimicrobial Resistance Monitoring System (NARMS) , approximately 35% of *S.* Kentucky isolated from poultry were resistant to streptomycin, 15% were resistant to a β-Lactam, extended spectrum cephalosporin and cephamycins, and 48% were resistant to tetracycline (11). Unlike most *Salmonella* pathogens that acquire resistance to multiple antibiotics, this organism has not had an increase in human pathogenicity in the U.S. However, multiple drug resistant (MDR) strains of *S.* Kentucky have been reported in French travelers presenting as gastrointestinal infections (30). Isolates from the French travelers were shown to be resistant to as many as 9 antimicrobials including ciprofloxacin. More importantly, these multidrug infections were more difficult to treat and required several rounds of antibiotics.

It has been proposed that resistance development in pathogens such as *Salmonella* is due to the use of antimicrobials in food animals (17). Subtherapeutic levels of antibiotics used for

growth promotion in poultry include chlortetracycline, penicillin, tylosin, and virginiamycin (22). A newer antimicrobial, fluoroquinolone, is used at therapeutic levels to treat major disease such as *E. coli* in poultry (22). Antimicrobial resistance in *Salmonella* has been reviewed extensively by McDermott which discusses the ability of this pathogen to acquire as well as transfer multiple resistance genes on transposable elements such as plasmids (21). More importantly, it has been shown that *S.* Kentucky is capable of passing resistance plasmids to pathogenic bacteria thus producing pathogens with multidrug resistance (MDR) and capable of causing human infections (13). Researchers have found that one of the plasmids found in *S.* Kentucky is similar to the avain pathogenic *Escherichia coli* (APEC) virulence plasmid thought to have only been found in *E. coli*. Acquisition of the APEC plasmid increases the microbes pathogenicity which may explain the increase of colonization of *S.* Kentucky in poultry (11). A group of phylogenetically similar *S.* Kentucky isolates as shown by PFGE, all contained an APEC-like resistance plasmid (13). Furthermore, 94% of *S.* Kentucky isolated from chicken contained an APEC-like plasmid that conferred resistance to tetracycline and streptomycin. *S.* Kentucky has increased in prevalence in chickens over the past few years and resistance to tetracycline and streptomycin is the most commonly found resistance phenotype. More importantly, Welch *et al* found that *S.* Kentucky isolated from retail meat products contained a plasmid (IncA/C incompatibility group) backbone previously found in multidrug resistant *S.* Newport and the plague bacterium *Yersina pestis (31).* The IncA/C backbone contains genes that encode for plasmid replication/maintenance and conjugative transfer genes as well as resistance to multiple antibiotics. This plasmid confers resistance to streptomycin, tetracycline, β-lactmases and quaternary ammonium compound resistance (*sugE*). This innocuous microbe is able to pass resistance genes to other foodborne pathogens, thus contributing to a deadly cycle. This warrants

further investigation since this microbe has increased in prevalence in a food item and has the propensity to not only cause foodborne illnesses but could also be more difficult to treat due to its drug resistance. Likewise, studying the genomic content of an organism rarely associated with human illness may help to discover genes that are deficient in *S.* Kentucky as compared to other more pathogenic serotypes.

In addition, since this organism has developed multidrug resistance in other countries and has the capability to cause a more severe infection in some humans, it may be necessary to study the genomic content of this organism (30). It is assumed that when bacteria acquire genes for resistance they also acquire virulence genes. Since *S.* Kentucky isolates in the U.S. are not associated with human illness, yet have acquired resistance to antibiotics, it would be interesting to compare the genetic content of this organism to other *Salmonella* pathogens to determine the differences within their genomes. Previous studies looking for virulence genes that would increase this bacterium's ability to colonize poultry found 24 virulence genes related to pathogenicity (16). It was discovered that *S.* Kentucky contained most of pathogenicity islands 1 through 5. It was also found that *S.* Kentucky isolates had a growth advantage over the other *Salmonella* serotypes when grown in low pH environments. This was especially true if acetic acid was present in the environment. It was stated that *S.* Kentucky can proliferate better than other *Salmonella* in the chicken cecum because it does not produce an acid adaptive response thus saves energy and resources for growth. Looking into the genetic content of *S.* Kentucky may reveal genes that allow this bacterium to establish itself in poultry as well as prevent it from becoming a successful human pathogen. Further studies concerning genetic content of *S.* Kentucky may also provide a look into the future of this bacterium concerning its potential to become more pathogenic.

The objective of this study was to determine the genetic content of *S.* Kentucky, isolates from the National Antimicrobial Resistance Monitoring System (NARMS). Comparative genomic analysis was used to compare the genomic content of *S.* Kentucky to the sequenced genomes of *Salmonella* to determine the differences within the strains as well as the evolutionary relationships between these strains. Unlike PFGE, comparative genomics determined the genes that were present or absent in various strains of *S.* Kentucky. Unique genes found only in *S.* Kentucky as compared to the *Salmonella* genomes probed on the micorarray chips were discovered with the genome alignment program Mauve (The University of Wisconsin-Madison, Madison, WI, USA). This information helped determine possible mechanisms leading to the increase in MDR *S.* Kentucky in poultry. Information concerning the genetic makeup of *S.* Kentucky provided a better understanding of the genes that enhance its ability to colonize poultry. Information included the evolutionary relationship of *S.* Kentucky to other strains of *S.* Kentucky as well as 5 other *Salmonella* serotypes, the genetic differences within various strains, and the genes that are unique to *S.* Kentucky. Some of these genes identified may also reveal new targets for novel antimicrobials or interventions to prevent colonization of poultry by *S.* Kentucky and avoid a potentially emerging pathogen from becoming a common human pathogen.

Material and Methods

***Salmonella* Kentucky stains.** Isolates (n=12) for this study were acquired from the National Antimicrobial Resistance Monitoring System (NARMS) to represent the most genetic diversity as determined by PFGE analysis. Antimicrobial resistance profile was determined and reported by the National Antimicrobial Resistance Monitoring System (NARMS) using the Sensititer system (Trek Diagnositc Systems, Inc., Westlake, OH) which included amikacin, amozicillin-

clavulanic acid, ampicillin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim-sulfamethoxazole as previously described (27). Isolates were defined as being susceptible, intermediate, or resistant following the Clinical and Laboratory Standards Institute breakpoints (8). PFGE analysis was performed following the CDC PulseNet protocol using the restriction enzyme XbaI (14). Bionumerics 5.10 software (Appled Maths, Sint-Martens-Latem, Belgium) was used to analyze images of gels, and dendrograms of the PFGE patterns were generated with Dice correlation coefficients with a tolerance of 1.5%. Dendrograms of multidrug resistant patterns as well as pan-suceptible patterns were generated from a large group of *S.* Kentucky from the VetNet database to determine similarity clusters (25). Isolates were selected from the larger group based on distance on the dendrogram, date strain was obtained, source, and location. PFGE patterns selected from the larger group of *S.* Kentucky were analyzed again to determine the similarity cluster of the selected group.

Microarray design. *Salmonella* microarray contained 5,660 PCR products which covered 95% of all genes in genomes of *S.* Typhimurium strain LT2, *S.* Typhimurium strain SL1344, *S.* Typhi strain CT18, *S.* Typhi strain Ty2, *S.* Paratyphi A strain SARB42, and *S.* Enteritidis strain PT4. Each multiserotype microarray contained the genomes in triplicate thus producing 3 identical replicate hybridizations on one chip.

G**enomic isolation, labeling and hybridization.** Genomic DNA was isolated following manufacturer's instructions using the GenElute bacterial DNA kit (Sigma, St Louis MO, USA). Genomic DNA (1.5 µg) from the control, *S.* Typhimurium LT2, and *S.* Kentucky were labeled overnight as previously described with cy5 and cy3, respectively (24). Prehybridization of *S.* Kentucky along with *S.* Typhimurium LT2, control, were hybridized as previously described

onto the *Salmonella* genomic chips overnight at 42°C in a hybridization chamber (24). Post hybridization washes were done as previously described. Genomic chips were scanned with the GenePix Personal 4100A (Molecular Devices, Sunnyvale, CA) using the GenePix Pro software to acquire an image.

Data acquisition. QuantArrray 3.0 software (Packard BioChip Technologies) was used to analyze the spot intensities. Data was normalized by subtracting the background noise from the intensity values resulting in the signal of the spot in each channel. The *Salmonella* genomic chip was printed in triplicate resulting in 3 data points for each spot. The median of the 3 spots was used to determine the absence or presence of the *S.* Newport genomes as compared to the other *S. enterica* genomes. Data was analyzed to determine the asbsence (0) or presence (1) values as previously described (23).

Bionumerics analysis. Microarray absence presence data was imported into the Bionmuerics program (V 6.0, Applied Maths Austin, TX, USA) using the import fields and character function. Imported data was manipulated using cluster analysis with unweighted-pair group method using arithmetic averages (UPGMA) to determine the phylogentic relationship based on the genetic content of the *S.* Kentucky used in this study. Statistic tools in Bionumerics were used to determine the percentage of genes absent or present in *S.* Kentucky as compared to the *Salmonella* genomes on the microarray chip.

Sequence alignment. All *Salmonella* genomes located on the microarray chips were aligned with the genome of *S.* Kentucky CVM29188 GenBank number ABAK00000000 using the Mauve genome alignment program (The University of Wisconsin-Madison, Madison, WI, USA). *S.* Kentucky was isolated in 2003 from a chicken breast and contains three plasmids of which one encode genes for resistance to streptomycin and tetracycline while another plasmid encodes

resistance genes to β-lactmase (*bla*$_{CMY-2}$) and quaternary ammonium compound resistance (*sugE*) (13). Alignments were used to determine the genes that were unique to *S.* Kentucky as compared to the other *Salmonella* genomes located on the microarray chips. Locally collinear blocks (LCBs) were identified by Mauve based on regions that are homologous and do not contain any rearrangements. LCBs were presented as colored blocks that represent the alignment of the genome with the other genomes. These colored blocks were either above or below (reverse orientation) a single black line based on its orientation. Regions located outside of the LCBs did not align with the other genomes and were thus unique to that organism. Inside the block of each LCB, Mauve created a similarity plot to show the similarity between the genomes. The higher the similarity plot, the more similar the sequence was to the other genomes and the more color within the LCBs. Areas with no similarity plot (no color) were considered unique to that genome. Genetic information from the areas that are colorless and thus unique to that organism will be recorded in Excel and placed in order according to location on the genome as well as gene function (10).

Results

Strain information: Strains selected from NARMS study included 12 isolates that were serotyped as *S.* Kentucky. Upon further PFGE analysis, 1 of the isolates was found not to be *S.* Kentucky and was left out of the study. Two of the *S.* Kentucky isolates were resistant to aminogycosides and tetracyclines while one was resistant to aminoglycosides, beta-lactam plus inhibitor, cephems, and penicilins. The remaining 7 isolates were sensitive to all antimicrobials used in sensititer testing. Ten isolates were isolated from chicken while one was isolated from turkey. Dates of isolation range from 2004 to 2005.

PFGE. To determine the relationship of the MDR *S.* Kentucky to each other as well as to pan-susceptible strains of *S.* Kentucky, isolates were analyzed with PFGE and dendrograms were built using Bionumerics software (Figure 4.1). Similarity clusters from PFGE patterns revealed that the branches of 6 *S.* Kentucky clustered together with approximately 80% identity. This cluster consisted of 5 pan-susceptible isolates and 1 isolate resistant to aminoglycosides, beta-lactam plus inhibitor, cephems, and penicillins.

Microarray analysis. The genomic content of 11 *S.* Kentukcy was compared with *S.* Typhimurium LT2, *S.* Typhi CT18, *S.* Typhi Ty2, *S.* Paratyphi A SARB42, and *S.* Enteritidis PT4 genomes. *S.* Kentucky had 68% to 74% of the genes present as compared to the *Salmonella* genomes on the microarray chip (Table 4.1). The microarray results were compiled into one Excel spreadsheet to compare the genetic variations within the *S.* Kentucky isolates. Initially, analysis was performed by finding large contiguous sections of absent/present probe hybridization. Further analysis included looking for the absence/presence of specific genes with functions that would correlate with niche or virulence development. Table 4.2 illustrates the genes that were absent in the study isolates as compared to the *Salmonella* genome microarray chip. The region STM0715-STM0727 which is involved in sugar transportation was absent in 10 of the *S.* Kentucky isolates from this study. Another sugar transport system which also transported sodium (STM1127-STM1133) was absent in 9 of *S.* Kentucky isolates. The *rfb* cluster which is involved in fructose and mannose metabolism was missing in 10 of the *S.* Kentucky isolates (STM2083-STM2089). Furthermore, all 11 *S.* Kentucky were missing the region STM3251-STM3256 which included the *agaR* gene responsible for transcriptional regulations of sugar metabolism. All *S.* Kentucky isolates were missing genes from the phosphotransferase system (STM4110 to STM4116) for fructose and mannitol metabolism. In

addition 9 isolates used in this study were missing the region STM0030-0038 which included enzymes involved in metabolism, DNA repair, biosynthesis of vitamins, coenyzmes, and antibiotics. Gene *thiL*, STM0419, encodes genes for biosynthesis and salvage of thiamine and was absent in 7 isolates. STM0854 to STM0861 encodes for genes involved in fatty acid biosynthesis, valine, leucine and isoleucine degradation, genes that form part of the electron-transfer system in mitochondria. This region was absent in 10 of *S.* Kentucky isolates. Interestingly, all *S.* Kentucky used in this study were missing region STM0608-0609, *ahp*, which encodes antioxidant genes.

Virulence genes include flagella which allows the bacteria to come into close contact with the host cell and pili which functions by adhering to the host cell. The *S.* Kentucky strains in this study were not missing any flagella or pili genes as compared to the 5 other *Salmonella* genomes. This suggests that these strains of *Salmonella* had the same attachment and movement capabilities as the pathogenic bacteria used as probes on the microarray chip. On the other hand, the *dgo* gene, region STM3827-3830, was missing from all *S.* Kentucky used in this study. The *dgo* gene is involved in the Major Facilitator Superfamily (MFS) which is used by bacteria as a nutrient uptake and efflux pump for antibiotics. Region STM4417 to STM4436 is another MFS used to transport various substrates including antibiotics, sugar phosphates, and amino acids across the cell membrane that is missing from ten of the *S.* Kentucky isolates. Two genes, *ompS1* and *cspB*, encode for putative porin gene and a putative cold shock protein, respectively, and were found to be missing from 5 *S.* Kentucky isolates. Putative genes in this case refer to the fact that the sequences match specific genes; however, the function is not fully understood.

Evolution of bacteria has been enhanced by prophages which have integrated into the genome of microbes. This co-evolution of microbe and phage has been extensively reviewed by

Brussow *et al* where it is stated that the acquisition or absence of genes for prophages often determines the virulence/pathogenicity of a microbe (6). *S.* Typhimurium used as a probe on the *Salmonella* microarray chip contained 4 functional prophages. Fels-1, Gifsy-1, and Gifsy-2 prophage were missing from all of the isolates in this study. Only 2 isolates had a small region, approximately 55%, from the Fels-2 prophage (STM2694 to STM2722). Region STM2230 to STM2244 was absent in all isolates. This region encodes genes necessary for tail formation as well as a homolog to the virulence gene *msgA.* Four *S.* Kentucky isolates contained 2 phage shock proteins (*pspE* and *pspD)* which have been found in only a few microbes including *E. coli* and *S.* Typhimurium (1). All isolates except for MH97765 were missing the *cdtB* gene which encodes host recognition and invasion, however all of the isolates in this study contained host recognition and invasion genes *invA.* All the *S.* Kentucky isolates also contained the iron acquisition (*iroN)* virulence gene. Table 4.3 demonstrates the genes absent/present and associated with a pathogenicity island. These strains of *S.* Kentucky contained a variety of genes from pathogenicity islands 1 through 5. Also, all the isolates from this study were missing the region STM3752-3755 which contains virulence genes *sugR* and *rhuM* and the region STM4489 to STM4498 which includes a phospholipase D gene and is usually associated with pathogenic bacteria.

Data analysis with bionumerics. Data was imported into the Bionumerics program and cluster analysis was performed with UPGMA based on the absence or presence of genes on the microarray chip. Comparison of the PFGE dendrogram with the CGH dendrogram revealed very similar clusters (Figure 4.2). CGH contained the same group of isolates as PFGE cluster A except one of the MDR *S.* Kentucky was found to cluster with the other MDR from this study.

Unlike PFGE, clusters from CGH data were grouped more closely together depending on their antimicrobial sensitivity.

Identification of unique genes in S. Kentucky sequences via Mauve analysis. *S.* Kentucky genome was compared to the *Salmonella* genomes used as probes on the microarray chip to determine genes that were unique to *S.* Kentucky. The sequenced CVM29188 *S.* Kentucky contained 4887 genes and 3 plasmids: pCVM29188 (141 genes), pCVM29188 (204 genes), and pcVM29188 (63 genes). According to results submitted to NCBI, strain CVM29188 contained a large number of pseduogenes as compared to *S.* Typhimurium. Mauve results revealed 162 genes and 129 hypothetical proteins that were unique to *S.* Kentucky as compared to other *Salmonella* genomes. Table 4.4 illustrates the genes with a known function that were unique to *S.* Kentucky. *S.* Kentucky contained unique genes involved in transcription, replication, recombination, and repair, defense mechanisms, signal transduction mechanisms, cell wall/membrane biogenesis, cell motility, and carbohydrate transport and metabolism. Some unique genes found in *S.* Kentucky include those encoding for 10 fimbrial proteins, 8 type VI secretion proteins, 4 phage proteins, 4 arsenic resistance proteins, 4 β-lacatmase proteins, 6 outer membrane lipoproteins, 2 quaternary ammonium compound-resistance proteins, *sugE*, and 11 transposase metabolic pathways.

Discussion

S. Kentucky is predominantly found in poultry yet is rarely associated with human infections. In recent years this microbe has acquired resistance to multiple antibiotics in particular aminogycosides and tetracyclines. These two antimicrobials are older drugs and thus have been used for years so it is not surprising that these organisms have developed resistance. Unfortunately, development of resistance to newer drugs such as ceftriaxone has been reported in

other countries involving human infections (9). Not only were those pathogens resistant to multiple antibiotics but they were also more difficult to treat and resulted in sequelae such as arthritis. It is evident from the previously published findings concerning travel-acquired *S.* Kentucky, that this organism is capable of becoming a more pathogenic microbe. Results from this study have found genes that are missing in *S.* Kentucky as compared to those located on the *Salmonella* whole genome microarray. Previous studies have shown that the loss of genetic functions can contribute to the development of niches such as the case of *S.* Kentucky and poultry (19). *S.* Kentucky genome contained 182 pseudogenes as stated by NCBI genome database. Pseudogenes are sequences of DNA that contain a point mutation or a stop codon that prevents the gene from being expressed. McClelland *et al.* found several bacteria that also had large numbers of pseudogenes to have evolved into human specific pathogens. It was suggested that the large numbers of pseudogenes was due to the evolution of the bacteria which eradicated genes from itself because these genes were no longer needed in the new niche (19).

Dendrogram of the isolates using PFGE results produced two distinct clusters. For the most part the data from the microarray hybridizations produced the same two clusters. Microarray dendrogram, however, clustered all the antibiotic resistant organisms together while the PFGE dendrogram did not. Notably, the dendrogram built using microarray data produced clusters that were more similar in comparison than those built with PFGE data. Clusters from microarray data were at least 80% or more similar while clusters from PFGE data were only 42% or more similar. Based on these results, microarray analysis is less discriminatory than PFGE. Previous genome sequencing of *Salmonella* serotypes found these microbes to range up to 97% identical within the different serotypes (20). Researchers found that CGH of various *Salmonella* serotypes were sometimes found to be genotypically close due to similar genetic content (23).

Current microarray data shows that *S.* Kentucky whether antibiotic resistant or pan-susceptible to be more genotypically related as compared to clusters from PFGE analysis.

The *Salmonella* microarray contained the genome of *S.* Typhimurium as well as several other *Salmonella* pathogens. *S.* Typhimurium contains 4 functional prophages, Fels-1, Fels-2, Gifsy-1 and Gifsy-2. Several of these prophages found in *S.* Typhimuruim are rarely found outside this organism (6). Since prophage and pathogenicity islands conferring virulence genes have been horizontally acquired by *Salmonella* these organisms vary greatly in their pathogenicity (12). In this study, *S.* Kentucky isolates were missing all of the Fels-1, Gifsy-1, and Gifsy-2 prophages and only two isolates (MH97765 and MH53737) contained a small region from Fels-2 (STM2694 to STM 2722). This region encoded genes for phage tail and capsid formation. PFGE and microarray dendrograms found these isolates to be the least similar as compared to the other *S.* Kentucky isolates used in this study. This difference is most likely due to the fact that isolates MH97765 and MH53737 contained several genes that were absent/present as compared to the other *S.* Kentucky used in this study. These isolates contained genes such as *rfb* which encodes for fructose and mannose metabolism and membrane genes that transported sodium/glucose. Thus, even though these organisms are the same serotype they contain very different genetic content most likely due to lateral gene transfer as shown by previous studies (20).

Another distinguishing feature concerning *S.* Kentucky is the fact that all the isolates in this study contained some form of SPI 1-5 (Table 4.3). Thus these strains of *Salmonella* have the genes necessary to be pathogenic. Previous studies also found that *S.* Kentucky contained these 5 pathogenicity islands (16). Interestingly, those researchers determined that *S.* Kentucky was statistically less invasive as compared to other *Salmonella* based on the invasion of chicken

embryo cells. Despite decreased pathogenicity, *S.* Kentucky was more proficient at establishing colonization in chickens than other *Salmonella (16)*. It was thought that by not producing acid tolerance response proteins when the environment became more acidic, that these organisms could spend more energy and resources on growth. Hinton *et al.* studied the microbiological changes in the ceca of broiler chickens which was found to have high levels of *Salmonella* and in particular *S.* Kentucky (15). Before broilers are transported for processing they go through the process of feed withdrawal to reduce the fecal material in the bird. Initially, the cecal pH of broilers increased significantly but after 12 hours the pH decreased (15). One study found that *S.* Kentucky was capable of outgrowing other serotypes at pH of 5.5; however, when transferred to a pH of 2.5 *S.* Kentucky did not grow as well as the other serotypes (15). Thus, *S.* Kentucky has been shown to have a reduced acid response which may allow this organism to spend more energy on growth in an acidic environment and may explain why *S.* Kentucky is prevalent in poultry when sampled at slaughter. Furthermore, the inability to grow at a pH similar to that seen in the human gastrointestinal tract may explain the lack of human illness associated with *S.* Kentucky. Since bacteria that elicit an acid tolerance response use their energy to make acid tolerance proteins, then the microbes are not focusing on growth. Thus, *S.* Kentucky has an advantage during the feed withdrawal due to the acidic environment. Also, since *S.* Kentucky has a chance to increase in numbers perhaps they mask the presence of other *Salmonella* making it more difficult to really determine the bacterial content of the cecum as well as other areas of the broiler with other Enterbacteriaceae. *S.* Kentucky may not be more prevalent in poultry as compared to the other bacteria but may have higher numbers in and on the bird at the time of slaughter making it more difficult to detect other serotypes.

Another source of decreased pathogenicity could be the missing regions STM3752 to STM3755 (*sugR* and *rhuM)* which contain virulence genes. Previous studies found that several *Salmonella* serotypes were also missing genes *sugR* and *rhuM* from the SPI-3 plasmid as compared to *S.* Typhimurium (2). Instead these organisms contained fimbrial operon in between two insertion sequence elements similar to genes found in *E. coli.* Two organisms from this study missing *sugR* and *rhuM, S.* Derby and *S.* Infantis, are in the NARMS top ten *Salmonella* isolated from poultry (11). Thus, it is possible that *S.* Kentucky which is also found in poultry has acquired the same genome replacement. Interestingly, it was discovered that regions containing *sugR* and *rhuM* genes was an integration site for many pathogenicity islands (2). In addition, Mauve analysis revealed unique genes associated with fimbrial proteins as well as transposase/insertion proteins in *S.* Kentucky as compared to the other serotypes. Aligning the sequences with the NCBI Basic Local Alignment Search Tool (BLAST) determined these unique sequences for fimbrial and insertion proteins to be similar with those found in *S.* Derby. Therefore it is possible that *S.* Kentucky has also acquired a new insertion at this region for attachment genes.

Interestingly, functions including membrane genes and region STM3827 to STM3830 and STM4417 to STM4436 were missing from all *S.* Kentucky isolates used in this study. These regions are Major Facilitator Superfamilies (MFS) and are used to transport various substrates including antibiotics, sugar phosphates, and amino acids across the cell membrane. Antibiotic efflux pumps such as MFS contribute to the resistance to multiple antibiotics since it can pump out several antimicrobials (28). Furthermore, several *S.* Kentucky isolates were missing genes that encode for porins (*ompS1* and *cspB*). Porins form holes in the cell outer membrane that allow small hydrophilic molecules to cross (4). These porins act as sensory transducers which

allows the bacterium to respond rapidly to environmental issues. As stated earlier, *S.* Kentucky does not produce an acid tolerance response as seen in other *Salmonella.* Perhaps the loss of important sensory porins prevents *S.* Kentucky from responding to an acidic environment. This may have allowed *S.* Kentucky to outcompete in low acid environments such as in poultry; alternatively, perhaps losing this sensory pathway has hindered this microbe in establishing colonization of the human gut which is even more acidic than poultry.

Recent sequencing of the *S.* Kentucky genome makes it possible to compare this organism to other sequenced *Salmonella* genomes. According to Mauve genome analysis, *S.* Kentucky contained 168 genes and 125 hypothetical proteins that were not found in the 5 other *Salmonella* genomes used on the microarray chip. *S.* Kentucky contained unique genes involved in transcription, replication, recombination, and repair, defense mechanisms, signal transduction mechanisms, cell wall/membrane biogenesis, cell motility, carbohydrate transport and metabolism, and metal and antibiotic resistance. Interestingly, *S.* Kentucky contained 5 unique genes involved in arsenic resistance. Since arsenic is found throughout the environment and used in poultry feed it is possible to have high enough levels of arsenic in the bird to inhibit microbial growth (29). Thus resistance to arsenic may allow *S.* Kentucky to survive in an environment that is too harsh for other serotypes or other microbes. In addition, *S.* Kentucky contained a gene, *sugE*, for quaternary ammonium compound resistance. Studies have shown that *sugE* encodes an efflux pump that confers resistance to a subset of quaternary ammonium disinfectants (7). Sanitizers used in the food processing industry contain quaternary ammonium compounds thus resistance to this substance may allow *S.* Kentucky to proliferate in these environments. Also, if *S.* Kentucky was not eradicated from the environment with the disinfectant then it can survive to contaminate bird carcasses during processing. More

importantly, this sequenced strain of *S.* Kentucky contained several genes for β-lactamase that were not found in the *Salmonella* used on the microarray chip. The protein sequences of the β-lactamases were compared using the NCBI BLAST program to determine if these genes were found in other microbes. All four β-lactamase genes matched β-lactamases found in the sequenced strain of *S.* Newport (SNSL254). Studies support these findings concerning *sugE* and β-lactamase genes found in *S.* Kentucky (13). Other studies found that *S.* Kentucky isolated from retail meats contained the IncA/C backbone which usually confers resistance to multiple antibiotics (18, 31). Antibiotic resistant *S.* Kentucky isolates in this study were only resistant to a few antimicrobials and these were usually streptomycin and tetracycline. Lindsey *et al* found that some strains of *Salmonella* were resistant to only 2 antibiotics (sulfamethoxazole and tetracycline) even though these organisms contained plasmids that conferred resistance to several antimicrobials. Therefore, it is possible that the isolates used in the current study with antimicrobial resistance also harbor the IncA/C plasmid and contained resistance to other antimicrobials that were not detected with sensititer because these genes were not expressed.

Another distinct feature of the sequenced *S.* Kentucky is that it contained 10 unique genes for fimbrial proteins. Fimbria allows the bacterium to attach to a host cell thus it is imperative in the colonization of an animal such as poultry. Comparing these sequences against other microbes in the NCBI databased using BLAST revealed that *S.* Kentucky shares fimbrial proteins with *Salmonella* pathogens *S.* Saintpaul, *S.* Dublin, and *S.* Derby. Furthermore, 8 unique genes were found to encode a type VI secretion system (T6SS). The T6SS encodes genes for adherence, cell invasion, macrophage survival and persistence in a host (5). Interestingly, *S.* Typhimurium LT2 also contains a T6SS; however, several T6SS genes were found to be unique to *S.* Kentucky using Mauve. Comparative analysis of various *Salmonella* serotypes found little

homology within each group suggesting these genomic islands varied and were acquired via lateral gene transfer (5). Evidence was found with Mauve supporting the ability of *S.* Kentucky to have had genes transferred laterally. There were 11 unique transposase genes which provide sites that recognize transposons (26). Transposons are mobile elements that carry genes like antibiotic resistance. More importantly, transposons can carry genes for several antimicrobial resistances. Since there are 11 unique sites that recognize various transposons this organism could easily acquire resistance to multiple antibiotics.

S. Kentucky has virulence genes similar to other pathogenic strains of *Salmonella* associated with human illness. However, this pathogen is rarely associated with human illnesses and yet has increased in prevalence in poultry. Approximately, 50% of *Salmonella* isolated from poultry in the U.S. carry *S.* Kentucky thus the chance of consuming chicken that is contaminated is great. Nevertheless, reports of infections in humans from *S.* Kentucky are rare. Perhaps there are colonization factors not found in this serotype that allow *Salmonella* pathogens to colonize the human gut. Also, since *S.* Kentucky has been reported to have a poor acid tolerance response maybe it is unable to survive the passage through the gastric content of the human gut. There are many possibilities that prevent this organism from causing human infection; however, since *Salmonella* acquire genes from other microbes it is possible that in the future *S.* Kentucky may gain the genes necessary to cause illness. In fact this may have occurred in *S.* Kentucky strains from in travelers in France (30). These strains were not only resistant to multiple antibiotics but they also caused a more severe disease that required treatment. Further investigation concerning the differences between *S.* Kentucky strains from the U.S. compared to those in other countries may reveal important virulence genes leading to an increased pathogenicity. Also, as stated earlier *S.* Kentucky contained unique genes associated with a transferable MDR IncA/C plasmid.

It would be interesting to see if this IncA/C plasmid is more common in *S.* Kentucky isolated from poultry. Furthermore, these microbes usually express resistance to streptomycin and tetracycline, thus it would interesting to see if they contained genes that encoded for more resistance. Since *S.* Kentucky is the single most predominate serotype isolated from poultry carcass, it would be imperative to determine if these microbes not only contained resistance genes for multiple antibiotics but also contained self-transferrable plasmid that could easily be transmitted to human pathogens.

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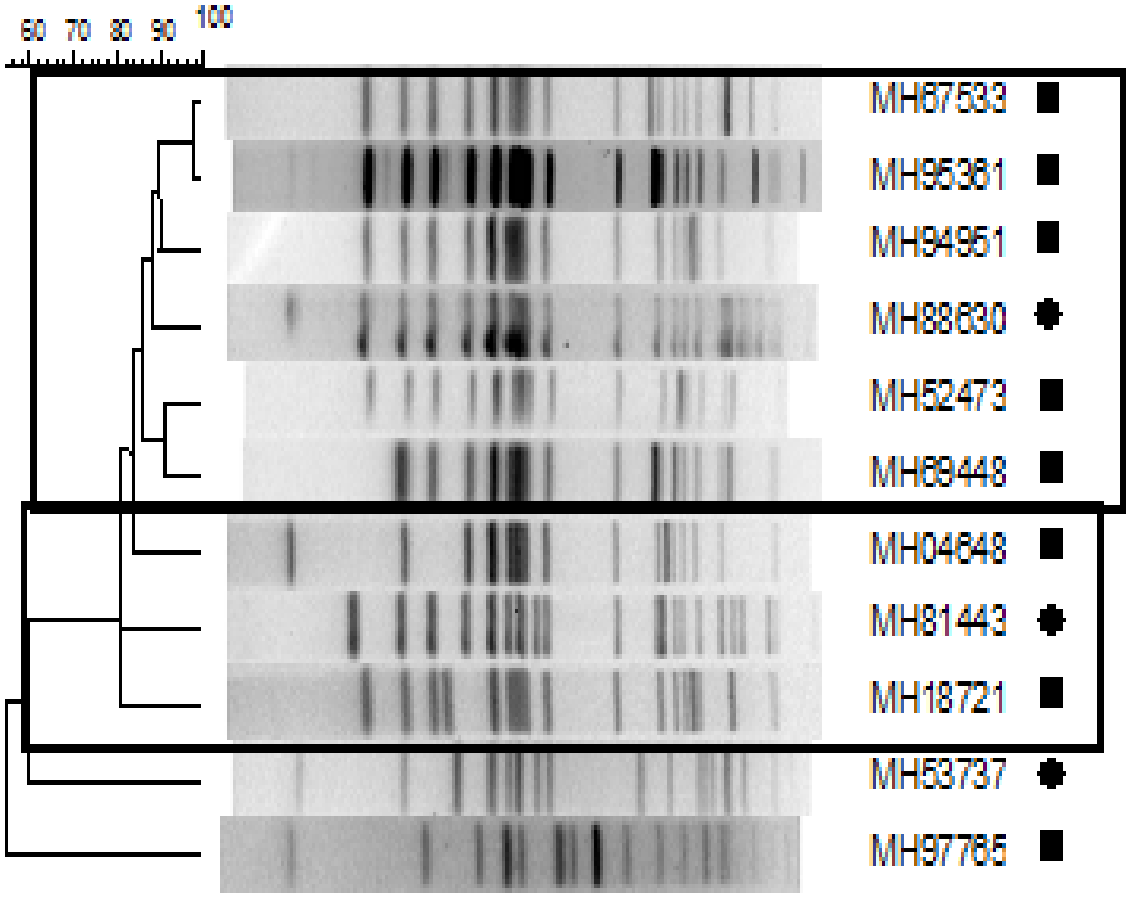

Figure 4.1. Cluster analysis from PFGE patterns with *S.* Kentucky isolates using Dice correlation with a tolerance of 1.5%. Pansusceptible isolates denoted with a square while antibiotic resistant strains are denoted with a circle.

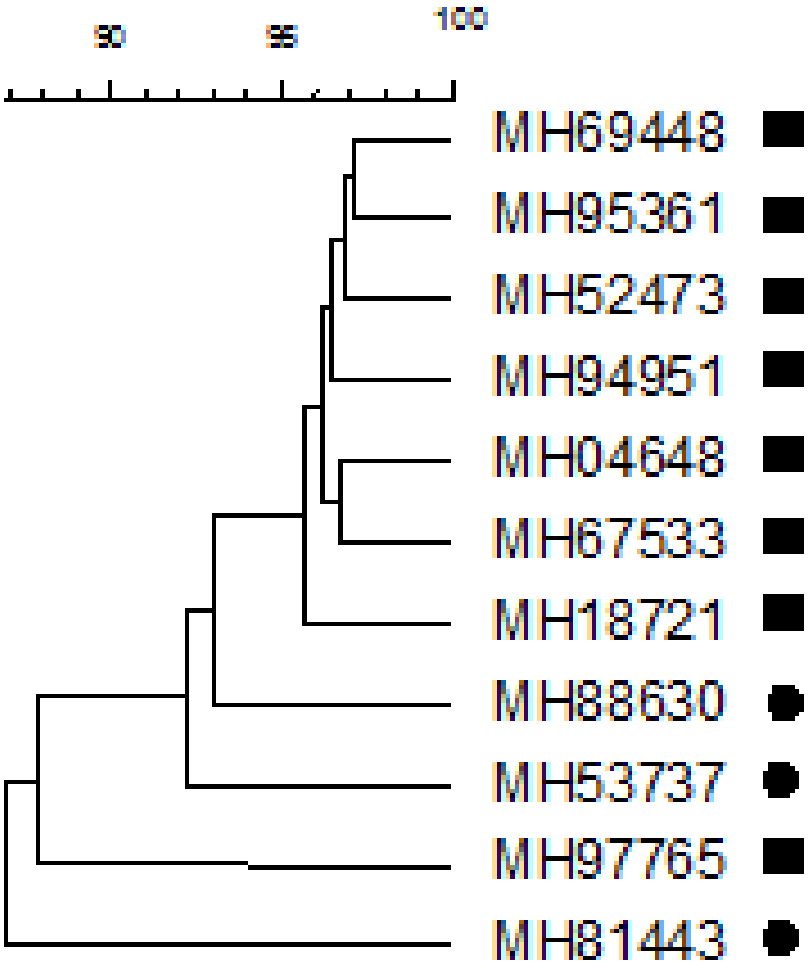

Figure 4.2. Cluster analysis of *S.* Kentucky CGH data using categorical clustering UPGMA.

Table 4.1. Percentage of genes present within *S.* Kentucky genes as compare to *Salmonella* whole genome microarray.

Kentucky Isolates	Present	Percentage Present
MH52473	4109	72.55
MH95361	4139	73.08
MH04648	4205	74.24
MH69448	4153	73.32
MH67533	4232	74.72
MH94951	4149	73.25
MH97765	4027	71.10
MH88630	4013	70.85
MH81443	3833	67.67
MH53737	4136	73.02
MH18721	4085	72.12

Table 4.2. *S.* Kentucky genes absent or present on the *Salmonella* whole genome chip.

cluster/gene	STM no.	**52473**	**95361**	**4648**	**69448**	**67533**	**94951**	**97765**	**88630**	**81443**	**53737**	**18721**
-[a]	0030-0038	-	-	-	-	-	-	+	-	-	+	-
stf	0195-0201	+	+	+	+	+	+	-	+	+	+	+
-[a]	0290-0295	-	-	-	-	-	-	+	-	-	-	-
-[a]	0326-0335	+	+	+	+	+	+	-	+	+	+	+
prp	0368-0371	+	+	+	+	+	+	+	+	-	+	+
yajF	0393	+	+	+	+	+	+	+	-	-	+	+
araJ	0394	+	+	+	+	+	+	+	-	-	+	+
sbcC	0395	+	+	+	+	+	+	+	-	-	+	+
thiL	0419	+	-	-	-	-	-	+	-	-	+	+
yba, mdl	0456-0461	+	+	+	+	+	+	+	+	-	+	+
ybb, all, gcl, gip, glx, arcC	0514-0525	+	+	+	+	+	+	-	+	+	+	+
ybb, glx, all, fdr	0524-0530	+	+	+	+	+	+	+	+	-	+	+
yfdH	0554-0558	-	-	-	-	-	-	-	-	-	-	-
-[a]	0571-0577	+	+	+	+	+	+	-	+	+	+	+
ahpC, ahpF	0608-0609	-	-	-	-	-	-	-	-	-	-	-
ybe	0564-0660	+	+	+	+	+	+	-	+	+	+	+
-[a]	0715-0727	-	-	-	-	-	-	+	-	-	-	-
-[a]	0761-0769	+	+	+	+	+	+	-	+	+	+	+
-[a]	0854-0861	-	-	-	-	-	+	+	-	-	-	-
-[a]	1127-1133	-	-	-	-	-	-	+	-	-	+	-
flg	1176-1201	+	+	+	+	+	+	+	+	-	+	+
pnt	1479-1492	+	+	+	+	+	+	+	+	-	+	+
-[a]	1549-1554	+	+	+	+	+	+	-	+	+	+	+
ans	1584-1585	-	+	+	-	+	-	-	-	-	-	-
ydc	1622-1626	+	+	+	+	+	+	+	+	-	+	+
-[a]	1633-1637	+	-	+	+	+	+	+	+	+	+	+
psp	1686-1687	+	+	+	-	+	-	-	-	+	+	+
yci, trp	1718-1725	+	+	+	+	+	+	+	+	-	+	+
yci, ton, cis	1733-1739	+	+	+	+	+	+	+	+	+	+	-
rtT	1758-1760	+	+	+	+	+	+	-	+	+	+	+
chaA,kdsA, ychA	1771-1773	+	+	+	+	+	+	+	-	-	+	+
hemK, prfA, lolB,ipk, prsA, ychM	1775-1781	+	+	+	+	+	+	+	+	-	+	+
ymgE, ycgR&Q, emtA	1793-1800	+	+	+	+	+	+	+	+	-	+	+
pagO & K, mig-3	1857-1872	-	-	-	-	-	-	-	-	-	-	-
yodD, dsrA	1984-1985	-	-	-	-	-	-	-	+	-	-	-
ompS1,cspB	1995-1996	-	-	+	-	+	+	+	-	-	+	+
pudB, pduC,pduD	2039-2041	+	+	+	+	+	+	+	-	-	+	+
rfb	2083-2089	-	-	-	-	-	-	+	-	-	-	+
oafA	2230-2244	-	-	-	-	-	-	-	-	-	-	-
-[a]	2903-2904	-	-	+	-	-	-	-	+	+	-	-
-[a]	3033-3034	-	-	+	+	+	-	-	+	+	-	-
-[a]	3117-3123	-	-	-	-	-	-	+	-	-	-	-
agaR	3251-3256	-	-	-	-	-	-	-	-	-	-	-
-[a]	3651-3654	-	-	-	-	+	-	+	-	-	+	-
sugR, rhuM	3752-3755	-	-	-	-	-	-	-	-	-	-	-
dgo	3827-3830	-	-	-	-	-	-	-	-	-	-	-
-[a]	3844-3846	-	-	-	-	-	-	-	-	-	-	-
-[a]	4110-4116	-	-	-	-	-	-	-	-	-	-	-
srfJ	4417-4436	-	-	-	-	-	+	-	-	-	+	-
-[a]	4489-4498	-	-	-	-	-	-	-	-	-	-	-

-[a] There is no gene name associated with the specified STM region.

Table 4.3. Hybridization results of virulence genes located in *S.* Kentucky.

cluster/gene	STM no.	Function	**52473**	**95361**	**4648**	**69448**	**67533**	**94951**	**97765**	**88630**	**81443**	**53737**	**18721**
cdtB	-[a]	Putative toxin-like protein	-	-	-	-	-	-	+	-	-	-	-
pagP	628	Regulatory system	+	+	+	+	+	+	+	+	+	+	+
pipA	1087	SPI3	+	-	+	+	+	+	+	+	+	+	+
sopB	1091	Homologous to *ipgD*	+	+	+	+	+	+	+	+	+	+	+
msgA	1241	Macrophage survival	+	+	+	+	+	+	+	+	+	+	+
pagC	1246	Macrophage survival	+	-	+	+	+	+	+	+	+	+	+
ttrC	1384	SPI2	+	+	+	+	+	+	+	+	+	+	+
sopE2	1855	TTSS	+	+	+	+	+	+	+	+	+	+	+
sopA	2066	SPI1;TTSS	+	+	+	+	+	+	+	+	+	+	+
invA	2896	Invasion protein	+	+	+	+	+	+	+	+	+	+	+
iroN	2777	Iron acquistion	+	+	+	+	+	+	+	+	+	+	+
orgA	2869	Flagellar biosynthesis/invasion	+	+	+	+	+	+	+	+	+	+	+
prgI	2873	Cell invasion	+	+	+	+	+	+	+	+	+	+	+
sopD	2945	SPI1;TTSS	+	+	+	+	+	+	+	+	+	+	+
tolC	3186	Organic solvent tolerance	+	+	+	+	+	+	+	+	+	+	+
misL	3757	Pathogenicity island 3	+	+	+	+	+	+	+	+	+	+	+
mgtC	3764	Mg2+ transport protein Pathogenicity island 3	+	+	+	+	+	+	+	+	+	+	+
sifA	1224	Replication in macrophages	+	+	+	+	+	+	+	+	+	+	+
sipB	2885	Cell invasion	+	+	+	+	+	+	+	+	+	+	+
sitC	2863	Iron acquistion	+	+	+	+	+	+	+	+	+	+	+
slyA	1444	Hemolysin transcriptional regulator	+	+	+	+	+	+	+	+	+	+	+
spaN	-[a]	TTSS	+	+	+	+	+	+	+	+	+	+	+
pefA	PSLT018	Fimbriae	+	+	+	+	+	+	+	+	+	+	+
spvC	PSLT038	Virulence plasmid	-	-	-	-	-	-	-	-	-	-	-
spvB	PSLT039	Growth within macrophage	-	-	-	-	-	-	-	-	-	-	-
spiA	-[a]	Survival in macrophage	+	-	+	+	+	+	+	+	+	+	+
ssaQ	-[a]	Secretion system SPI2	+	+	+	+	+	+	+	+	+	+	+

-[a] Virulence gene not a STM gene thus no STM number.

Table 4.4. Genes unique in CVM29188 *S.* Kentucky identified with Mauve.

Gene		CDS	
	locus tag	CDS (product)	protein_id
1-229	A0001	DNA topoisomerase IV, B subunit	ZP_03078724.1
257-838	A0002	esterase YqiA	ZP_03077731.1
838-1878	A0003	cyclic 3',5'-adenosine monophosphate phosphodiesterase	ZP_03075945.1
2679-2996	A0006	quaternary ammonium compound-resistance protein SugE	ZP_03078472.1
2993-3526	A0007	outer membrane lipoprotein blc	ZP_03078024.1
3620-4765	A0008	beta-lactamase	ZP_03078752.1
5089-6351	A0010	TnpA	ZP_03075937.1
6616-8103	A0011	putative membrane protein	ZP_03078648.1
198030-198105	A0207	tRNA-Val	
198109-198184	A0208	tRNA-Lys	
198323-198400	A0209	tRNA-Lys	
264381-265304	A0276	transcriptional regulator, LysR family	ZP_03075144.1
265769-267100	A0278	major facilitator superfamily MFS_1	ZP_03075453.1
267151-267900	A0279	3-oxoacyl-[acyl-carrier-protein] reductase	ZP_03075049.1
267914-268759	A0280	transketolase	ZP_3075648.1
268759-269751	A0281	transketolase, central region	ZP_03076362.1
269751-272420	A0282	aldehyde-alcohol dehydrogenase 2	ZP_03076859.1
277320-277925	A0286	integrase, catalytic region	ZP_03075850.1
278174-278575	A0287	ISPsy11, transposase OrfA	ZP_03075055.1
362872-363150	A0374	transposase subfamily	ZP_03076735.1
364265-365185	A0378	transcriptional regulator, LysR family	ZP_0307433.1
365403-366575	A0379	putative ROK family protein	ZP_03074789.1
367349-368317	A0380	glucokinase	
368412-370079	A0381	glucose-methanol-choline oxicoreductase	ZP_03076592.1
370168-371223	A0382	lolE protein	ZP_03074978.1
371321-372445	A0383	putative oxidoreductase YcjS	ZP_03074520.1
376163-377371	A0389	transposase (IS4 family)	ZP_03078612.1
377478-378644	A0390	conserved hypothetical protein	ZP_03076199.1
378841-380187	A0391	porin B	ZP_03075683.1
381664-383007	A0395	benzoate MFS transporter	ZP_03078908.1
426184-426585	A0432	ISPsy11, transposase OrfA	ZP_03075777.1
426834-427439	A0433	integrase, catalytic region	ZP_03076983.1
855263-855658	A0901	protein MalY	ZP_03078667.1
855605-856258	A0902	maltose regulon regulatory protein Mall	ZP_03078015.1
950012-950863	A1000	protein TolA	ZP_03077881.1
952745-953653	A1002	LysR substrate binding domain protein	ZP_03077392.1
953827-954807	A1003	2-ntiroporpane dioxygenase NPD	ZP_03075743.1
1285904-1286077	A1339	mehtyl-accepting chemotaxis sensory transducer	ZP_03075893.1
1316434-1318155	A1366	tail fiber protein	ZP_03077665.1
1322744-1324891	A1373	putative phage portal protein	ZP_03078440.1
1324891-1326603	A1375	large subunit terminase	ZP_03078038.1
1326584-1327378	A1376	putative prophage terminase small subunit	ZP_03076688.1
1327848-1328312	A1378	bacteriophage lysis protein	ZP_03077650.1
1328620-1329159	A1379	lysozyme	ZP_03077032.1
1333610-1333972	A1384	antitermination protein Q	ZP_03076649.1
1334463-1335062	A1386	IrsA	ZP_03075363.1
1336660-1337082	A1389	replication P family protein	ZP_03077756.1
1341180-1344194	A1396	exodeoxyribonuclease VIII	ZP_03075980.1

Gene		CDS	
	locus tag	CDS (product)	protein_id
1345337-1345657	A1398	gp46	ZP_03075291.1
1461095-1461256	A1537	putative cytoplasmic protein	ZP_03076702.1
1477964-1478266	A1561	integrase	ZP_03077646.1
1478285-1479334	A1562	arsenical-resistance protein	ZP_03076996.1
1479352-1481112	A1563	arsenical pump-driving ATPase	ZP_03076856.1
1481156-1481518	A1564	arsenical resistance operon trans-acting repressor ArsD	ZP_03076644.1
1481552-1482028	A1565	arsenate reductase	ZP_03075920.1
1482025-1482399	A1566	arsenic resistance trasncriptionl regulator	ZP_03075426.1
1552195-1553352	A1643	glycosyl transferase, group 1	ZP_03075899.1
1553339-1554349	A1644	second mannosyl transferase	ZP_03076595.1
1554349-1555548	A1645	O-antigen polymerase	ZP_03076327.1
1555642-1556685	A1646	rhamnosyl transferase	ZP_03075611.1
1557348-1558310	A1648	glycosyl transferase, group 2 family protein	ZP_03077139.1
1558303-1559643	A1649	putative membrane protein	ZP_03078826.1
1620852-1620986	A1702	IS1-family insertion element protein	ZP_03075578.1
1623286-1624152	A1707	protein YibA	ZP_03074690.1
1624362-1624595	A1708	protein RhsD	ZP_03074773.1
1791168-1792430	A1870	TnpA	ZP_03075579.1
1792754-1793899	A1872	beta-lactamase	ZP_03078527.1
1815379-1823976	A1896	ShdA	ZP_03075587.1
1824406-1825614	A1898	transposase (IS4 family)	ZP_03078471.1
2025207-2026244	A2098	putative membrane protein	ZP_03077591.1
2028538-2028870	A2103	plasmid stabilization system protein, RelE/ParE family	ZP_3074505.1
2028901-2029134	A2104	conserved domain protein	ZP_03076414.1
2029294-2029443	A2105	transposase	ZP_03075808.1
2029622-2030635	A2106	transposase for insertion sequence element A	ZP_03076891.1
2030884-2031684	A2108	integrase core domain protein	ZP_03075647.1
2031703-2031972	A2109	DNA-binding prophage protein	ZP_03076128.1
2033198-2034427	A2111	HTH domain protein	ZP_03076278.1
2205458-2206081	A2263	transposase B	ZP_03075117.1
2208452-2213410	A2266	nuclease-related domain family	ZP_03077679.1
2213669-2214127	A2268	protein YpjI	ZP_03076990.1
2214213-2214671	A2269	antirestriction protein	ZP_03078955.1
2214680-2215162	A2270	DNA repair protein, RadC family	ZP_03075225.1
2215411-2215728	A2272	putative antitozin module of toxin antitoxin system	ZP_03075759.1
2215818-2216078	A2273	toxin of the YeeV-YeeU toxin-antitoxin system	ZP_03077390.1
2413446-2414492	A2484	putative secreted protein	ZP_0307589.1
2547625-25481234	A2626	mannitol operon repressor	ZP_03077439.1
2548348-2549319	A2627	frutcose-1,6-bisphosphatase class II	ZP_03078571.1
2549316-2550590	A2628	L-sorbose 1-phosphate reductase	ZP_03077892.1
2550614-2551993	A2629	pts system mannitol-specific eiicba component	ZP_03078362.1
2552021-2552464	A2630	mannitol-specific cryptic phosphotransferase enzyme iia componenet	ZP_03075445.1
2663858-2664175	A2762	quaternary ammonium compound-resistance protein SugE	ZP_03074757.1
2664172-2664705	A2763	outer membrane lipoprotein blc	ZP_03075406.1
2664799-2665944	A2764	beta-lactamase	ZP_03078886.1
2666268-2667530	A2766	TnpA	ZP_03077542.1
2705217-2706479	A2806	TnpA	ZP_03077533.1

Table 4.4 continued

Gene		CDS	
	locus tag	CDS (product)	protein_id
2706803-2707948	A2808	beta-lactamase	ZP_03076024.1
2708042-2708209	A2809	outer membrane lipoprotein blc	ZP_03075409.1
3050534-3050917	A3173	acetyltransferase, gnat family	ZP_03075227.1
3051113-3052216	A3175	high-affinity branched-chain amino acid ABC transporter, periplasmic Leu?Ile?Val-binding protein LIvJ	ZP_03076155.1
3342083-3343222	A3458	outer membrane protein	ZP_03076002.1
3559222-3561048	A3676	Hsp90xo protein	ZP_03077710.13
3868366-3868833	A3972	Transposase	ZP_03074780.1
3869715-3870005	A3976	PefB	ZP_03076298.1
3870040-3870579	A3977	K88 fimbrial protein A	ZP_03075637.1
3870589-3873027	A3978	CshB porin	ZP_03077557.1
3873050-3873841	A3979	chaperone protein ClpE	ZP_03076010.1
3873874-3874368	A3980	K88 minor fimbrial subunit F aeF	
3874586-3875401	A3981	putatvie K88 minor fimbrial subunit Fael	ZP_03076707.1
3875634-3876431	A3982	minor fimbrial subunit	Zp_03078518.1
3876459-3877223	A3983	minor fimbrial subunit	ZP_03075417.1
3878799-3879650	A3986	SrgB	ZP_03074868.1
3982852-3983268	A4101	VagD	ZP_03076715.1
3983265-3983495	A4102	conserved domain protein	ZP_03078931.1
4022511-4023761	A4143	integrase	ZP_03075933.1
4032344-4034317	A4153	DNA sulfur modification protein DndD	ZP_03077429.1
4034314-4035975	A4154	putative sulfurtransferase DndC	ZP_03074912.1
4036401-4037525	A4155	cystein desulfurase DndA	
4037522-4038331	A4156	restriction endonuclease family	ZP_03075491.1
4038432-4038698	A4157	transposase subfamily	ZP_03076821.1
4064394-4065854	A4192	type 1 restriction enzyme EcoKl specificity protein	ZP_03078499.1
4356270-4357331	A4477	putative fimbrial protein	ZP_03078344.1
4357343-4357942	A4478	putative fimbrial protein	ZP_03076139.1
4357961-4358518	A4479	putative fimbrial protein	ZP_03077635.1
4358545-4359129	A4480	putative fimbrial protein	ZP_03077091.1
4359152-4361749	A4481	outer membrane usher protein HtrE	ZP_03078596.1
4361817-4362575	A4482	chaperone protein EcpD	ZP_03075169.1
4362662-4363267	A4483	major fimbrial subunit	ZP_03074677.1
4459193-4461076	A4580	type VI secretion protein, family	ZP_03076489.1
4461092-4461586	A4581	type VI secretion system lysozyme-related protein	ZP_03075995.1
4461583-4462407	A4582	SciE protein	ZP_03074967.1
4462394-4463284	A4583	putatvie cytoplasmic protein	ZP_03078782.1
4463664-4466303	A4584	type VI secretion ATPase, ClpV1 family	ZP_03077974.1
4466403-4466945	A4585	type VI secretion protein, family	ZP_03076748.1
4466969-4468477	A4586	type VI secretion protein, EvpB/family	ZP_03075641.1
4468828-4469109	A4587	putative cytoplasmic protein	ZP_03076263.1
4469363-4469848	A4588	hemolysin-coregulated protein	ZP_03074637.1
4470152-4470637	A4589	putative cytoplasmic protein	ZP_03075269.1
4470673-4471005	A4590	putative periplasmic protein	ZP_03078704.1
4471148-4471633	A4591	hemolysin-coregulated protein	ZP_03077040.1
4471745-4472236	A4592	type VI secretion lipoprotein, family	ZP_03077647.1
4472240-4473583	A4593	type VI secretion protein, family	ZP_03076011.1

Table 4.4 continued

Gene		CDS	
	locus tag	CDS (product)	protein_id
4473580-4474884	A4594	SciP protein	ZP_03074884.1
4476329-4480198	A4597	type VI secretion protein IcmF	ZP_03076116.1
4484305-4486524	A4605	Rhs-family protein	ZP_03074651.1
4487017-4491516	A4607	YD repeat protein	ZP_03077384.1
4494881-4497115	A4613	Rhs-family protein	ZP_03076183.1
4778891-4779157	A4896	transposase subfamily	ZP_03075197.1
4779190-4779990	A4897	integrase core domain protein	ZP_03075971.1
4780154-4780681	A4898	putative inner membrane protein	ZP_03076701.1
4780966-4781718	A4900	YgeR	ZP_03077034.1
4781989-4782534	A4901	isopentenyl-disphosphate delta-isomerase	ZP_03078228.1
4782610-4784127	A4902	lysyl-tRNA syntehtase LysS	ZP_03078742.1
4785340-4787073	A4904	single-stranded-DNA specific exonulcease RecJ	ZP_03074582.1
4787079-4787792	A4905	thiol:disulfide interchange protein DsbC	ZP_03075705.1
4787816-4788712	A4906	tyrosine recombinase XerD	ZP_03076161.1
4788825-4789346	A4907	flavodoxin	ZP_03077223.1
4789399-4789812	A4908	putative inner membrane protein	ZP_03077601.1
4789793-4790059	A4910	TPR repeat protein	ZP_03076190.1
4790309-4791289	A4911	tRNA-modifying protein YgfZ	ZP_03076158.1
4791405-4792064	A4912	channel protein, hemolysin III family	ZP_03075335.1
4792228-4792539	A4913	protein YqfB	ZP_03075271.1
4792697-4794130	A4914	6-phospho-beta-glucosidase BglA	ZP_03075765.1

Table 4.4 continued

CHAPTER 5

CONCLUSIONS

Salmonella Newport

Recent development of multidrug resistant (MDR) *S.* Newport has been associated with dairy cattle while pan-susceptible strains are usually linked to vegetable contamination. More worrisome, is the fact that MDR *S.* Newport has increasingly been linked to human infections and which may cause a more severe infection that requires medical treatment. The majority of MDR pathogens acquire resistance through the conjugation of plasmids. These plasmids can contain resistance for multiple antibiotics as well as virulence genes that can encode for toxins, flagella, and pili. The genome content of *Salmonella* serotypes varies greatly as it relates to pathogenicity. Certain serotypes can cause gastroenteritis while others can invade the blood stream. These variations in diseases are due to differences in virulence genes within each serotype. *S.* Newport from this study had several genes related to virulence that were similar to genes found in *S.* Typhimurium, *S.* Typhi, *S.* Paratyphi, and *S.* Enteritidis. Gentically *S.* Newport was found to be more similar with Comparative Genomics Hybridization (CGH) than with PFGE. The percentage of similarity used in the cluster analysis to differentiate the *S.* Newport microarray data found the isolates to be 92% or more similar, while PFGE results was only 80% similar. These results suggest that PFGE is more discriminatory than CGH; however, it also suggests that PFGE is unable to discriminate serotypes that are closely related based on genetic content. CGH results demonstrated that *S.* Newport contained phage genes that were previously rarely found outside of *S.* Typhimurium. Gifsy-1 and Gifsy-2 prophage were found in most *S.*

Newport isolates and 3 even contained genes that encoded approximately 72% of the Fels-2 prophage. Prophages encode important virulence factors such as *sodC* which increase *S.* Typhimurium's pathogenicity and thus may contribute to increased virulence of *S.* Newport. Other notable virulence genes include those that encode for fimbriae, flagella, and membrane proteins. Strains from this study were missing several important adhesion proteins such as *stc* and *lpf*. Losing these adhesion properties may prevent *S.* Newport from being ubiquitous in nature as is *S.* Typhimurium. Losing genes plays a large role in niche development in bacteria and in particular aids in the evolution of human specific *Salmonella* serotypes. In addition, MDR *S.* Newport and pan-susceptible strains both contained genes from *Salmonella* Pathogenicity Islands (SPI) 1-5 which are found in pathogenic serotypes. SPI 1-5 contain several virulence genes involved in adhesion, macrophage survival, toxin production, flagellum, and iron acquisition.

Pan-susceptible strains of *S.* Newport are usually not found to cluster with MDR strains as shown by PFGE. On the other hand, MDR *S.* Newport strains are often indistinguishable with PFGE due to their clonal nature making it difficult to determine epidemiology relationships. Genetic differences between these 2 susceptibility types were seen with CGH anaylsis. Several pan-susceptible strains of *S.* Newport were missing genes from the phosphotransferase system (PTS) located in region STM0571 to STM0577 which is used for fructose and mannose metabolism. Interestingly, 5 MDR *S.* Newport isolates have the region STM2741 to STM2768 which is absent in the other isolates. This region encodes genes for fructose and mannose metabolism as well an ATP-binding protein involved in virulence. Eleven MDR *S.* Newport, which includes all isolates from human sources, were missing the *yih* cluster that encode outer

membrane porin genes and sugar transport proteins. Since porins allow small molecules to cross the cell membrane perhaps this loss has limited MDR *S.* Newport to dairy cattle colonization.

To determine genes that were unique to *S.* Newport as compared to those found on the microarray chip, Mauve analysis was performed. There were approximately 100 genes and 79 hypothetical proteins that were unique to *S.* Newport. Interesting genes included arsenic resistance, carbohydrate transport and metabolism, cell motility, and phage proteins. Arsenic resistance may enable this organism to grow in an arsenic rich environment since this metal is often found in the environment as well as dairy cattle feed. Metabolism of carbohydrates may also contribute to *S.* Newport's niche development in dairy cattle since it would allow this pathogen to use a new sugar source. Phage proteins that were found to be unique provide evidence that *S.* Newport underwent lateral gene transfer and may also have acquired some virulence genes. Also, *S.* Newport contained unique defense mechanisms as part of a type I restriction-modification system. Alteration of EcoRI sites may prevent enzymatic degradation by other microbes.

Salmonella **Kentucky**

Almost half of the *Salmonella* isolated from poultry carcasses are *S.* Kentucky yet this microbe is rarely associated with human illness. To better understand this phenomenon, we used CGH to compare the genetic content of *S.* Kentucky to 5 other *Salmonella* serotypes, which are known human pathogens. In this study CGH revealed *S.* Kentucky to be less diverse as compared to PFGE. Clusters from microarray data were at least 80% or more similar while clusters from PFGE data were only 42% or more similar. *S.* Kentucky isolates in this study were missing the Fel-1, Gifsy-1, and Gifsy-2 prophage. Two isolates contained a small region

STM2694 to STM2722 in the Gifsy-2 prophage and these 2 isolates were found to be the least similar to the other *S.* Kentucky isolates used in this study by CGH and PFGE. Interestingly, *S.* Kentucky contained some form of SPI 1-5 thus this serotype appears to have genes necessary for human colonization, attachment to host cell, cell invasion, and macrophage survival. Yet this organism is not known to be a human pathogen. Studies have shown that *S.* Kentucky does not mount an acid tolerance response which is believed to allow them to proliferate over other microbes that are spending energy on proteins for survival rather than growth. This occurrence may allow *S.* Kentucky to dominate over other bacteria in poultry; however, a lack of acid tolerance response may prevent *S.* Kentucky from establishing itself in the human host since the human stomach is much more acidic. Also, the *Salmonella* in this study were missing virulence genes *sugR* and *rhuM* which were found to missing in several other serotypes. It was discovered that these other serotypes contained insertion sequences with fimbrial operons located between them. Mauve analysis revealed unique fimbrial and insertion sequences in *S.* Kentucky thus it is possible that the same genome insertion occurred in these microbes. In addition, two regions, STM3827 to STM3830 and STM4417 to STM4436, encoding Major Facilltator Superfamilies (MFS) were missing from all *S.* Kentucky isolates. MFS are used to transport various subtrates including antibiotics, sugars, and amino acids across the cell membrane. Notably, MFS contribute to resistance to multiple antibiotics since several drugs can be pumped out of the microbial cells. Other membrane associated proteins missing from several *S.* Kentucky isolates were porins *ompS1* and *cspB.* These porins act as sensory transducers which allow bacteria to respond rapidly to environmental cues. Perhaps the absence of these porins prevents *S.* Kentucky from eliciting an acid tolerance response.

Analysis with Mauve revealed 168 unique genes and 125 unique hypothetical proteins in *S.* Kentucky. Genes that were unique to *S.* Kentucky included transcription, replication, recombination, defense mechanisms, signal transduction, cell wall/membrane biogenesis, cell motility, carbohydrate transport and metabolism, and metal and antibiotic resistance. Noteworthy genes included 5 genes that encoded arsenic resistance. Poultry like dairy cattle consume large quantities of arsenic from the environment as well as from food thus this resistance would allow *S.* Kentucky to flourish in an arsenic rich environment. Also, these isolates contained a quaternary ammonium compound resistance gene and several β-lactamase genes. All four unique β-lactamase matched those found in *S.* Newport which has been shown to contain these genes on a transferable plasmid. These studies found that *S.* Newport which contained a similar MDR plasmid found in the human pathogen *Yersina pesits,* had an IncA/C backbone that conferred resistance to multiple antibiotics. *S.* Kentucky is usually resistant to 2 antimicrobials, streptomycin and tetracycline; however, this incident was also reported in other serotypes which contained the IncA/C plasmid. They contained genes for other antimicrobial resistances yet only expressed resistance to sulfamethoxazole and tetracycline. In addition, *S.* Kentucky contained 8 unique genes for a type VI secretion system (T6SS) which encodes genes for adhesion, cell invasion, macrophage survival and persistence in a host. There were also 10 unique fimbriae genes thus this microbe had unique attachment/adherence proteins as compared to the other serotypes.

Comparison of Genetic Content in *S.* Newport versus *S.* Kentucky.

Genetically, *S.* Kentucky and *S.* Newport are very similar yet these organisms have developed different niches and have vast differences in pathogenicity. MDR *S.* Newport is predominately associated with dairy cattle and is the third most commonly isolated *Salmonella*

serotype from humans. On the other hand, *S.* Kentucky is the most common serotype isolated from poultry and is rarely associated with human illness. Furthermore, both microbes have developed resistance to multiple antibiotics. To determine how these organisms differ based on genetic content we compared the CGH results. CGH revealed some interesting differences. One of which was the fact that *S.* Newport contained the Gifsy-1 and Gifsy-2 prophage while *S.* Kentucky did not. The majority of the Fels-2 prophage was found in a few of the *S.* Newport isolates and only 2 of the *S.* Kentucky isolates contained a small region found in the Fels-2 prophage. Gifsy-2 prophage contains a virulence factor *sodC*, which makes *S.* Typhimurium more pathogenic. The *sod* gene encodes for a superoxide dismutase which converts toxic superoxide to less toxic peroxide. *S.* Kentucky isolates were also missing 2 hydroperoxide reductase genes (*aphC* and *aphF*) which also protect the cell during peroxidase activity. Thus *S.* Newport is able to survive the oxidative burst produced by macrophage. Perhaps this difference allows *S.* Newport to cause disease in humans while *S.* Kentucky is left defenseless against the host macrophage. *S.* Kentucky was also missing regions STM0715 to STM0727, STM0854 to STM0861, and STM1127 to STM1133 involved in cell wall biogensis, transcription, and carbohydrate transport and metabolism. In addition, *S.* Kentucky isolates were missing *pagO* and *pagK*, genes regulated by the PhoPQ system. Their function is unknown however PhoPQ usually regulates genes involved in intracellular survivial, invasion, phagosome alteration, and resistance to antimicrobials. Thus one could deduce that these missing genes had a similar function.

Sugar metabolism is extremely important in the development of niches. Both organisms were missing the *rfb* operon which is involved in fructose and mannose metabolism as well as region STM2230 to STM2243 which encodes phage genes. Region STM3117 to STM3123

which encodes genes involved in the TCA cycle was absent in *S.* Kentucky. An *agaR* operon located in region STM3251 to STM3256 was absent in both serotypes. Also, region STM3752 to STM3755 which encodes genes *sugR* and *rhuM* was absent in *S.* Newport and *S.* Kentucky isolates. These genes are predicted to be involved in virulence. Additionally, region STM3827-3830, an MFS cluster, was absent in *S.* Kentucky while another MFS cluster (STM4417 to STM4436 was absent in both serotypes. As stated previously, MFS transport various substances included antibiotics across the cell membrane. All *S.* Newport from this study were missing a region that encodes genes for degrading plant cell walls to release carbon source for growth (STM4010 to STM4020). This region also included genes for carbohydrate transportation and metabolism thus this serotype has lost a source of sugar which may explain the differences in *S.* Kentucky's and *S.* Newport's environmental niches. Both serotypes were missing a phosphotransferase system for fructose and mannitol metabolism (STM4110 to STM4116).

S. Kentucky has a majority of the virulence genes found in *S.* Newport as well as other human pathogens. Obvious differences between these 2 organisms included genes for survival during peroxidase activity in macrophage. This lack of response by *S.* Kentucky may prevent it from causing human disease like that of *S.* Newport. It would be interesting to compare the MDR *S.* Kentucky isolates from travelers in France to MDR *S.* Newport isolates in the U.S. to investigate genetic differences or similarities. Also, comparison of the MDR *S.* Kentucky from France to strains in the U.S. may reveal interesting differences that could lead to a better understanding of these microbes ability to become a pathogen associated more often with human disease. Bacteria are everywhere and in almost everything. The purpose of studying foodborne pathogens is not to eradicate bacteria from the food system but rather prevent the establishment of bacteria that have the potential to cause human harm. Learning why a bacterium establishes a

niche in a particular food sources is important to prevent future problems with foodborne pathogens.